DISTRIBUTION AND GENETIC VARIABILITY OF WHITEFLY- TRANSMITTED GEMINIVIRUSES OF COTTON

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Abstract

Whitefly-transmitted geminiviruses (subgroup III, Geminiviridae) are emerging viral pathogens of cotton, worldwide. Virtually nothing is known about the genetic variability, biological characteristics, or the molecular epidemiology of these new virus pathogens of cotton. The genetically variable core region (550bp) of the coat protein gene was examined as a potentially informative molecular marker by which to identify and track the global distribution of WFT geminiviruses of cotton. This is an essential step toward assessing the risks that these emerging viruses pose to cotton production efforts. Preliminary analyses of geminivirus isolates from North America, Central America and the Caribbean Basin, and Sudan indicate that the core region of the coat protein gene permitted predictions about relationships between virus isolates from cotton, based on subgeographical and major geographical origins, and has potential for distinguishing between virus strains and quasi-species. From these experiments, a database of coat protein gene sequences has been established to permit identification of additional isolates from cotton, and to facilitate the tracking of WFT geminiviruses for molecular epidemiological and subsequent risk assessment objectives. Using this latter information, it will become possible to identify the most important geminiviruses against which disease resistance efforts should be targeted. Further, the availability of a broad suite of cotton geminivirus isolates from both Eastern and Western Hemispheres will permit testing of germplasm and/or genetically engineered cultivars for virus-specificand/or broad spectrum resistance for the first time.

Introduction

Whitefly-transmitted (WFT) geminiviruses pose serious constraints to the production of cotton grown in subtropical and temperate world regions (Brown, 1992). Recent and unprecedented infestations of the whitefly vector, *Bemisia tabaci* (Genn.) have contributed directly to increased inoculum levels of WFT geminiviruses now present in cotton growing areas throughout the world. In the US and other New World locales, the introduction of the B biotype of *B. tabaci* (also *B. argentifolli*) has precipitated a disastrous situation. The inherently virulent characteristics of the B biotype have fostered the rapid establishment of this whitefly as a major pest and geminivirus vector in the sunbelt states of the US and elsewhere throughout the world. In many affected areas, uncontrollable infestations in cotton by the B biotype have reduced yields as a result of direct feeding damage, honeydew contamination of cotton fiber, and an increase in diseases caused by WFT geminiviruses (Bedford et al., 1994; Brown et al. 1995a, b).

The increased frequency of insecticide resistance in whitefly populations has created yet another dilemma (Byrne and Devonshire, 1993; Dittrich and Hassan,1985) which promises to further increase virus inoculum levels, particularly in locations in which pest populations cannot be reduced to pest threshold levels. Given that the vector threshold level is less than one whitefly per plant, the outlook is less than optimistic. In the past, reducing whitefly levels and cultural practices helped reduce of virus disease incidence (Idris, 1990), but the expansion of irrigated agricultural practices has permitted increased cultivation of cotton, and vegetable crops in the same locale, inadvertently providing a year-round reservoir for whitefly populations.

The recent increase in whitefly vector population levels has resulted in a greater frequency of disease, and the expanded distribution of WFT geminiviruses in cotton in Chad, India, Mali, Nigeria, Pakistan, and Sudan (Brown, 1992; Idris, 1990; Katiyar,1987; Sundaramurthy,1992). Indeed, as a result of a widespread WFT geminivirus disease, the 1993 Pakistan cotton crop was declared a disaster, and the causal agent has been identified as the cotton leaf curl virus (Hameed and Khalid,1994; Mansoor et al., 1993). The disease situation is also approaching intolerable levels in India as well (A. Varma, pers. comm.).

It is known that *B. tabaci* populations from different world locations can transmit geminiviruses from both Old and New World sites (Bedford et al., 1993; 1994; Brown et al., 1995a), indicating the ability of *B. tabaci* to transmit subgroup III geminiviruses is an inherent feature of this whitefly species, worldwide; albeit, clear differences in biology and behavior have been recognized for different biotypes of *B. tabaci* (Bedford et al 1993; 1994; Brown et al., 1994; Brown et al., 1995a, b, c). This observation suggests an increased potential for movement and possible introduction of WFT geminiviruses into new locations, through whitefly-infested and/or virus-infected plants transported by international trade.

Finally, an important, and often disregard outcome of increasing levels of the whitefly vector, is the greater potential for whitefly mediated transmission events between hosts. This phenomenon results in greater probabilities of novel recombination and pseudo-recombination events between viruses in mixed infections (Bisaro, 1994). And, these rather common events can yield new strains or viral

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quasi-species that may feasibly emerge as new viral pathogens.

New World geminiviruses

In Central America and the Caribbean Basin, a whitefly-associated virus-like disease of kenaf was reported previously (Granillo et al., 1974), and only since 1989-92, has cotton production in Guatemala, Nicaragua (P. Anderson, per. comm.), and the Dominican Republic (Brown et al., 1991) been compromised due to whitefly-transmitted geminivirus epidemics. Following the introduction and establishment of the B biotype into the region beginning in approximately 1987 (Costa and Brown, 1991; Costa et al, 1993), widespread geminivirus epidemics have become commonplace (Brown, 1994; Brown and Bird, 1992).

A WFT-geminiviruses is known to cause cotton leaf crumple disease (CLCV) in the Arizona and California in the US, and in Mexico (Brown, 1992; Dickson et al; 1954), and losses have not been significant in most years, because typically, CLCV infections occur mid-season when plants are less susceptible to damage. Infection by CLCV results in yield loss, regardless of the age of plants at time of infection, however severe symptoms and the greatest losses are associated with early season infections (Brown et al 1987). Because infestations in cotton by the B biotype frequently reach threshold levels earlier in the season, CLCV has the potential to pose a new and serious threat to cotton production in the SW US.

