Immunodetection of Two Curtoviruses Infecting Sugar Beet

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ABSTRACT

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Beet leafhopper-transmitted curly top virus is a serious problem in many different crops in the semiarid western United States, including sugar beet, tomatoes, and beans. Curly top is caused by a genetically diverse complex of phloem-limited curtoviruses. Due to the phloem restriction of curtoviruses and the lack of a convenient laboratory host–vector system for curly top virus propagation and purification, no commercial immunodetection tests are available for curtoviruses. Routine diagnostics for curly top rely either on visual symptoms or on polymerase chain reaction (PCR) tests. Lack of an enzyme-linked immunosorbent assay (ELISA) system is one of the factors hampering development and screening of the curly top resistant germplasm in, for instance, sugar beet and bean breeding programs. To fill in this gap, we developed an ELISA-based detection system for curtoviruses which utilizes virus-specific antibodies generated against bacterially expressed capsid protein (CP) of *Beet mild curly top virus*. Bacterially expressed CP was affinity purified and used as an antigen for antibody production in two animal species. Specificity of the resulting antisera was tested in Western blots and various triple-antibody sandwich (TAS)-ELISA formats with sugar beet, bean, and *Nicotiana benthamiana* leaf tissue. We demonstrate reliable detection of two curtoviruses in different crops in TAS-ELISA format, suitable for large-scale screening of germplasm in breeding programs.

Curly top, caused by any of three *Curtovirus* species (*Beet severe curly top virus* [BSCTV], *Beet mild curly top virus* [BMCTV], and *Beet curly top virus* [BCTV]), has been a problem for the sugar beet industry as well as for the bean industry in the United States since the early twentieth century (2,3,25). Curtoviruses are transmitted by the beet leafhopper (*Circulifer tenellus* Baker), which is widespread in semiarid areas of the western United States. Management strategies may include vector control in overwintering areas, seed- or crop-applied insecticides, and/or development of resistant cultivars (2,10,24). Resistant sugar beet and bean varieties have been successfully introduced (10,24), but losses to curly top are still considerable. Maintaining resistance to curly top in sugar beet is troublesome because of yield drag and its quantitative nature (24).

To facilitate curly top resistance breeding and possible management strategies, good detection and quantification methods are needed. Virus detection for curly top in sugar beet is typically based on visual inspection of fields to observe virus symptoms in the foliage (23). However, visual detection only facilitates selection of plants with reduced foliar symptoms and does not address virus titer level, i.e., actual resistance. In addition, this type of detection often requires laboratory retesting of suspicious samples, and needs reliable and economically feasible laboratory diagnostic tools. The most commonly used assay for this purpose is enzyme-linked immunosorbent assay (ELISA), which detects the free or assembled capsid protein (CP) of the virus (4). However, due to the phloem-limited nature of the virus infection, and difficulty in obtaining quantities of virions sufficient for antiserum production from any known host plants, good specific ELISAs suitable for virus detection on a large scale have not been developed for curtoviruses.

At least three independent productions of curly top virus–specific antisera have been reported since the early 1980s (9,16; H.-Y. Liu, *personal communication*). These antisera were developed in rabbits against BCTV virions purified from shepherd's purse and are still in use on a small scale. However, stocks of these sera are nearly depleted, and since antisera were raised in rabbits, immunodetection of curtoviruses with these three sera is restricted either to Western blots, direct immunolabeling protocols, or simple double-antibody sandwich (DAS)-ELISA formats (9). No commercial antibodies are available at the moment for immunodetection of curly top virus. There are other methods of testing focused on the nucleic acid detection, such as the polymerase chain reaction (PCR) assay (17). However, these are, at the moment, more expensive and less amenable to scale-up, and may be hampered by the virus diversity in the field (19,22,25).

