PROCEEDINGS

OF THE

33rd ANNUAL MEETING

6-12 July 1997

Proceedings Edited
by
Nelson Semidey and Lucas N. Aviles

Published by the Caribbean Food Crops Society

Proceedings of the Caribbean Food Crops Society. 33:366-372. 1997

BIOLOGICAL AND MOLECULAR CHARACTERISTICS OF TOMATO-INFECTING GEMINIVIRUSES TRANSMITTED BY THE Bemisia tabaci (GENN.) SPECIES COMPLEX IN PUERTO RICO

J.K. Brown[1], M. Sosa[2], and J. Bird[2]. [1]Dept. of Plant Science, University of Arizona, Tucson, AZ 85721; [2]College of Agricultural Sciences, Agri. Exp. Station, Rio Piedras, P.R. 00928.

ABSTRACT. Whitefly-transmitted geminiviruses (Subgroup III, Geminiviridae) have long recognized as pathogens of weed species and bean crops in Puerto Rico, and were recently shown to cause diseases of tomato. At least five symptoms phenotypes were associated with infected tomato in P.R. during 1991-1996. Geminivirus etiology was demonstrated by whitefly transmission and polymerase chain reaction (PCR) detection using subgroup III universal PCR primers. At least three distinct genotypes were discerned in infected tomato based upon comparison of key virus sequences with those of well-characterized subgroup III viruses. Each virus genotype has a unique experimental host range and causes differential symptoms in diagnostic test species. The precise relationships between P.R. viruses that are indigenous to weed hosts and those found in bean and tomato are under investigation in order to identify the most economically important viruses to target for resistance efforts, to map their distribution in host plants, and to evaluate the potential for recombination or reassortment in the formation of new and emerging geminiviruses.

INTRODUCTION

Whitefly-transmitted (WFT) geminiviruses have been reached pandemic proportions in food and fiber crops throughout the Caribbean Basin and the southwestern U.S. during the last decade (Bird and Maramorosch, 1978; Brown, 1994; and Brown and Bird, 1992). Although both regions were historically known to have WFT geminiviruses endemic to crop and weed hosts, disease problems have accelerated since the introduction and establishment of the B biotype whitefly vector in 1987-88 (Brown et al., 1995). As a result, newly emerging viruses are prominent in agroecosystems in Arizona and Puerto Rico (Brown et al., 1991; 1995). The goal of this project are to map the distribution of whitefly-transmitted geminiviruses in cultivated and weed species in Puerto Rican agroecosystems using molecular sequences from target regions of the viral genome. From these sequences we will predict the evolutionary histories and hence, determine the genetic relationships of viruses in crop and weed hosts. In this project, an approach was developed in which polymerase chain reaction (PCR) (Saiki et al., 1988) and primers specific to subgroup III, Geminiviridae (Wyatt and Brown, 1996; Idris et al., submitted) were implementes to obtain and investigate geminivirus sequences in an expedient and high resolution manner. This approach permits the tracking of WFT geminiviruses in crop and weed hosts using molecular sequence data. A second objective is to determine the identity and distribution of the wild hosts of geminiviruses that have an economic impact on cultivated crops. In this way, it will be possible to identify the most economically important geminiviruses, and thus, to determine the particular viruses that will be targeted in the development of resistant crop varieties. Disease resistant cultivators can then be developed through combinatorial strategies utilizing host plant genes through plant breeding methods, and by virus-derived resistance in trangenic plants.

Identification of the specific indigenous hosts of WFT geminiviruses that infect cultivated species is also necessary for effective implementation of geminivirus disease control based upon effective crops management practices. Collectively, the control of geminivirus diseases will be possible through implementation of management practices based upon molecular epidemiological information and through the development of resistant varieties. These approaches will lead to less reliance on hard chemistries to control the whitefly vector to reduce virus disease incidence in crop plants, and ultimately, to the sustainable production of quality food crops for local and export markets.

OBJECTIVES

- To utilize polymerase chain reaction (PCR) for routine detection of WFT geminiviruses in Puerto Rico.
- To map the geographic distribution of WFT geminiviruses in agroecosystems in Puerto Rico.
- To predict the evolutionary histories of whitefly-transmitted geminiviruses in weed and cultivated species using molecular sequences from target regions of the viral genome.

