Characterization of the Beet Yellow Stunt Virus Coat Protein Gene

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ABSTRACT

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The beet yellow stunt virus (BYSV) genome contains at least nine open reading frames (ORFs) that code for proteins ranging from 6 to 66 kDa. Based on amino acid sequence comparisons, the coat protein (CP) was previously identified as the product of ORF7. We expressed the product of ORF7 in bacteria and confirmed that ORF7 codes for the BYSV CP by immunoblotting. BYSV is a phloem-limited virus, and virus CP antigen of a quality sufficient for diagnostic antisera production has not been available. To produce BYSV antigen free of plant host contaminants, ORF7 was cloned into a pMAL bacterial expression vector. The resulting fusion protein was affinity-purified and used as an antigen to raise anti-BYSV CP antisera in rabbits and guinea pigs. Using these antisera, an indirect double-

antibody sandwich (DAS) enzyme-linked immunosorbent assay (ELISA)-based diagnostic system was developed. This indirect DAS-ELISA format enabled reliable detection of BYSV in tissue extracts from virus-infected lettuce diluted up to 5,000 times. The diagnostic system developed may enable large-scale epidemiological studies of BYSV using simple serological techniques. The antisera raised had a titer exceeding 1×10^5 in immunoblots and easily detected the 23.7-kDa BYSV CP in virus-infected lettuce and sowthistle plants. In these two plant species, BYSV CP was detected as two closely migrating bands during electrophoresis, which may suggest posttranslational CP modifications. To further characterize the BYSV CP gene, the 5'-untranslated region (UTR) of the BYSV CP subgenomic RNA (sgRNA) was cloned and sequenced. The CP-encoding, approximately 1.9-kb sgRNA has an AT-rich, 66-nucleotide-long 5'-UTR colinear to the genomic sequence upstream of ORF7.

The potentially destructive, aphid-transmitted beet yellow stunt disease was first described in California by Duffus (9,10). Symptoms in sugar beet were similar to the beet yellowing disease caused by beet yellows virus (BYV) and beet western yellows virus (BWYV); however, the pathogen, beet yellow stunt virus (BYSV), had a different host range, differed in vector specificity, and was serologically unrelated to both BYV and BWYV (10,11,29). BYSV causes twisting and stunting in beet and yellowing, severe stunting, and eventual collapse in lettuce. The principal vector of BYSV, the aphid *Hyperomyzus lactucae* (Linnaeus), preferentially feeds on sowthistle, which is a widespread weed in California and the main natural reservoir of BYSV. At times of heavy aphid infestations, *Myzus persicae* (Sulzer), a less efficient BYSV vector, visits weeds adjacent to commercial crops, causing severe disease outbreaks and substantial losses in beet and lettuce. The virus was reported to occur in California, Oregon, and Washington (10,12); it also was found in England (35).

BYSV has flexuous, thread-like virions approximately 1,400 nm long composed of a single RNA of about 18 kb encapsidated with a coat protein (CP) of 23.7 kDa (12,18,19,29). Based on virion morphology, cytopathology, and transmission characteristics, BYSV has been classified among definitive closteroviruses (3,4,11,12). The virus is transmitted by aphids in a semipersistent manner;

attempts to transmit BYSV by mechanical inoculation were unsuccessful (cf. 12). Until now, studies on BYSV incidence and epidemiology have been hampered due to the lack of efficient detection tools. Like other closteroviruses, BYSV is a phloem-associated virus (13), and its concentration in infected plants is low. Purification of BYSV and subsequent production of diagnostic antisera has proven to be difficult, resulting in antisera that cross-react with host proteins and, thus, are not satisfactory for reliable virus detection. However, methods of molecular genetics have overcome these difficulties for other closteroviruses, e.g., BYV (2), citrus tristeza virus (CTV) (26,28), sweet potato chlorotic stunt virus (16), and grapevine leafroll virus-3 (24), by using bacterially expressed virus antigens for antisera production.

Recently, we cloned and sequenced approximately 10.5 kb of the BYSV genome (18,19) and, based on amino acid sequence analysis, identified an open reading frame (ORF) encoding the BYSV CP (19). We initiated this work to characterize the CP gene and to express the BYSV CP antigen in bacteria for subsequent production of BYSV-specific antisera suitable for reliable, enzyme-linked immunosorbent assay (ELISA)-based virus detection.

