Characterization of a New Potyvirus Naturally Infecting Chickpea

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ABSTRACT

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During the 1999 to 2001 growing seasons, symptoms consisting of mosaic, stunting, yellowing, wilting, shortening of internodes, and phloem discoloration were observed in chickpea (*Cicer arietinum*) grown in the Department of Chuquisaca in southern Bolivia. In some fields, approximately 10% of the plants exhibited viruslike symptoms and suffered greatly reduced seed yields. Lentil (*Lens culinaris*) was also observed to be infected but not pea (*Pisum sativum*) or faba bean (*Vicia faba*) growing in nearby fields. Infected chickpea tissue reacted positively to the potyvirus group-specific monoclonal antibody (MAb), but there was no serological reaction with antisera to the potyviruses *Bean yellow mosaic virus*, *Clover yellow vein virus*, *Cowpea aphid-borne mosaic virus*, *Pea seedborne mosaic virus*, *Bean common mosaic virus*, or *Bean common mosaic necrosis virus*. Western blots of total protein extracts probed with the potyvirus MAb revealed a single band ca. 32 kDa. Comparative sequence analysis of cDNA clones generated from the putative coat protein gene consisted of 282 amino acids (31.9 kDa) and showed moderate identities of 67, 66, 63, 63, and 61% with the coat proteins of potyviruses *Pepper severe mosaic virus*, *Pepper yellow mosaic virus*, *Potato virus Y*, *Plum pox virus*, and *Pepper mottle virus*, respectively. Phylogenetic analysis of the coat protein amino acid sequence revealed that this virus is a unique member of the family *Potyviridae* and is phylogenetically most closely related to a group of Solanaceae-infecting potyviruses rather than to other legume-infecting potyviruses. The proposed name for the new causal agent is Chickpea yellow mosaic virus.

Chickpeas (*Cicer arietinum* L.) have been cultivated in different regions of Bolivia for several hundred years. They are an important cash crop for small area farmers in the highlands (2,400 to 3,000 m above sea level) of the Department of Chuquisaca in southern Bolivia. In March to May 1999 to 2001, unusual viruslike symptoms were observed in local chickpea land races in farmers' fields around Escana, Santa Rosalia, Sucre, and Yotala. Symptoms consisting of yellowing and stunting also were observed in lentils (*Lens culinaris* Medik.) growing in Sucre and Yotala. From inoculation studies performed in Sucre, a virus was presumed to be the

causal agent of symptoms observed in lentil, and it appeared to be identical to the pathogen infecting chickpea. No virus symptoms were observed in pea (*Pisum sativum* L.) or faba bean (*Vicia faba* L.) growing adjacent to virus-infected chickpea or lentil. Symptoms on virus-infected chickpeas consisted of mosaic, stunting, yellowing, wilting, shortening of the internodes, and phloem discoloration. In some fields, approximately 10% of the plants exhibited the characteristic virus symptoms. Pod formation and seed yields from infected plants were greatly reduced, and seeds were often small, deformed, and discolored. In addition to local land races, virus symptoms were observed in chickpea cultivars and lines originating from the United States and the International Center for Agricultural Research in the Dry Areas (ICARDA), Syria, that were planted in observation trials in Sucre and Yotala. The U.S. cultivars consisted of Dwelley, Evans, Myles, and Sanford, and ICARDA lines included ILC 72 and ILC 3279. Lentil, pea, and chickpea infected by mechanical inoculation from symptomatic chickpea at the laboratory in Sucre further suggested that a viral agent was the cause of the disease observed in the field. Symptoms observed were unlike symptoms described for any of at least 22 different viruses reported in chickpea (4,12,13,15,17,18,21). The

objective of this study was to characterize the causal agent on the basis of host range, symptomatology, serological relationships, and nucleotide sequence. The name Chickpea yellow mosaic virus (CpYMV) is proposed.

MATERIALS AND METHODS

Virus source, host range, and seed transmission. Symptomatic chickpea plants were collected from the field and inoculated to *Chenopodium amaranticolor* Costa & Reyne and *C. quinoa* Willd. Inoculation buffer was 50 mM potassium phosphate, pH 7.4, containing 20 mM sodium sulfite. Diatomaceous earth was included as an abrasive. Local lesions appeared in 5 to 7 days after inoculation, and three serial transfers from *C. quinoa* were made subsequently. The virus was then inoculated to and maintained in pea for additional studies. Thirty-two plant species were selected for the host range study.