MATERIALS AND METHODS

Virus Detection and Cloning by Polymerase Chain Reaction: WFT geminiviruses clones were obtained from DNA extracts of representative tomato and weed isolates by polymerase chain reaction using primers pairs to target three separate regions of viral genomes as described.

Total nucleic acid extraction: Nucleic acids were extracted from symptomatic tomato leaves (12 d post-inoculation) using the method of Doyle (Doyle and Doyle, 1987) with slight modifications. Leaves were ground to power while frozen in liquid nitrogen and immediately suspended into cetyltrimethylammonium bromide (CTAB) buffer. Homogenates was extracted with an equal amount of chloroform: isoamyl alcohol (24:1), and the phases were separated by centrifugation at 9000 x g for 10 min at 4C and the supernatant was precipitated overnight with two thirds vol. isopropyl alcohol. Nucleic acids were pelled by centtrifugation at 9000 x g for min. at 4C, washed with 1 ml wash buffer containing 76% ethanol and 0.2 M sodium acetate, and pelled again by centrifugation. Pellets were dried and resuspended in 10 mM Tris-1mM EDTA (TE) buffer pH 8.0 (1:1) (wt/v).

Polymerase Chain Reaction (PCR), cloning and sequencing of PCR products: Two fragments of the DNA-A and DNA-B genomic components were amplified by the polymerase chain reaction (PCR) (Saiki et al. 1988) using degenerate primers designed to target specific regions of subgroup III (J.K. Brown and S. D. Wyatt, unpublished data). Degenerate PCR primers were designed around conserved regions identified by multiple sequence alignment of the published sequences of representative subgroup III geminiviruses. PCR primers for amplification of the large IR., and flanking sequences on DNA-A were AV1303[5'CGTCCA(A/G)ACTT(G/T)GAA (A/G) TT(G/C)AG3'] and AC515[5'CT(G/T)GGCTT(C/T)CT(A/G) TACA(A/T)GGGC 3']. The coat protein gene

and its flanking sequences were amplified with AC1303[5' CT(G/C) AA(C/T)TT(A/C)AAGT(C/T)TGG ACG 3'] and AV168[5' ATTACCGGATGGCCGC 3']. Primers were synthesized at The Biotechnology Facility at the Univ. of AZ, Tucson.

PCR reaction were performed in a Perkin Elmer DNA Thermal Cycler (Norwaik, CT). The 25 ul reaction mixtures contained 0.5 ul total DNA extracted from infected tomato plants, 2.5 or 3.0 mM $MgCl_2$, 1x reaction buffer (10 mM Tris-HC1 50 mM KC1), 150 uM dNTPs, and 20 pmol of each primer. Tag DNA polymerase was used according to the manufacturer's specification (Perkin Elmer). Reaction mixtures were covered with 40 ul mineral oil to prevent evaporation. DNA amplification was carried out for 30 cycles of denaturalization at 95C for 1 min., primer annealing at 58C for 1 min., primer extension at 27C for 1 min. with a final cycle for 5 min. at 72C, afetr which the temperature was reduced to and held at 4C until samples were removed and electrophoresed. Experiments included a negative PCR control of total nucleic acids extracted from virus-free, healthy tomato leaves, an internal PCR control containing complete PCR reaction mixture without DNA template, but with distilled, autoclaved water, and a positive control to which nucleic acids at 1.0 mg/ml from infected tomato plants were added. PCR products were electrophoresed in 1% agarose minigels in TAE buffer (Tris-acetate-EDTA), ph 5.0. Gels were stained with ethidium bromide and PCR products were viewed by UV transsillumination.

Cloning and sequencing of PCR products: PCR products of the expected sizes were cloned into the plasmid vector (PCR® 2.1) included in the TA Cloning Fit (Invitrogen®, San Diego, CA) per manufacturer's instructions. Fragments containing putative DNA-A and DNA-B IR sequences were identified in several clones by miniprep screening and were confirmed positive for inserts using the respective PCR primers used for initial amplification. The DNA sequences of the cloned inserts were obtained by automated sequencing carried out by the Molecular Genetics Facility, Univ. of Georgia (Athens, GA) using automated Applied Biosystems' 373A (version 2.1.1) DNA Sequencer. Two clones of each viral insert were sequenced in both directions, using the respective PCR primers.