MATERIALS AND METHODS

Virus source and purification of double-stranded RNAs (dsRNAs). A California isolate of BYSV described previously (9, 18,19,29) was maintained in sowthistle (*Sonchus oleraceus* L.) or in lettuce (*Lactuca sativa* L.) in Salinas, CA, under greenhouse conditions. Inoculations were performed with *H. lactucae* aphids as described previously (10). dsRNAs from BYSV-infected sowthistle plants were isolated by the method of Valverde et al. (32) and analyzed in agarose gels as described previously (18).

Mapping of the 5' terminus of the CP subgenomic RNA (sgRNA) and cloning of the CP gene. The 5'-untranslated region (UTR) of the CP sgRNA was cloned using reverse-transcription polymerase chain reaction (RT-PCR) amplification and the poly-

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adenylated dsRNA as a template, essentially as described for the p20 sgRNA of CTV (17). Briefly, total dsRNAs isolated from BYSV-infected sowthistle plants were polyadenylated using yeast poly(A)-polymerase (United States Biochemical Corp., Cleveland) as described previously (18). The polyadenylated RNA was used for the first strand cDNA synthesis with Superscript II reverse transcriptase (BRL, Gaithersburg, MD). The resulting cDNA was used for amplification with the oligo(dT) containing primer M111 (Table 1) (18) and the specific minus-sense primer M190 (Table 1), which is complementary to an internal region of the BYSV CP gene 300 nucleotides (nt) downstream of the AUG codon. The amplification profile included 1 cycle of 94°C for 2 min; 2 cycles of 94°C for 1 min, 37°C for 1 min, and 72°C for 1 min; 30 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min; and a final extension cycle of 72°C for 10 min. The amplified DNA products were blunt-ended with T4 DNA polymerase, phosphorylated with T4 polynucleotide kinase, digested with *Xho*I restriction enzyme, and resolved in low-melting-point agarose gels. The DNA products were extracted from the agarose gels and ligated into *Xho*I/*Eco*RV-digested pBluescript SK (Stratagene Inc., La Jolla, CA), resulting in the clone pYS-34 (data not shown). Transformation into competent DH5 α cells (BRL), DNA manipulation, and sequencing were performed as described previously (18).

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size was revealed in bacterial extracts analyzed by electrophoresis; control extracts from cells carrying the original pMAL-c2 vector did not have this protein band (data not shown). The resulting 67-kDa fusion protein was purified from *E. coli* extracts by affinity column chromatography using amylose resin and analyzed by immunoblotting using BYSV-specific antiserum raised against partially purified BYSV virions. When electroblotted onto nitrocellulose membranes and probed with anti-BYSV serum, this 67-kDa fusion protein reacted strongly, while a similar fusion protein containing the CP of CTV did not (Fig. 2). This apparent strong serological reactivity toward the BYSV-specific antiserum confirmed our original identification of the ORF7-encoded protein product as the BYSV CP (19).

Mapping of the 5' terminus of the CP sgRNA. RT-PCR performed on the polyadenylated BYSV dsRNA using primers M111 and M190 produced a single abundant amplification product of approximately 350 bp (data not shown). This PCR product was cloned into the pBluescript SK vector, sequenced, and found to span the 5'-terminal portion of the CP gene with a 66-nt-long UTR upstream of the initiator AUG codon (Fig. 3). This presumed 5'-UTR of the CP sgRNA started with an A, like the 5'-UTRs of sgRNAs mapped in two other closteroviruses, BYV and CTV (2, 17). Also, like the 5'-UTRs of other closteroviruses, it was very AT-rich (41 of 66 nt were either A or T).

Bacterial expression of the BYSV CP and production of antisera. The BYSV CP constructs described were designed to produce a full-length CP and a CPn (Fig. 1). Both recombinant proteins were used to immunize two different animal species,

rabbit and guinea pig, in order to have a set of antisera suitable for the I-DAS-ELISA format, i.e., for antigen trapping and subsequent antigen detection.