Seed transmission assays were conducted using seed collected from infected pea cultivar 8221 and chickpea cultivar Dwelley mechanically inoculated with the virus. Plants were grown to maturity, and the seed was then collected from mature pods. Plants from the collected seed were allowed to grow for 10 to 14 days and then tested individually by enzyme-linked immunosorbent assay (ELISA).

Serology, virus purification, viral coat protein, and nucleic acid analysis. Preliminary examination of leaf-dip preparations with an electron microscope revealed long slightly flexuous particles ca. 760 nm in length determined by internal size standards. As a result of these observations, pea and chickpea plants infected with the virus were evaluated by ELISA using the universal potyvirus group-specific monoclonal antibody (Agdia, Inc., Elkhart, IN), and antisera were elicited with the following members of the family *Potyviridae*: *Bean common mosaic virus* (BCMV), *Bean common mosaic necrosis virus* (BCMNV), *Bean yellow mosaic virus* (BYMV), *Clover yellow vein virus* (CYVV), *Cowpea aphid-borne mosaic virus* (CABMV), and *Pea seedborne mosaic virus* (PSBMV). In addition, tissue samples were tested for carlaviruses *Pea streak virus* (PeSV) and *Red clover vein mosaic virus* (RCVMV), and potexviruses *Clover yellow mosaic virus* (CYMV) and *White clover mosaic virus* (WCMV).

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The virus was purified from approximately 50 g of infected pea tissue collected 19 to 21 days postinoculation using a modified procedure of Veerisetty and Brakke (27). The final sucrose gradient described in their procedure was eliminated and replaced with a cesium gradient. The partially purified virus was adjusted to 40 mg/μl (wt/vol) cesium chloride and centrifuged for 15 to 18 h at 125,000 × *g* at 12°C. The light scattering band was collected, diluted in resuspension buffer (16.5 mM Na₂HPO₄ and 1.8 mM sodium citrate, pH 8.0), and centrifuged for 1 h at 250,000 × *g*.

Total protein preparations for Western blot analysis were made from inoculated chickpea, pea, and lentil exhibiting symptoms of the virus. Tissue was macerated in 150 mM Tris-HCl, pH 6.8, containing 2% sodium dodecyl sulfate, 5% 2-mercaptoethanol, and 10% glycerol. Samples were boiled for 5 min, chilled on ice, and then centrifuged for 5 min at 12,000 × *g*. Sample preparations were resolved on 12% discontinuous sodium dodecyl sulfate (SDS) polyacrylamide gels (20) and then electroblotted onto 0.45-μm nitrocellulose membranes (26). The blots were probed overnight at room temperature using the potyvirus group-specific monoclonal antibody at a dilution rate of 1:1,000. The blots were then washed and probed with goat anti-mouse alkaline phosphatase (Bio-Rad, Hercules, CA) at a 1:2,000 dilution, washed after 1 h, and then visualized with nitro blue tetrazolium and 5-bromo-4-chloro-3-indoyl phosphate substrate (Sigma Chemical Co., St. Louis, MO) (3). Protein molecular weight was determined by the use of low-molecular-weight prestained protein standards (Bio-Rad, Hercules, CA). Protein samples from purified virions were prepared in a similar manner. Protein bands in polyacrylamide gel electrophoresis (PAGE) gels were visualized after staining with a 2% aqueous solution of silver nitrate. Molecular weight was determined by use of molecular weight standards (Sigma).

RNA was extracted from purified virions (1 mg/ml) in 260 μl of resuspension buffer by the addition of 3 μl of 0.1 M dithiothreitol, 7 μl of proteinase K (10 mg/ml), and 30 μl of 10% SDS. Extracts were incubated 30 min at 37°C. The proteinase K was denatured using an equal volume of Tris-saturated phenol (pH 8.0) and chloroform. After separation by centrifugation, the viral nucleic acid was concentrated by ethanol precipitation.

The molecular size of the viral RNA was determined by agarose gel electrophoresis after denaturation with glyoxal and dimethylsulfoxide (DMSO) (23). RNA was electrophoresed in 1.2% agarose using 10 mM sodium phosphate buffer, pH 6.8. A 0.24- to 9.5-kb RNA ladder (Gibco, Gaithersburg, MD) was used for molecular size standards. Following electrophoresis, glyoxal was removed from gels by agitation for 15 min in TAE buffer (40 mM

Tris, pH 8.0, 20 mM sodium acetate, 1 mM EDTA). Gels were stained with ethidium bromide and observed on a transilluminator at 300 nM. Images were recorded and stored using an AlphaImager 2000 (Alpha Innotech, San Leandro, CA).