BCTV is the type member of the genus *Curtovirus*, family *Geminiviridae*. BCTV has a single-stranded, circular DNA genome of ca. 2.9-kb encoding seven genes in virion- and complementary-sense orientations. Curtoviruses form small, quasi-isometric geminate particles (18 × 30 nm) containing a single species of CP (5,9,15). This CP is encoded by the V1 gene in the virion-sense orientation, and is composed of 254 amino acids (18). The 29.4-kDa CP of all three curtoviruses causing curly top in sugar beet, i.e., BCTV, BMCTV, and BSCTV, are very conserved at the amino acid sequence level (ca. 95% identity) (21), and thus should provide an ideal target for virus detection using a protein-based technique like ELISA.

In this report, we describe production of curtovirus-specific antisera in two animal species, rabbit and guinea pig. An abundant, bacterially produced BMCTV CP antigen was used to immunize both animals. Both antisera were found capable of capturing curly top virus when used as coating antibodies in a triple-antibody sandwich (TAS)-ELISA protocol. Hence, we describe a complete ELISA-based detection protocol applicable for curly top virus detection in infected sugar beet, tomatoes, beans, and *Nicotiana benthamiana*, which can be scaled up for testing resistant germplasm in many breeding programs.

MATERIALS AND METHODS

Virus sources. The virus-infected garden bean sample (var. Blue Lakes) used for BMCTV CP amplification and cloning was provided by Marvin Miller (Integrated Biological Systems, Nampa, ID). This leaf sample was collected in August 2008 west of Caldwell, ID, and submitted to the University of Idaho Plant Virology laboratory for identification. It was tested for the presence of curly top virus using the PCR procedure of Soto and Gilbertson (17) and found positive. Subsequent sequencing of the amplified 1.1-kb PCR fragment revealed that this was the BMCTV curly top species. Curly top virus–infected bean and sugar beet leaf samples were from the virus-infected plants maintained in Kim-

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berly, ID by the USDA-ARS Northwest Irrigation and Soils Research Laboratory (NWISRL) and the Beet Sugar Development Foundation (BSDF). Additional curly top virus–positive sugar beet leaf samples were from the curly top insectary maintained by the BSDF in Twin Falls, ID. These virus-infected samples had been exposed to viruliferous beet leafhoppers (at least six per plant) from a colony previously documented to carry BCTV, BMCTV, and BSCTV (25). To verify broad specificity of the antibodies produced, plant samples infected with individual curtoviruses were tested: these samples were provided by W. Wintermantel (USDA-ARS, Salinas, CA). Specifically, leaf samples of sugar beet, tomato, and *Nicotiana benthamiana* infected with BSCTV (strain CFH), and *N. benthamiana* infected with BMCTV (strain Worland) were used in our TAS-ELISA and PCR assays.

Bacterial expression of the BCTV CP gene. The entire CP gene of BMCTV was amplified from DNA extracts of a BMCTV-infected bean leaf using a PCR protocol (13) and two specific primers, CTf 5′-GGT*GAATTC*ATGAGRAAATAT ACAAGAAATACGT-3′ and CTr 5′-CCA *CTGCAG*TTATTAATARAAATARCATCT ACA-3′. The amplified BMCTV CP gene was cloned into a pMAL expression vector (New England Biolabs, Ipswich, MA) between *Eco*RI and *Pst*I sites, and resulting fusion protein (FP) carrying maltose-binding fragment (MBP) was affinity-purified from bacterial extracts using amylose resin column as described previously (8). Purity of the ca. 72-kDa FP antigen was evaluated on 4 to 20% gradient PAGE with subsequent staining with Coomassie blue. Antigenic specificity of this FP was verified by Western blots probed with the BCTV-specific antibodies kindly provided by H.-Y. Liu (USDA-ARS, Salinas, CA); this old antiserum was raised against the Logan strain of curly top virus which is now reclassified as BCTV. Yields of 1 to 3 mg per liter of liquid bacterial culture were recorded, and this purified 72-kDa FP antigen was stored at –20°C until use.