The antisera obtained were tested in immunoblotting against proteins from BYSV-infected sowthistle and lettuce plants (Fig. 4). Both antisera proved to be highly BYSV-specific, with titers in immunoblots exceeding 1×10^5 for both the anti-BYSV CPn from guinea pig and for the anti-BYSV CP from rabbit. The antisera to bacterially expressed proteins demonstrated low reactivity to normal plant proteins, resulting in a very low background in immunoblots (Fig. 4A and B). Interestingly, the approximately 24-kDa BYSV CP in infected lettuce leaf tissue was detected as a pair of very close bands of approximately equal abundance (Fig. 4). In sowthistle tissue, the upper band was barely visible, while the lower band was abundant (Fig. 4, lanes 1 to 3). High specific reactivity of both antisera towards BYSV-infected plants indicated their potential suitability for ELISA-based BYSV detection.

Development of an ELISA-based BYSV detection system. To develop an I-DAS-ELISA, we tested both rabbit and guinea pig antisera for their trapping abilities, since both antisera seemed to be satisfactory when used as intermediate detecting antibodies in immunoblotting (Fig. 4). The rabbit anti-CP serum was found to efficiently capture the virus from BYSV-infected lettuce when diluted $1:10^4$. The anti-CPn guinea pig serum, when used as a secondary detecting antibody, detected BYSV infection in lettuce sap at up to a $1:5,000$ dilution (Fig. 5). The optimal working dilution of the guinea pig antiserum was found to be $1:10,000$. In the reverse combination, use of the guinea pig antiserum as primary trapping antibody and the rabbit antiserum as secondary detecting antibody resulted in high background and an insufficient difference between infected and healthy plants (data not shown). We believe that the most plausible explanation might be competition for the same epitopes between these two antisera. An important factor for successful I-DAS-ELISA detection was found to be relatively high dilutions of antisera used for trapping as well as for detection ($1:10,000$ in this case).

Fig. 2. Immunoblot analysis of the beet yellow stunt virus (BYSV) (lane 1) and citrus tristeza virus (CTV) (lane 2) coat proteins expressed in *Escherichia coli* as 67-kDa fusion constructs and separated by electrophoresis in 8 to 20% gradient polyacrylamide gels. Proteins were transferred onto nitrocellulose membranes and probed with **A**, anti-BYSV serum (dilution $1:2,000$) or **B**, anti-CTV serum (dilution $1:20,000$). Lanes 1 and 3, 2 μ g of the BYSV fusion protein; and lanes 2 and 4, 2 μ g of the CTV fusion protein. Positions of marker proteins are indicated.

Fig. 3. Sequence analysis of the 5'-untranslated region for the beet yellow stunt virus (BYSV) 1.9-kb subgenomic (sg) RNA upstream of open reading frame 7. The presented sequence of the pYS-34 clone corresponds to the plus-strand of the BYSV genome. The 5' terminal nucleotide of the sgRNA is indicated by the arrow. Reading (5' to 3') is from the bottom to the top.

DISCUSSION

The genome of BYSV contains at least nine ORFs that code for proteins ranging from 6 to 66 kDa (18,19). Of these putative BYSV proteins, only the CP has been experimentally assigned a specific structural function (29). Based on amino acid sequence comparisons, the CP was identified as a protein product of ORF7, which was separated from the 3' terminus of the BYSV genome by two downstream ORFs (19).

In closterovirus genomes, the CP gene is located internally and is normally expressed via translation of the corresponding sgRNA (7,8). The size of the CP-encoding sgRNAs varies in monopartite closteroviruses, from 1.6 kb in BYV (2,14) to 3.2 kb in CTV (15). In bipartite closteroviruses, the CP-encoding sgRNAs are about 3 kb (20,21,34). Mapping the 5' terminus of the BYSV CP-encoding sgRNA 66 nt upstream of the respective start codon (Fig. 3) gives the exact size of the sgRNA as 1,864 nt based on the published BYSV sequence (19). The 5'-UTR of the BYSV CP-encoding sgRNA is colinear to the BYSV genomic sequence upstream of ORF7 (Fig. 3) (19). This colinearity confirms our previous conclusion that closterovirus transcription follows an alphavirus-like mechanism (17).