Electron microscopy. Virus samples were prepared from leaf dip preparations or purified virions. Samples were applied to carbon-coated Formvar 300-mesh grids and stained with an aqueous solution of 2% uranyl acetate. Preparations were observed using a JEOL 1010 transmission electron microscope. Particle lengths were determined using an internal size standard.

Synthesis of cDNA. First-strand cDNA synthesis was carried out using the method described previously by Wyatt et al. (28). Second-strand synthesis was performed essentially using the procedure of Gubler and Hoffman (6). Double-stranded cDNA was blunt-end ligated into *EcoRV*-digested pBluescript II SK+ vector. Plasmids were transformed into *E. coli* DH10β competent cells. Cells were grown on a selective medium containing X-Gal and IPTG as described by Sambrook et al. (25). White colonies containing plasmids with inserts of at least 2 kb in size, as determined by restriction enzyme mapping, were amplified in Luria Broth media containing 50 μg/μl ampicillin. The plasmids were isolated by alkaline lysis (25) and the double-stranded DNA prepared for sequencing. Sequence reactions of the forward and

complementary strands were analyzed using an automated dye termination system (ABI, Inc.). Sequence data were further analyzed using Align Plus (Scientific and Educational Software, Cary, NC). Coat protein sequences of all members of the *Potyviridae* for which these data are available were aligned using PILEUP (Accelrys, San Diego, CA). Based on a preliminary phylogenetic analysis using the neighbor-joining method, to reduce computing requirements the 25 taxa closely related to CpYMV were chosen for more detailed phylogenetic analysis, paying particular attention to legume-infecting members of the group. In order to further simplify the analysis, only one member of a subgroup (e.g., the BCMV subgroup of the genus *Potyvirus*) was used. A Bayesian inference of phylogenetic relationships was done using the software MrBayes (Huelsenbeck and Ronquist: <http://morphbank.ebc.uu.se/mrbayes/>). The log likelihood sum reached a plateau after about 3,000 generations, and data from 7,000 additional generations were collected. A consensus tree was calculated, as were clade probabilities. To verify the robustness of the inference, the multiple sequence alignment was also analyzed using maximum parsimony.

RESULTS

Virus purification. The virus preparation after centrifugation in cesium chloride resulted in two light-scattering zones at 2.5

Fig. 1. Mosaic symptoms on leaves of chickpea cv. Dwelley infected with Chickpea yellow mosaic virus.

and 3.6 mm below the meniscus. The upper band was a nonfluorescing zone and was determined to be a nonviral contaminant as determined by observation using electron microscopy. The fluorescent zone at 3.6 mm consisted of flexuous rod-shaped particles and averaged 2.48 OD₂₆₀

units for 50 g of tissue. The average A_{260}/A_{280} ratio was 1.34.

Host range. The virus induced systemic infection in several legume species, including *Cicer arietinum*, *Lens culinaris*, *Medicago sativa* L., and *Pisum sativum*. Local lesions occurred without systemic spread

in *Chenopodium quinoa*, *C. amaranticolor*, and *Phaseolus vulgaris* L. Symptoms in pea included pronounced mosaic, mottle, and greening of veins. Symptoms on chickpea were consistent with those observed naturally infected in the field (Fig. 1). No stunting of plants that occurred in

Fig. 2. **A**, Western blots of Chickpea yellow mosaic virus probed with the potyvirus group-specific monoclonal antibody. Lane M: prestained protein molecular weight marker, lane 1: nucleoprotein prepared from purified virions, lane 2: total protein preparation from chickpea (*Cicer arietinum*) cv. Dwelley, lane 3: total protein preparation from pea (*Pisum sativum*) cv. Dark Skin Perfection, lane 4: healthy chickpea. **B**, Silver-stained virus protein preparations of Chickpea yellow mosaic virus purified from pea and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Lane M: protein molecular weight marker, lane Vp: virus protein.