Immunization and antisera testing. Polyclonal antisera against affinity-purified, bacterially expressed FP were produced in rabbits, chickens, or guinea pigs after three to five immunizations with Freund's adjuvant. One animal of each species was used for immunization. Specificity of polyclonal antibodies was initially tested against BMCTV-infected or healthy bean leaf tissue in Western blots, and subsequently against virus-infected sugar beet tissue in Westerns or TAS-ELISA. The titer of antibodies produced in a chicken was too low for an ELISA-based detection system, and immunization was not continued after three injections. However, good antivirus titer of antibodies produced in rabbit and guinea pig allowed collection of multiple production bleedings 2 to 4 weeks after a booster immunization. The two antisera, UID16 (rabbit) and GP-1 (guinea pig), were therefore given further consideration in the development of the TAS-ELISA–based system. Plant and bacterial extracts were analyzed using 4 to 20% gradient polyacrylamide gel electrophoresis (PAGE) (Bio-Rad, Hercules, CA) followed by Coomassie staining or Western blots. For Western blot analyses, proteins separated by PAGE were transferred to nitrocellulose membranes and probed with an antibody under testing as described previously (7).

Plant sample extraction, ELISA procedure, and PCR testing. ELISA protocol followed the previously published methodology (14) with some modifications. One-gram samples of green leaf tissue were homogenized in 10 ml of extraction buffer (1× phosphate-buffered saline, 0.5% Tween 20, 0.3% dry milk, 2% polyvinylpyrrolidone). Homogenates were loaded into wells of Nunc MaxiSorp microtiter plates (Nunc, Rochester, NY) either directly, or after precoating with UID16 or GP-1 antisera at 1:10,000 dilution. Plates were incubated with plant extracts, washed with phosphate-buffered saline plus Tween 20 (PBST), and an intermediate detecting antiserum (GP-1 or UID16) at 1:10,000 dilution was applied to the wells in PBST buffer. After incubation for 4 h at 37°C, plates were washed extensively with PBST, and anti-guinea pig (A-7686, Sigma-Aldrich, St. Louis, MO) or anti-rabbit (A-3687, Sigma-Aldrich) IgG-conjugates with alkaline phosphatase at 1:30,000 dilution in PBST with 0.3% dry milk or 0.2% bovine serum albumin (BSA) were added, and the plates were incubated 4 h at 37°C. The plates were washed with PBST, and 0.6 mg/ml of *p*-nitrophenyl phosphate in 0.1 M diethanolamine buffer, pH 9.8, was added as a substrate. The yellow color reaction was monitored by measuring absorbance at 405 nm using an ELISA reader (SpectraMax 190, Molecular Devices, Sunnyvale, CA). All samples were always loaded in duplicates, and virus-positive and -negative control samples were included into each ELISA experiment. A signal was considered to be positive if it was 3 times higher than the negative control. PCR testing was performed using primers described in and according to the protocol of Soto and Gilbertson (17).

RESULTS

The soluble nature of the bacterially expressed FP, and the relatively good yield (1 to 3 mg per liter of bacterial culture) of the BMCTV-specific antigen, allowed us to immunize three animal species (rabbits, guinea pigs, and chickens). However, only the rabbits and guinea pigs produced immune responses suitable for detection of curly top viruses using indirect and TAS-ELISA. Initial characterizations of the specificity and relative titer of the antisera produced were performed using virus-infected and healthy sugar beet leaf samples. Figure 1 shows a Western blot of curly top virus–infected and healthy sugar beet leaves for one of the antisera, GP-1, which was later developed as a detecting antibody source for TAS-ELISA. A single prominent band was revealed by this antiserum only in extracts from virus-infected plants (Fig. 1). The apparent mobility of this band in this Tris-sodium dodecyl sulfate (SDS) electrophoretic system corresponded to an approximately 32-kDa protein, which is about 9% higher than the calculated molecular weight of 29.4-kDa for the curtovirus CP. This deviation may indicate anomalous mobility of the CP in the Tris-SDS gels (6) or post-translational modifications of the protein, or both. There were no significant nonspecific background reactions in healthy sugar beet tissue (Fig. 1) other than a weak band at ca. 60-kDa that might represent an abundant plant protein. The rabbit antibodies from the UID16 serum also reacted in Westerns with a single major virus CP band; however, these rabbit antibodies displayed some nonspecific reactions with healthy sugar beet proteins, and thus were utilized as coating (capturing) antibodies in ELISA.

