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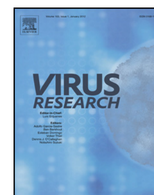

Genotyping of *Cucumber mosaic virus* isolates in western New York State during epidemic years

Characterization of an emergent plant virus population

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ABSTRACT

In the early 2000s an epidemic of cucumber mosaic virus (CMV) spread within the Midwestern and Eastern US affecting snap and dry bean (*Phaseolus vulgaris* L.) cultivation. Fifty one CMV isolates from this period were partially characterized from varied hosts by sequencing a section from each of the three genomic RNAs. Aside from one subgroup II strain from pepper, all isolates, including those from snap bean, fell within the IA subgroup. The nucleotide sequence diversity of virus populations sampled at multiple sites and at different years was significantly higher than that of a population from single site in a single year, although in general the number of polymorphisms was low (<11%). Complementary DNA (cDNA) clones of Bn57, a representative isolate from snap bean, were engineered for the production of infectious in vitro RNA transcripts initiated from a T7 promoter. Infections from these cDNAs resulted in symptoms consistent with those of the original field isolate, indicating that a satellite RNA is not involved in symptom expression in snap bean. These infectious clones were used to assess symptom determinants and the effects of virus infection on plant growth. Inoculations with pseudorecombinants derived from Bn57 and the non-bean infecting strain Fny confirmed RNA2 as a specific determinant for snap bean infection. Bn57, along with almost all isolates identified in this study contained the Y631 locus in the 2a protein, a determinant for systemic infection in bean. The presence of this locus extended to all non-bean hosts except two pepper infecting isolates. Infection by Bn57 in snap bean had a significant effect on pod number and mass with a 55 and 41 percent reduction in greenhouse assays, respectively. To our knowledge Bn57 is the first CMV strain isolated from *P. vulgaris* to be fully sequenced and cloned, providing a useful tool for analyses of CMV-host interactions.

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1. Introduction

Recent decades have seen the emergence of cucumber mosaic virus (CMV) as a serious pathogen of common bean (*Phaseolus vulgaris* L.) in the Great Lakes region of the United States. This sudden increase in virus incidence coincided with the introduction of the soybean aphid (*Aphis glycines*) from Asia (Losey et al., 2002; Ragsdale et al., 2011). Severe virus symptoms were observed initially in the Midwest in 2000, and the incidence of virus diseases increased to the east and south reaching New York (NY) State the following years (Larsen et al., 2002; Shah et al., 2006). Reported field incidence and associated crop loss were sometimes as high as 100% (Larsen et al., 2002; Nault et al., 2006), the impact on yield of CMV infection being both dependent on cultivar (Taylor and Shail, 2006) and plant stage at the time of inoculation (Larsen et al., 2002). The overall total economic cost in NY State alone from 2001 to 2009 was estimated to be more than $10 million (Reiners et al., 2010).

CMV has perhaps the largest host range of any virus with at least 1,241 susceptible species in 101 plant families, including both monocots and dicots. It can be transmitted by over 80 species of aphid in a non-persistent manner (Edwardson and Christie, 1991). The genome of CMV consists of three segments RNA1, RNA2 and RNA3 of approximately 3.3, 3.0 and 2.2 kilonucleotides in length, respectively. RNA1 is monocistronic and encodes an open reading frame (ORF) (1a) containing the replication associated methyltransferase and helicase domains. RNA2 is bistronic with a large

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ORF (2a) containing a RNA-dependent RNA polymerase (RdRp) domain and a small overlapping ORF (2b) encoding a suppressor of RNA silencing. RNA3 is bicistronic: the movement protein (MP) associated ORF separated by the intergenic region (IGR) from the three-prime proximal coat protein (CP) ORF. All five viral proteins have been implicated in movement, but aside from the MP their role is assumed to be indirect (Jacquemond, 2012; Palukaitis and Garcia-Arenal, 2003). Phylogenetically, CMV can be subdivided into two main subgroups I and II which share 70–75% nucleotide identity and as such they taxonomically approximate two separate species. Subgroup I can be further subdivided into IA and IB; the latter, being predominantly Asian in distribution (Jacquemond, 2012). Natural admixtures between these different subgroups usually occur infrequently in the form of reassortants and recombinants (Bonnet et al., 2005; Fraile et al., 1997; Kim et al., 2014; Lin et al., 2004; Maoka et al., 2010; Nouri et al., 2014).

In this study we describe the collection from Upstate NY of 51CMV isolates collected during and immediately after the epidemic years of the early 2000s. Using partial sequences from each genomic RNA segment we analyzed the evolutionary trends underlying the virus population. From these data we identified a typical virus variant (Bn57) from snap bean and produced infectious clones for which infection in snap bean was partly characterized.