The 5'-UTR of the BYSV CP sgRNA is 14 nt longer than the respective 5'-UTR of the BYV CP sgRNA, which is 52 nt long (2). Inspection of the intergenic region between ORF6 and ORF7 of BYSV (104 nt) (19) and comparison with the corresponding intergenic region between ORF5 and ORF6 of BYV (72 nt) (1) revealed no statistically significant conservation of the nucleotide sequence (data not shown). Even the overall base composition of the BYV and BYSV intergenic regions differs significantly, 71% A+T in BYV and 58% A+T in BYSV (1,19). Nevertheless, in both BYV- and BYSV-infected plants, the CP-encoding sgRNA is the most abundant sgRNA species (7,8,14,18,29), which may in-

Fig. 5. Indirect double-antibody sandwich enzyme-linked immunosorbent assay detection of beet yellow stunt virus (BYSV) in infected lettuce extracts. The rabbit anti-coat protein (CP) serum was used for coating (dilution 1:10,000), and the guinea pig anti-N-terminal half of the CP serum was used for detection as an intermediate antibody (dilution 1:10,000). Each measurement is an average of the readings from two wells. ● = BYSV-infected lettuce; ○ = healthy lettuce.

Fig. 4. Immunoblot analysis of the total proteins extracted from individual beet yellow stunt virus (BYSV)-infected or healthy plants. The denatured proteins from the plant extracts were separated by electrophoresis in 8 to 20% gradient polyacrylamide gels, transferred onto nitrocellulose membranes, and probed with **A**, the guinea pig anti-N-terminal half of the coat protein (CP) serum (dilution 1:20,000) or **B**, with the rabbit anti-CP serum (dilution 1:10,000). Samples from sowthistle or lettuce plants are marked above the gel, numbers indicate individual BYSV-infected sowthistle plants, healthy controls are designated by the letter H. The BYSV-infected lettuce sample is designated by the letter I; Positions of the BYSV CP and marker proteins are indicated.

dicating similar regulation of CP expression. In other monopartite (e.g., CTV [15,25]) and bipartite (e.g., lettuce infectious yellows virus [20]) closteroviruses having additional ORFs downstream of the CP gene, the CP-encoding sgRNA may not necessarily be the most abundant sgRNA species. This could reflect the larger size of the respective sgRNA and the increased number of genes between the CP gene and the 3' end of the genome (cf. 6).

Symptoms of BYSV as well as other closteroviruses in the field are relatively nonspecific and can be easily confused with insect damage, nutritional deficiencies, or premature senescence, and, thus, laboratory methods are usually required for reliable diagnosis (11, 23,33). On the other hand, the strong association of closteroviruses with phloem tissue hampers purification of the viral antigens necessary for antisera production. Viral antigens purified from infected plant tissue are often contaminated with host proteins, which may result in high background reactivity of the diagnostic antisera toward plant host antigens. Recent progress in molecular genetics has made it possible to produce closteroviral CPs in bacteria, thus overcoming shortages of virus-specific antigens and potential contamination with plant proteins (2,16,24,26). Here, we presented data on the expression of BYSV CP antigen in bacterial cells, use of this antigen for antisera production, and development of an efficient immunodiagnostic assay of BYSV in lettuce. Using bacterially expressed constructs of BYSV ORF7, we have experimentally identified ORF7 as the BYSV CP gene and thus confirmed our previous computer-assisted CP identification (19). In spite of the BYSV phloem tropism, the amount of virus antigen present in infected lettuce 3 weeks postinfection is apparently quite sufficient for detection in the I-DAS-ELISA format. BYSV was successfully detected in lettuce extracts diluted up to 5,000 times, and this diagnostic system might be used for routine field detection.

The double bands of the CP closely migrating during electrophoresis, characteristic of BYSV infection in lettuce and to a lesser extent in sowthistle, can be interpreted either as products of proteolytic degradation or posttranslational modifications. Interestingly, BYSV virions purified from *Chenopodium capitatum* plants were demonstrated to contain a single immunoreactive CP species migrating as a single band in SDS-polyacrylamide gel electrophoresis (29). A similar situation has also been shown for BYV. In BYV-infected beet, New Zealand spinach, and *Stellaria media* plants, the CP was detected as a double band, while BYV virions purified from the same plants contained a single species of the CP (30; A. V. Karasev, unpublished data).

In the infected plant, the CP is the most abundant viral protein, and the CP gene is apparently the most highly expressed virus gene. Characterization of CP gene expression not only provides important information on BYSV replication mechanisms, but also helps to create new tools for virus detection. Here, we have shown that production of antisera against inexpensive, bacterially expressed BYSV antigens allows development of highly sensitive and reliable ELISA-based detection systems suitable for routine virus diagnostics. This approach will undoubtedly be of particular use for immunodetection of many other phloem-limited viruses.

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