CpYMV-CP.AA	(1)	ADQQRAELDAGAQREKKEKDKDKDKNTSTQSGSSDQGKALTTTNRDRDVN
PF00767.AA	(1)	-----RDRDVN
CpYMV-CP.AA	(51)	AGTSGPFSIPRIKAMTSKLNIPRIKGKEVVNLDHLLIYEPKQVDLSNTRA
PF00767.AA	(7)	AGTSGTFSVPRLKKISAKMRLPKVKGKAVLNLDHLLKYKPDQVDISNTRA
CpYMV-CP.AA	(101)	THSQFEFWHTAVCEGYGIQESDSQVIMNGLMVWCIENGTSPTNNGNWTMM
PF00767.AA	(57)	TQEQFNTWYEGVKEDYDVTDEQMSIILNGLMVWCIENGTSPTNNGTWTMM
CpYMV-CP.AA	(151)	DGEEQVEYPLKIVIENAKPTFRQIMAWFSDLAEEYIEKRNAKESYMPRYG
PF00767.AA	(107)	DGEEQVEYPLKPIIENAKPTLRQIMAHFSDAAEAYIEMRNSEKPYMPRYG
CpYMV-CP.AA	(201)	QIRNLRDKSLARYAFDFYEITSKTPDRAREAHLQMKAAALKNTQNKMFGL
PF00767.AA	(157)	LQRNLTDMSLARYAFDFYEITSKTPVRAREAHMQMKAAALRGKQNRFLGL
CpYMV-CP.AA	(251)	DGSVGNAAEESTERHTVEDVTSNMHSMGLGVRNF
PF00767.AA	(207)	DGNVGTQEEDTERHTADDVNRNMHSLGVR--

Fig. 3. Alignment of Chickpea yellow mosaic virus deduced coat protein amino acid sequence (CpYMV-CP.AA) with potyvirus coat protein contained in the GenBank Conserved Domain Database (pfam 00767). Conserved sequence is highlighted in shaded blocks.

chickpea and lentil was observed in pea. Local lesions were noted on inoculated leaves of alfalfa, but systemic symptoms were absent. No symptoms were produced on, and no virus was detected in: *Beta vulgaris* L., *Cassia occidentalis* L., *Cucumis sativus* L., *Cucurbita pepo* L., *Datura tatula* L., *Glycine max* (L.) Merr., *Gomphrena globosa* L., *Lycopersicon esculentum* Mill., *Nicotiana tabacum* 'Occidentalis', *N. tabacum* 'Rustica', *N. tabacum* 'Samsun', *N. tabacum* 'Xanthi', *Trifolium*

pratense L., *T. repens* L., *Trigonella foenum-graecum* L., *Vicia faba*, and *Vigna unguiculata* (L.) Walp.

Serology, viral coat protein, and nucleic acid analysis. Chickpea and pea tissue exhibiting the described symptoms reacted positively in ELISA to the potyvirus monoclonal antibody; however, reactions using antisera elicited with the potyviruses BCMV, BCMNV, BYMV, CYVV, CABMV, and PSbMV were negative. Reactions were also negative when sympto-

matic tissue was tested for PeSV, RCVMV, CYMV, and WCMV.

When total protein sample preparations on Western blots were probed with the potyvirus monoclonal antibody, a single band ca. 32 kDa was observed (Fig. 2). In contrast, two distinct bands ca. 32 and 28 kDa were produced from purified virus preparations (Fig. 2). Similar results were observed when purified virus protein preparations were resolved by SDS-PAGE and silver stained (Fig. 2). Purified virion

Fig. 4. Bayesian phylogenetic inference based on the coat protein amino acid sequence of representative members of the genus *Potyvirus*. Branch lengths are proportional to distance, and the numbers presented on certain branches of the tree reflect clade probabilities, when >50%, indicating the level of support for branches of the tree.

RNA denatured in glyoxal and DMSO resolved as a single band in agarose gels (data not shown). The molecular size was determined to be approximately 9.45 kb.

Seed transmission. Four hundred seed collected from infected pea 'Dark Skin Perfection' and 50 seed from infected chickpea 'Dwelley' were germinated and observed to be symptomless during a 21-day examination period. ELISAs performed 10 days after emergence were negative for the virus when evaluated using the universal potyvirus monoclonal antibody.

cDNA synthesis and sequence analysis.

In cDNA synthesis and cloning experiments, 3'-end cDNA clones of the viral RNA consisted of a poly-A tail 12 to 26 nucleotides in length. The deduced amino acid sequence obtained through the coat protein (GenBank accession no. AF527897) region was consistent with the genome arrangement of the *Potyviridae*. The putative coat protein gene beginning 235 nucleotides upstream from the poly-A tail consisted of 846 nucleotides encoding 282 amino acids.