Once the specificity of these two BMCTV-specific antisera was confirmed, we focused our efforts on development of the ELISA-based system for curtovirus detection. First, the antivirus titer was determined for each of the antisera, using indirect ELISA protocol. In this case, plant extracts from curly top virus–infected and healthy sugar beet leaves (diluted 1:10 with the extraction buffer) were loaded directly into ELISA plate wells, and antiserum under testing was titrated as detecting antibody. This titration curve was directly

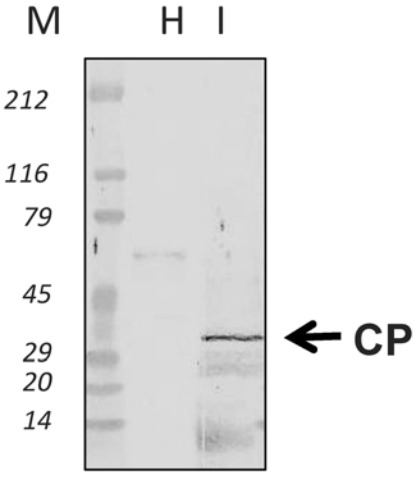

Fig. 1. Detection of the curly top virus capsid protein (CP) in infected (I) sugar beet in Western blots probed with GP-1 antiserum diluted 1:10,000. H = healthy plant. Arrow marks the CP position. M = protein markers.

compared to the titration of the corresponding preimmune serum; the titer was determined as the dilution of the serum producing an ELISA signal that exceeds the preimmune serum signal by at least threefold. The guinea pig serum GP-1 was determined to have a titer against curly top virus in excess of 700,000. The rabbit UID16 serum was found to have a similar titer against curly top virus in the same type of assay. From titration curves, the optimal working dilutions of both UID16 and GP-1 antisera for TAS-ELISA were calculated as 1:10,000.

Both UID16 and GP-1 antisera were able to capture curly top virus antigen from the infected plant tissues; however, the combination of UID16 as coating antibody and GP-1 as detecting antibody produced the lowest background and was selected as the best for further development. Figure 2 represents an example of virus detection in sugar beet leaves using this combination of UID16 for coating and GP-1 as an intermediate, detecting antibody. The antigen was captured by the UID16 antiserum diluted 1:10,000, and the detecting antibody was titrated against either curly top virus–infected or healthy sugar beet leaf extract (Fig. 2). This graph shows that the detection system developed has high sensitivity and was able to reliably detect the virus up to approximately 1:500,000 (Fig. 2).

Since curly top–infected sugar beet samples from USDA-ARS NWISRL in Kimberly, ID, were known to be infected with a mixture of all three viruses, BCTV, BMCTV, and BSCTV (22,25), a separate experiment was performed to test if the detection specificity is broad enough to detect individual virus species. *N. benthamiana* plants infected with BMCTV and BSCTV (kindly provided by W. Wintermantel, USDA-ARS, Salinas, CA) were tested in TAS-ELISA format side-by-side with the healthy *N. benthamiana* tissue (Fig. 3). Both virus species were easily

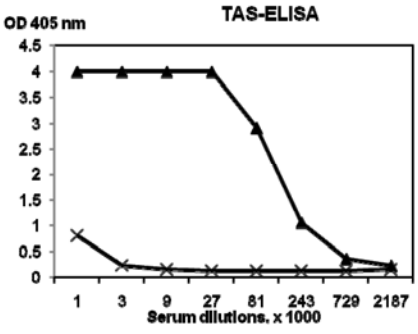

Fig. 2. A titration curve for curly top virus in sugar beet leaves. Detection by triple-antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA): virus antigen was captured by the UID16 PAb at 1:10,000 dilution. The GP-1 PAb was used as detecting antibody; this antibody was titrated along the x-axis. Infected (▲) and healthy (×) leaf extracts were diluted 1:10 prior to loading onto the ELISA plate.