Computer Analysis of Viral DNA: Viral sequences were assemble from multiple DNA sequences in forward and reverse directions with the aid of EditSeg in DNASTAR software package (DNASTAR, Madison, WI), and DNA Strider 1.2 alias. The amino-acids of STLCV AR1, in frame, were predicted from the nucleotide sequence using DNA Strider 1.2 program (designed and written by C. Mark, Cmaissiart al' Energie Altomique). IR and AR1 sequences of representative viruses in the family were extracted from the GenBank Sequence and formatted for alignment by pairwise (Wilbur-Lipman) or comparsons in DNASTAR. Parsimony analysis was conducted with PAUP Version 3.0 (Swofford, 1991) using bootstrap sampling with random TBR and 50% majority rule.

RESULTS AND DISCUSSION

WFT geminivirus isolates were collected from weed and crop species throughout Puerto Rico, and viral DNA extracts were obtained. Isolates were verified to be WFT geminiviruses based on PCR amplification for the diagnostic fragment (550 bp) of the coat protein gene (data not shown). This PCR approach relies upon PCR primers that specifically amplify the

diagnostic size product only from subgroup III geminiviruses (Wyatt and Brown, 1994; 1996).

Using the two primer described above, PCR products were also obtained for virus genomic fragments of the entire coat protein gene and the large intergenic region of the A component of bipartite viruses or the single component (chromosome) of monopartite genome viruses. PCR fragments were cloned and sequenced for virus isolates from the right half of the A component in the positive sense orientation (V168/C1303) and for approximately 2/3 of the A component in the negative sense orientation (C515/V1303) (data not shown).

A phylogenetic reconstrution generated from sequences (approx. 450 bp) of the 5' end of the V168/1303 fragment predicts three distinct geminiviruses were present in tomato in P.R. from 1991-96 (Fig. 1) Isolates of the newly described tomato yellow twist geminivirus (TYTwV) (Bird and Brown, unpublished data; this report) (PR59TYTwV168.1/168.2 and PR43tomnov 95168.2/3) appear to be unique from other WFT geminiviruses studies to date (III), and this virus is most closely related to the well-characterized potato yellow mosaic (described from Venezuela) and bean dwarf mosaic (described from Brazil) viruses. By this analysis, tomato virus isolates PR12Mtomfeb95168.1/2 and PR4tomnov95168.1 appear most closely related to the abutilon mosaic/tomato mottle virus (ToMoV) cluster. Interestingly, PR43tomnov95168.1 was obtained from a mixed infection in tomato that contained TYTwV. All three isolates share greater than 93% similar in sequence to the corresponding region of tomato mottle geminivirus described recently from Florida (Simone et al., 1990; Abouzid et al., 1992), and are tentatively identified as ToMoV (Brown et al., 1995). Tomato isolates PR23Stom91.168.1 and Prtom91sev168.1 appear most closely related to merremia mosaic geminivirus (PR37Merquin 168.1/2), a WFT previously recognized in M. quinquefolia, a common weed in PR (Bird and Maramoroscch, 1978). By this analysis, these virus isolates are placed between Sinaloa tomato leaf curl virus (STLCV) and the bean-infecting Bean calico mosaic geminivirus (BCMoV) described from Sonora, Mexico (Brown et al., 1988; Brown et al., in preparation).

The leafhopper transmitted geminiviruses, beet curly top and pseudo curly top viruses were included here as outgroups in the phylogeny. WFT geminiviruses are sufficiently divergent from their leafhopper transmitted relatives (Subgroups I and II) that they partition in a separate location on distinct branches within the Geminiviruses phylogenetic tree. The inclusion of these two distantly related viruses also confirms that all other viruses studied here, appear to be members of the WFT subgroup III within the Geminiviridae. The results of efforts to identify and track tomato-infecting geminiviruses in P.R. from 1991 to 1997 using molecular sequences indicate that the exotic tomato yellow leaf curl virus (TYLCV) has not been detected. This is despite its recent introduction from the Middle East into Dominican Republic and Jamaica, as well as in several Eastern Caribbean countries beginning in about 1992 (Brown and Bird, unpublished report; Brown et al., submitted). In conclusion, at least three distinct geminiviruses exhibiting unique symptom phenotypes of geminiviruses infect tomato crops in Puerto Rico. In 1991, the predominant virus appeared to be the isolate most closely related to merremia mosaic virus. By 1995, it appears that TYTwV and ToMoV are the most prevalent viruses in tomato. Finally, if TYLCV is introduced and becomes established, this will constitute a fourth geminivirus that threatens production of tomato for local and export markets in P.R. Additional investigations of the identities and relationships of WFT geminiviruses in P.R. are underway. These studies will