2. Materials and methods

2.1. Plant sampling

Plants were collected over a five year period (2009–2014) sampling in and around cultivated bean fields located in the NY counties of Ontario, Orleans, Steuben and Tompkins (Fig. S1). In late July, 2009, 80 bean samples were collected from a single field displaying virus symptoms located in Geneva (Ontario County) NY. A structured sampling was undertaken, irrespective of whether the plants showed symptoms. The field was divided into four sections; samples were taken every ten plants in every other row moving diagonally across the field. In total twenty bean samples per section were collected. Weed samples were also collected in or next to the same field including one mayweed (*Anthemis arvensis*) plant (Mayweed-3). The same field was again surveyed in July, 2010, and while this time no symptomatic bean infection was noted, a symptomatic horsenettle (*Solanum carolinense*) plant (Horsenettle-1) was collected from the center of the field. Also in 2009 a collection was made of bean plants displaying symptoms in various fields in Steuben County. The following year, symptomatic plants were collected from various fields in Ontario County. In 2010 and four years later, additional cultivated plants showing virus-like symptoms (and shown to be infected by CMV) were collected. Archival material in the form of frozen or lyophilized leaf tissue with CMV either from bean or from summer squash growing in NY was also included in the analyses. The only non-NY sample was an archival bean collected from Wisconsin. This was included as a representative sample of an isolate of CMV from the early 2000s where the disease in bean was first noted (Fig. S1).

2.2. ELISA

CMV infection for all samples was tested using either a plate trapped antibody-enzyme linked immunosorbent assay (PTA-ELISA (Mowat and Dawson, 1987)) or a triple antibody sandwich-enzyme linked immunosorbent assay (TAS-ELISA; Agdia Inc., Elkhart, IN). PTA-ELISA was carried out using an in-house anti-CMV polyclonal antibody. TAS-ELISA was carried out using commercial reagents according to the manufacturer's instructions. Each assay included two healthy bean controls, one known CMV-infected bean positive control, and one purified CMV virion positive control. Assays were considered positive if OD405 nm values were three or more times that of the healthy control and greater than 0.1.

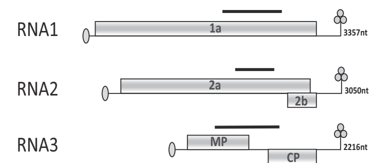

Fig. 1. The relative positions (black bars) of sequences on each genomic RNA (RNAs 1, 2 and 3) (Fny strain as reference) analyzed. RNA1, nucleotide (nt) positions 1895-2572; RNA2, 1577-1995; and RNA3, 454-1349. Open reading frames–gray shaded boxes. MP–movement protein, CP–capsid protein. Length of each RNA in nt shown on right. Oval – 5′ cap structure, cloverleaf – tRNA-like structure.

2.3. RNA extraction, RT-PCR and IC-RT-PCR

RNA extraction and sample storage. RNA extraction was done with the Qiagen (Valencia, CA) RNeasy Plant Mini Kit. Buffer RTL (Qiagen, Hilden, Germany) was used as the lysis buffer and extractions were eluted with 30 μl RNase-free water. RNA extracts were stored at −70 °C. Infection in all ELISA-positive samples was initially confirmed using the universal detection primers p766 (5′-GCCCCTTACTTTCTCATGGATGC-3′) and p767 (5′-GACTGACCATTTTAGCCGT-3′) in combination with internal control primers AtropaNad2.1a and AtropaNad2.2b (Thompson et al., 2003) (Table. S1). Sequencing primer pairs (F1 and R1, F2 and R2, and F3 and R3) (Integrated DNA Technologies, Coralville, IA) were designed to flank variable regions of interest in each of the genomic RNAs (Fig. 1). Their position was chosen based on conserved positions in sequence alignments between representatives of CMV subgroups IA, IB, and II; strains Fny (Acc. no. RNA1, D00356; RNA2, D00355; and RNA3, D10538), NT9 (Acc. no. RNA1, D28778; RNA2, D28779; and RNA3, D28780), and Q (Acc. no. RNA1, X02733; RNA2, X00985; and RNA3, J02059), respectively. Reverse transcription (RT) was carried out using random hexamers (Promega, Madison, WI) and Superscript III (Life Technologies, Grand Island, NY) following the manufacturer's directions. PCR was done using cloned *Pfu* DNA polymerase AD (Agilent Technologies, Santa Clara, CA) according to the manufacturer's instructions. An immunocapture RT-PCR (IC-RT-PCR) was used to amplify the RNA of archival lyophilized samples. Plant material was prepared in 2.0 ml tubes. 0.2 g of each sample was flash frozen in liquid nitrogen and pulverized with a pestle. 300 μl PBS + 0.1 % Tween® was added to each tube and the tubes then centrifuged at 4 °C for 3 min. CMV in-house polyclonal antibody (1:500 dilution in coating buffer (0.1 M sodium carbonate, pH 9.5)) was used to coat 0.3 ml reaction tubes for 2 h at room temperature. Tubes were then washed with PBS + 0.1% Tween® and distilled water. 100 μl of the supernatant from each plant preparation was added to each tube and incubated at 4 °C overnight. Following incubation, the tubes were washed with PBS-Tween® and distilled water. Thermocycler conditions for all PCRs were: 1 cycle of 94 °C for 5 min, 34 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 5 min, and 1 cycle of 72 °C for 10 min.

2.4. Nucleic acid sequence analysis

PCR amplicons derived from primer pairs F1 and R1, F2 and R2, and F3 and R3 were sequenced at the Cornell core facility in both directions and twice using the same primers used in PCR. Contigs were assembled using the Vector NTI® suite (Life Technologies, Grand Island, NY). Trimmed and edited sequences were aligned with T-Coffee (Tommaso et al., 2011). Substitution model selection and neighbor-joining, maximum likelihood (PhyML) (Guindon et al., 2010) and Bayesian (MrBayes) (Ronquist et al., 2012) inferred

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