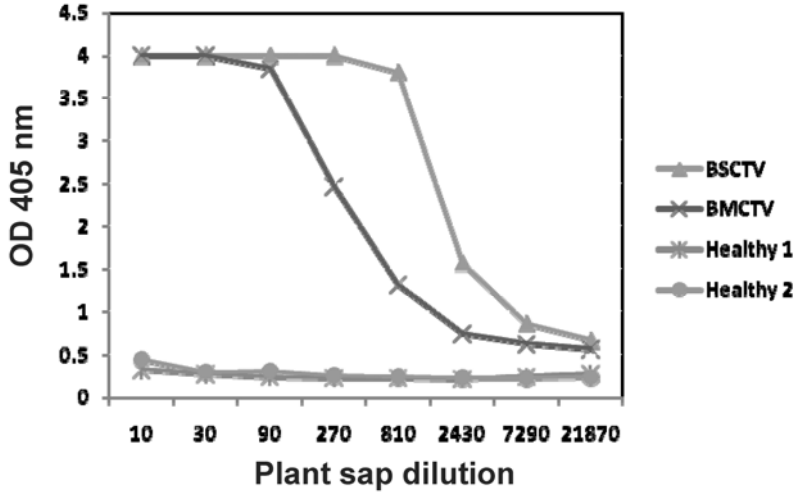

Fig. 3. Titration curves for *Nicotiana benthamiana* leaf extracts from two individual plants infected by *Beet severe curly top virus* (BSCTV) and *Beet mild curly top virus* (BMCTV). Virus antigens were captured with UID16 antiserum diluted 1:10,000 and detected with GP-1 antiserum diluted 1:10,000.