permit us to identify the specific virus genotypes against which genetically engineered for the expression of viral derived genes or gene products will result in disease resistant tomatoes, thereby, achieving sustainable production of tomatoes in Puerto Rico. Work is presently underway to obtain additional sequences of this region for distinct P.R. and US/Mexico viruses, and also for the large intergenic region, the full length coat protein (Padidam et al., 1995), the 550 bp core region of the coat protein (Brown and Wyatt, in preparation; Wyatt and Brown, 1996), and the 5' end of the coat protein gene (Padidam et al., 1995), all regions of the genome that may be potentially useful as gene markers for inferring virus phylogenies. Present and future work in this project will involve compilation of a comprehensive map of geminivirus distribution in Puerto Rico. Finally, we will obtain full-length infectious clones of the most economically threatening tomato infecting viruses which will serve as sources of genes for transgenic resistance efforts.

REFERENCES

Abouzid, A.M., J. E. Polston, and E. Hiebert. 1992. The nucleotide sequence of tomato mottle virus, a new geminivirus isolated from tomatoes in Florida. J. of Gen. Virology 73:3225-3229.

Bird, J., and K. Maramorosch. 1978. Viruses and virus diseases associated with whitefies. Adv. Virus Res. 22:55-110.

Brown, J.K. 1994. The current status of *Bemisia tabaci* (Genn.) as apest and vector in world agroecosystems. FAO Plant Prot. Bull. 42: 3-32.

Brown, J.K. and J. Bird. 1992. Whitefly-transmitted geminiviruses in the Americas and the Caribbean Basin: past and present. Plant Dis. 76: 220-225.

Brown, J.K., D.R. Frohlich and R. Rosell. 1995. The sweetpotato/silverleaf whiteflies: biotypes of *Bemisia tabaci* (Genn.), or a species complex ? Ann. Rev. Entomology. 40: 511-534.

Brwon, J.K., J. Bird, G. Banks, M. Sosa, K. Kiesler, I. Cabrera, and G. Fornaris. 1995. First report of whitefly-transmitted geminivirus epidemic in tomato in Puerto Rico. Plant Dis. 79:1250.

Brwon, J.K., A.M. Idris, and D. Fletcher. 1993. Sinaloa tomato leaf curl virus, (Stlcv), a new geminivirus infecting tomato and pepper in northwestern Mexico. Plant Dis. 77:1264.

Brown, J. K., E. Jiménez-García., and M.R. Nelson. 1988. Bean calico mosaic virus, a newly described geminivirus of bean. Phytopathology 78:1579.

Brown, J.K., R. Lastra, and J. Bird. 1991. First documentation of whitefly-transmitted geminiviruses causing widespread disease in cotton, tobacco, and tomato in Dominican Republic and in tomato in Puerto Rico. Fitopatologia 26:47.

Doyle, J.J. and J. L. Doyle. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull. 19:11-15.

Padidam, M., R. N. Beachy, and C. M. Fauquet. 1995. Classification and identification of geminiviruses using sequence comparisons. J. Gen. Viro. 76:249-263.

Saiki, R.K., D.H. Gelfand, S. Stoffel, S.J. Scharf, R. Higuchi, G.T. Horn, K.B. Mullis, and H.A. Erlich. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239:487 - 491.

Simone, G. W., J. K. Brown, E. Hiebert, and R. C. Cullen. 1990. Geminiviruses associated with epidemics in Florida, tomatoes and pepper. Phytopathology. 80:1063.

Swofford, D.L. 1991. PAUP: phylogenetic analysis using parsimony, version 3.0s. Champaign, IL: Illinois Natural History Survey.

Wyatt, S.D., and J.K. Brown. 1996. Detection of subgroup III geminivirus isolates in leaf extracts by degenerate primers and polymerase chain reaction. Phytopathology 86:1288-1293.

ACKNOWLEDGMENTS

This project is funded primarily by the Puerto Rico Economic Development Administration, San Juan, Puerto Rico, with additional support and/or valuable assistance from the Agricultural Experiment Station, University of Puerto Rico, Rio Piedras, P.R. The Experiment Station, University of Arizona, Tucson, AZ, and The United States Department of Agriculture, Office of International Development & Foreign Agriculture Services.