Analysis of the deduced amino acid sequence at the 5'-region of the coat protein gene revealed a motif similar to several other potyviruses and suggests the potential cleavage site that represents the N-terminus of the gene (Fig. 3). The N-terminus begins with an alanine residue that is preceded by the probable NIB/CP cleavage site VQHQ as identified by analogy to consensus sequences and genome arrangement in other potyviruses. The sequence DAG that is typically associated with aphid transmissible potyviruses (2) is located eight amino acids downstream from the coat protein N-terminus. An 11-aa sequence RDRDVNAGTSG that is highly conserved with other coat proteins within the *Potyviridae* (GenBank PF00767) was identified using BLAST (1), and begins 45 amino acids downstream from the putative N-terminus of the coat protein (Fig. 3). The 3'-end sequence LGVRNF occurs immediately prior to a TAA ochre codon. The estimated molecular weight of the putative coat protein is 31,953.

The coat protein amino acid sequence was analyzed for phylogenetic relationships with other members of the *Potyviridae*. Topologies of trees predicted using maximum parsimony (data not shown) or Bayesian inference were similar (Fig. 4). This analysis indicated that CpYMV was on a distinct branch of the phylogenetic tree, whose clade probabilities were well supported. Other viruses that shared the same branch of the tree were *Potato virus Y*, *Pepper mottle virus*, and other related viruses of the Solanaceae. CpYMV did not show any obvious phylogenetic relationship to legume-infecting viruses such as BCMV or BYMV.

DISCUSSION

Results obtained in this study indicate that CpYMV is a new and unique virus

that affects a limited number of crop plants in the family Leguminosae. The virus was observed to be most severe in naturally infected chickpea fields in the Department of Chuquisaca in Bolivia. It is presently not known if the virus occurs within other countries of South America. Symptom expression on experimentally infected pea was dramatic on leaves, but the virus produced little stunting and had no observed deleterious effects on pod and seed production. Although the sample size examined was relatively low, the virus was not seed-transmitted in pea or chickpea. These results are unusual for a potyvirus that commonly infects legumes. For example, BCMV, BCMNV, BYMV, PSbMV, CABMV, and *Southern bean mosaic virus* are each transmissible by seed from at least one common legume host (5,7,8,11,14,16,19,22). It is possible that CpYMV may be transmitted by seed of alfalfa or lentil, identified as additional systemic hosts of this virus, but these species were not evaluated further. Natural reservoir hosts for the virus are currently unknown. However, because alfalfa is grown as a crop in the Sucre region of Bolivia, it may be considered a perennial host if natural infection in the field can be verified. Although vector transmission studies were not conducted, CpYMV is presumed to be aphid-borne, and a consistent presence of the pea aphid (*Acyrtosiphon pisum*) was observed within infected fields of chickpea in the Chuquisaca region. While not conclusive, these observations provide circumstantial evidence that the pea aphid may be involved in transmission of the virus. Peas growing in areas near chickpea fields where infections were observed did not exhibit symptoms similar to those observed in pea inoculated experimentally. It is possible that the indigenous pea aphid biotype present in Sucre was not able to transmit CpYMV to pea plants growing in the area. There is also the potential that pea lines being grown in the field in Sucre have resistance to the virus through selection by farmers. Local farmers have been growing the same pea lines (land races) for years and usually save their own seed for subsequent planting. Therefore, it is not possible to identify the lines or where they originated.

Comparison of the deduced amino acid sequence of the CpYMV putative coat protein, while clearly a member of the genus *Potyvirus*, has shown that CpYMV is distantly related to all other potyviruses for which coat protein amino acid sequence data are available, with the largest percent identity at 67% with PepSMV. The coat protein sequence is more distantly related to potyviruses primarily affecting legume crops including BYMV (62% identity), BCMV (58%), and PSbMV (57%). The low percentage of identities is consistent with the lack of cross-reactivity with antibodies to these viruses. The predicted coat

protein amino acid molecular weight of 31,953 is also consistent with the ca. 32-kDa band produced on Western blots of total nucleic acid extractions from tissue infected with CpYMV. The additional 28-kDa protein band visualized from Western blots of purified virus preparations is likely a degradation product artifact resulting from proteolysis of the virus coat protein (9,10,24). Based on the presence of the highly conserved potyvirus sequence RDRDVNAGTSG within the coat protein, the positive reaction to the potyvirus monoclonal antibody, particle size and morphology, and coat protein molecular weight, sufficient evidence exists to place CpYMV as a distinct species of the genus *Potyvirus*, family *Potyviridae*.

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