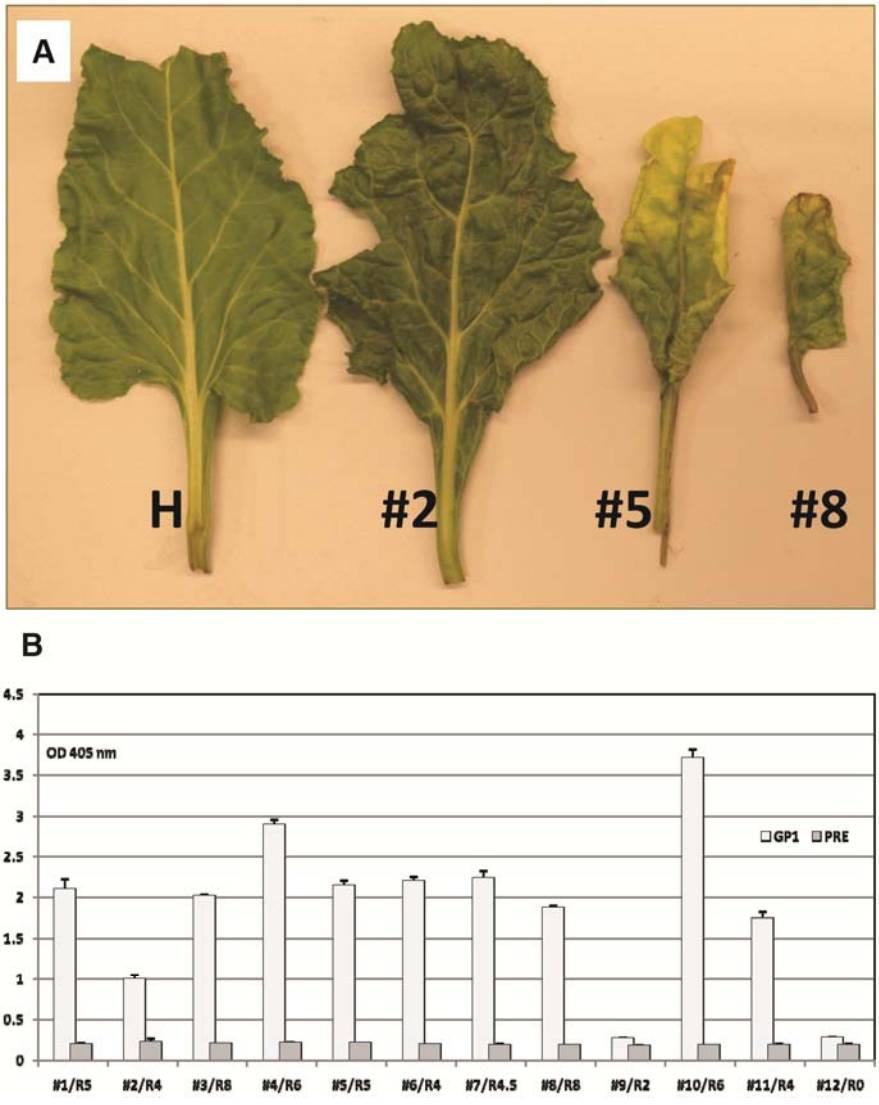

Fig. 4. Analysis of field sugar beet samples infected by curly top virus. **A,** Visual symptoms of curly top infection in the foliage; plant numbers correspond to enzyme-linked immunosorbent assay (ELISA) samples on panel B. **B,** Detection of the virus capsid protein (CP) with triple-antibody sandwich (TAS)-ELISA. ELISA plates were coated with UID16 and detection was performed with GP-1 antiserum diluted 1:10,000; PRE = detection with the pre-immune antiserum. Bars represent standard deviation for each data point in ELISA. Individual plant numbers on the x-axis are followed by the disease rating (scale 0 to 9; 0 = healthy, 9 = dead) for that plant.

detected with our antibodies, and for both the titer was quite high and sufficient to reliably detect the virus in *N. benthamiana* leaf extract diluted more than 5,000-fold (Fig. 3). The high sequence identity in CP amino acid sequences between BSCTV and BMCTV is likely what allowed the TAS-ELISA assay to reliably detect both virus species even though the original CP antigen came from BMCTV.

To test performance of this detection system in a field situation, 12 curly top virus–infected field samples from sugar beet germplasm screening experiments conducted at the USDA-ARS NWISRL in Kimberly, ID, were randomly collected, and scored based on the symptoms displayed, prior to TAS-ELISA (Fig. 4). These samples were scored visually using an arbitrary scale developed for sugar beet (0 to 9; 0 = healthy, 9 = dead) (23). The samples with visual scores between 0 and 8 were subjected to the TAS-ELISA testing using the antisera developed. As can be seen from Figure 4A and B, detection of curly top virus in sugar beet is very straightforward and reliable. It was generally consistent with the visual scoring, producing higher ELISA signal in samples with higher disease score (Fig. 4B). In one case, plant no. 9 which was scored 2, the virus titer was not detectable by TAS-ELISA. This was the lowest visual score in the experiment, however, and other plants, which scored between 4 and 8, showed readily detectable virus signal in TAS-ELISA (Fig. 4A and B). There was a positive quantitative trend between the virus titer and the visual score, but plant samples with the most advanced curly top symptoms, scored 8 (Fig. 4A, sample 8), apparently had virus concentration declining (Fig. 4B, samples 3 and 8). In this case, it probably reflects general wilting and necrosis in the advanced stages of curly top disease (Fig. 4A), leading to the overall drop in virus replication.