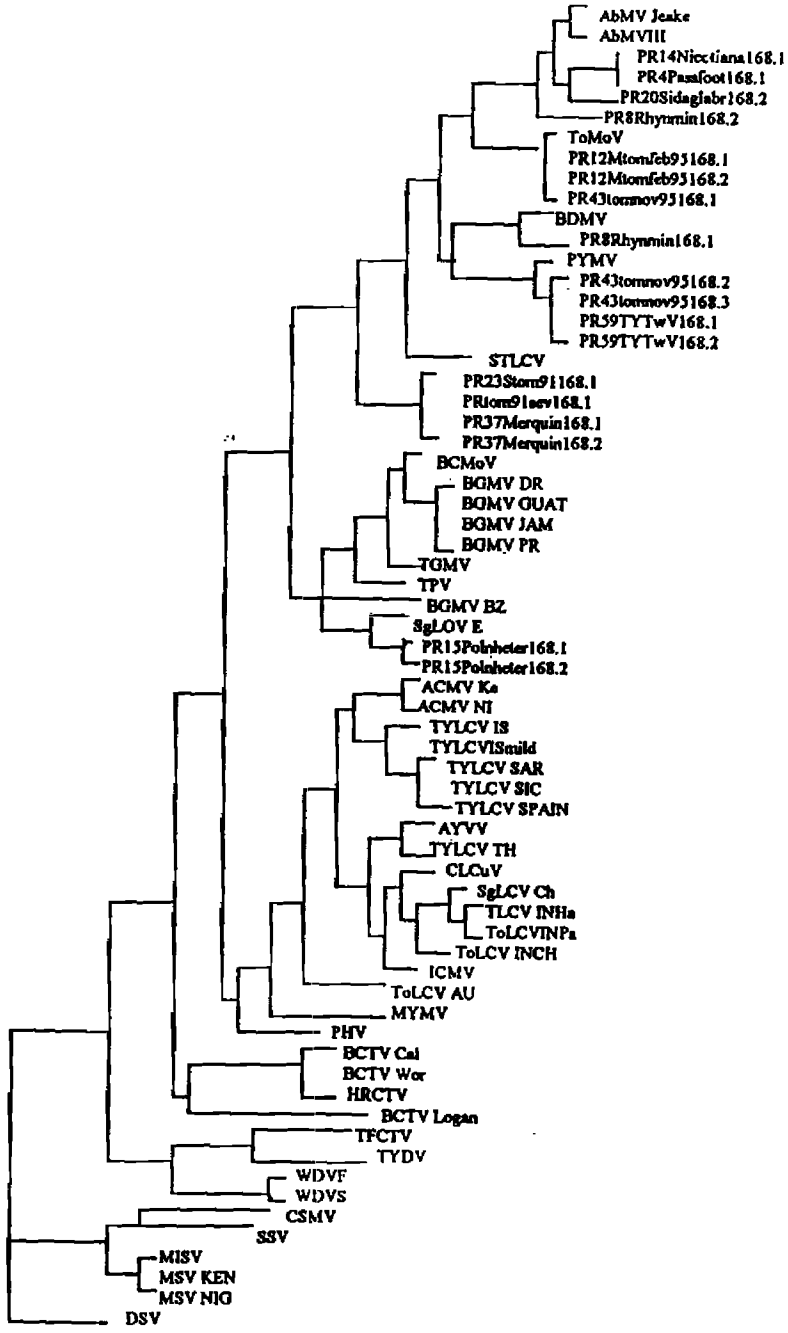

Figure 1. Phylogenetic reconstruction of WFT geminiviruses and relationships between P.R. viruses and selected well-characterized viruses in the Subgroup III, Geminiviridae. Digitaria streak virus (DSV), a monocot-infecting, leafhopper-transmitted geminivirus was designated as the outgroup for rooting the tree. Phylogenetic reconstruction was a accomplished by multiple sequence alignment using Clustal software, and parsimony analysis using PAUP. The tree (phylogram w/o bootstrap confidence values) shows the results of a 10 replicate bootstrap sampling (10 replicates each) in which a single shortest tree of 2121 in length was identified. Sampling was by TBR with random swapping and all trees saved during sampling conformed to the 50% majority rule (Consistency index (CI) = 0.483; Homoplasy index (HI) = 0.517; CI excluding uninformative characters = 0.455; HI excluding uninformative characters = 0.545; Retention index (RI) = 0.705; Rescaled consistency index (RC) = 0.341.