In order to address the reliability of the developed TAS-ELISA assay, a side-by-side curly top virus detection experiment was performed on the same sugar beet leaf samples using our TAS-ELISA protocol and the PCR-based assay described by Soto and Gilbertson (17). Of the 18 tested individual plants, 13 were found positive by ELISA, of which 12 were positive by PCR; only one sample was found positive by ELISA and negative by PCR (Fig. 5). All the negative samples by ELISA were also found negative by PCR (Fig. 5). Overall reliability of the TAS-ELISA was thus at least as good as that of the PCR assay.

This newly developed TAS-ELISA was also tested on dry bean leaf tissue collected from the germplasm screening experiments conducted at the USDA-ARS NWISRL in Kimberly, ID (Table 1). The two samples from curly top–infected plants with advanced symptoms were scored as 4.5 on the arbitrary scale (0 = healthy; 5 = dead) (26); they were also found virus-positive by ELISA, producing signal exceeding three times the signal with control preimmune serum when the GP-1 antiserum was used as an intermediate detecting antibody. No correlation between symptom severity and TAS-ELISA signal was noted (Table 1).

DISCUSSION

Bacterially produced antigens represent a useful alternative to traditional purified virus preparations as antigens when virus purification is a significant problem (1,8,11–13,27). This is especially true for phloem-limited or phloem-restricted viruses such as closteroviruses, luteoviruses, and here, geminiviruses. Not surprisingly, there have been multiple examples of production of recombinant capsid proteins in bacteria with subsequent development of specific antibodies for immunodetection (1,8,11–13,27). However, successful development of a complete detection system for a phloem-limited virus, especially based on antibodies against recombinant antigens and used in TAS/DAS-ELISA format, is anything but an easy or even predictable project. Although the use of such a recombinant antigen often solves the problem of an intermediate detecting antibody, usually the capturing antibody still remains a problem (1,12,13), since in many cases antibodies to recombinant proteins fail to bind native virus particles or capsid proteins, a property crucial for capturing the antigen at the first stage in TAS- or DAS-ELISA.

Here, we described successful development of a system for immunodetection of a group of curtoviruses infecting sugar beet, which is based on antisera against bacterially produced, recombinant BMCTV CP, suitable not only for Western blots but also for TAS-ELISA format (Figs. 1 to 5). The ability of the antisera generated, UID16 and GP-1, to capture curly top virus antigen from plant extracts is especially valuable, since it allows us to use the most advanced, "sandwich" formats of the ELISA protocol, both DAS- and TAS-ELISA, for detection of curtoviruses in field samples. Our data establish a TAS-ELISA assay for curly top virus detection and quantification since current curtovirus detection methods based on visual rating systems (10,23,26) or on laboratory PCR detection (17) have limitations. Visual rating systems were noted to be subjective, especially for curly top infection in beans (10). PCR detection, on the other hand, is relatively expensive and may be difficult to use on a large scale to screen germplasm in poorly equipped labs.

In characterizing this newly developed TAS-ELISA system for the detection of curtoviruses, three issues have been addressed here: (i) How broad is the specificity of this ELISA-based detection system, i.e., can we detect other species from the

Table 1. Detection by triple-antibody sandwich enzyme-linked immunosorbent assay of curly top in bean leaves—GP-1 antiserum versus preimmune antiserum

	OD 405 nm	SD	Disease rating
Bean 1	0.78	±0.02	4.5
Preimmune	0.18	±0.01	
Bean 2	2.53	±0.04	4.5
Preimmune	0.16	±0.01	

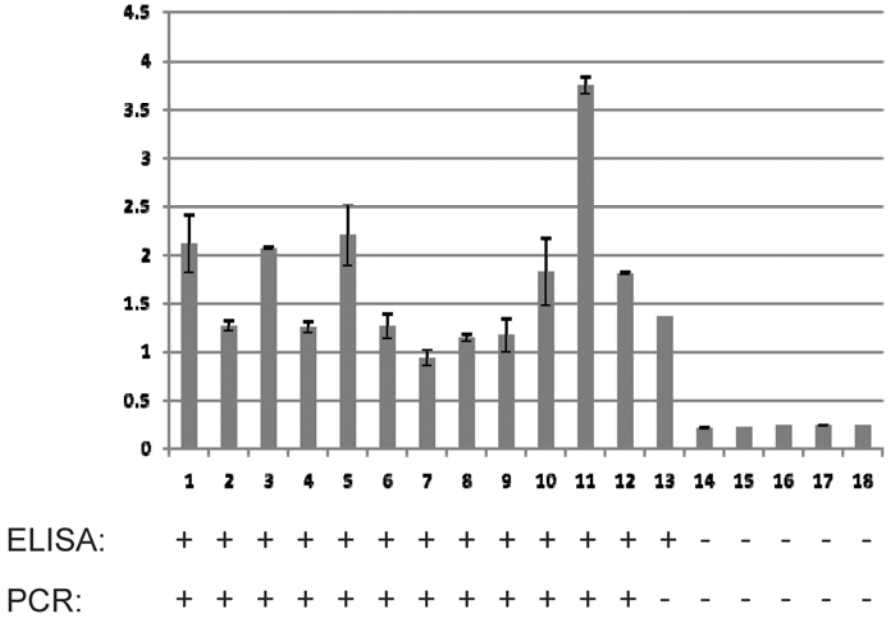

Fig. 5. Analysis of sugar beet samples infected with *Beet severe curly top virus* (BSCTV) (samples 1 to 13) and noninfected plants (samples (14 to 18) by triple-antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA) and polymerase chain reaction (PCR). Graph represents ELISA signals for individual sugar beet samples with standard deviations. A summary comparison between ELISA and PCR data for the same samples is given below the graph.

curtovirus complex, in addition to homologous BMCTV? (ii) How reliable and sensitive is this assay compared to the traditional visual and PCR based assays? (iii) Is this assay applicable to multiple crops that may be affected by the virus? The specificity of this ELISA seems quite broad, since both homologous BMCTV antigen and a heterologous BSCTV antigen were detected in the same host plant, *N. benthamiana* (Fig. 3). These results should not be considered surprising since there is very high sequence identity in the CP amino acid sequence (ca. 95%) among all three species, BCTV, BMCTV, and BSCTV (21).

Reliability of TAS-ELISA was first tested in sugar beet leaf samples against the conventional visual rating (Fig. 4A and B). Initial experiments suggested that a visual disease rating in susceptible varieties of sugar beet was consistent with the apparent virus titer as determined by TAS-ELISA (Fig. 4), in accordance with previously reported data (28). However, it was reported previously that curtovirus replication does not always result in symptom expression in sugar beet (20), and virus titer quantification may become a necessity in breeding programs under certain circumstances. Reliability of the TAS-ELISA was also tested in sugar beet leaf samples against the PCR-based detection described by Soto and Gilbertson (17) (Fig. 5). Of the 13 positive sugar beet samples identified by ELISA, one was missed by PCR, leading to a conclusion that this newly developed TAS-ELISA system for curtovirus detection is at least as reliable as the PCR-based assay.

The last issue addressed here was a more general applicability of this assay to other crops that may be affected by the virus, such as beans and others. The data presented demonstrate that this TAS-ELISA works very well, not only in sugar beet (Figs. 2, 4, and 5), but also in *N. benthamiana* (Fig. 3) and beans (Table 1). These species represent plants from three different families, Chenopodiaceae, Solanaceae, and Fabaceae, suggesting a broad applicability of this immunodetection system to a wide range of crops that may be affected by curtoviruses. Given the availability of such a universal detection system, large-scale germplasm screenings for curly top virus resistance are now possible in these and other crops, e.g., sugar beet, tomatoes, and beans.

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