In 1992, a previously undescribed leaf distortion disease of cotton was documented in Texas (author, this report), and the causal agent was found to be a WFT geminivirus, distinct from the CLCV. In Guatemala, a previously unidentified yellow mosaic disease of cotton was widespread in 1991-92, (Brown, 1993). The disease which was shown to be caused by a whitefly-transmitted geminivirus (Brown and Xie, unpublished), was in part responsible for the reduction in cotton acreage's planted in the South Coast area of Guatemala in 1993 and 1994. In the Dominican Republic, an uncharacterized WFT geminivirus was discovered, widespread in cotton, beginning in 1990. The disease is now known to be directly related to the introduction and country-wide distribution of the B biotype (Brown, 1993; Brown et al., 1995a). Similar disease symptoms were associated with whitefly-infested cotton in Puerto Rico when Sea Island cotton was widely cultivated on the South Coast near Poncho, previously, (J. Bird, per. comm.), and in Nicaragua beginning at least in 1991 (Brown and Anderson, unpublished).

Old World geminiviruses

The Eastern Hemisphere has a long history of virus-like disorders in cotton, and many of these diseases are attributed to uncharacterized, whitefly-transmitted viruses. Cotton leaf curl disease of cotton is well documented in India (Varma, per. comm.), several cotton growing areas in Africa (Brown, 1992), and has reached epidemic proportion in Pakistan for the first time (Mansoor et al., 1993). Leaf curling, and major, and minor vein thickening symptoms have also been observed for many years in cotton plantings in Mali, Chad, and Sudan (Brown, 1992, Idris, 1990), and threaten to increase in incidence concomitantly with rising whitefly vector population levels. The cotton crops in many locations in the Eastern Hemisphere were a total loss in 1992, 1993, and 1994. The effect of such dramatic losses in these countries is felt in the world market cotton pricing structure, and the impact is now of great consequence for US producers as well. Further, the mobile nature of germplasm, transplants, and ornamentals through global trade channels now permits ample opportunity for transport of exotic whitefly vector populations (Brown et al., 1995b, c) and exotic geminiviruses, and upon establishment in new areas, can threaten production of cotton crops.

Due to the rapid development of this situation, it has not been possible for cotton breeders to develop cotton cultivars with resistance to a multitude of emerging WFT geminivirus diseases in a timely fashion. In addition, most emerging geminiviruses of cotton have not been identified or studied in detail. Thus, the most threatening viruses must be identified, and prioritized with respect to disease resistance efforts. Understanding the identity and distribution of newly emerging geminiviruses are essential prerequisites to developing virus-resistant cotton cultivars for disease control.

The objectives of this study are to identify and characterize emerging whitefly-transmitted geminiviruses that may pose global or regional threats to cotton production. This effort is underway through a systematic collection and investigation of geminivirus isolates from cotton producing areas, worldwide. Here, are reported the preliminary results of a global survey, and partial genetic characterization of these isolates investigated in the AZ laboratory, through 1995. The objectives of this effort are to identify the most virulent, widespread geminivirus pathogens of cotton, to apply molecular genetics approaches to catalog and track the viruses; and ultimately to initiate efforts toward the development of geminivirus-resistant cotton in cotton cultivars relevant to the respective regions.

Materials and Methods

WFT geminivirus isolates were collected from field-infected material and transported to the UA laboratory, as fresh or dried leaf samples, or as total nucleic acid preparations (isolated in the country of origin). Viral core coat protein gene fragments were obtained using a polymerase chain reaction (PCR) protocol developed in the UA laboratory (Wyatt and Brown, 1996). Primers were designed to flank a variable region of the gene between approximately nucleotides 514-1048, under the auspices of

the working hypothesis that, because this region contains stretches of both conserved and variable sequences, it may be informative for virus identification, and to distinguish strains and virus species (quasi-species). As such, this region could be highly useful for virus identification, and for molecular epidemiological tracking of emerging viruses. These PCR primers have been shown through exhaustive testing, to amplify an expected 550 base pair fragment of the core region of the coat protein gene from all suspect and known WFT geminivirus infected plants (Brown et al., 1994; Wyatt and Brown, 1996).

Three DNA sequences per isolate were obtained from 550 bp fragments by automated fluorescent DNA sequencing (Molecular Genetics Facility, The University of Georgia, Athens, GA 30602). Sequences were aligned and edited. A progressive multiple sequence alignment was done using Wisconsin GCG (PileUp), and aligned sequences were formatted for parsimony analysis. Nucleotide alignments were made and genetic distances were calculated with PAUP (Swofford, 1993). A heuristic search of 100 replicates, tree bisection-reconnection (TBR) random, branch swapping yielded a single most parsimonious tree with a length of 1563 (Fig. 1a). One hundred replicates of the bootstrap method with heuristic search using TBR random, branch swapping, consistently identified the same large single island of most parsimonious trees from which a strict consensus tree was derived. In order to obtain some estimate of the robustness of the consensus tree, we also performed a bootstrap analysis under various conditions, resulting in confidence levels of 100% at all nodes (Fig. 1b).

Results and Discussion

The symptom phenotypes associated with different cotton geminivirus isolates studied here showed a range of foliar stunting and distortion, mild mosaics, yellow mosaics, mild to severe leaf curling or crumpling, and/or stunting of the entire plant. Prior to this survey, only two WFT geminiviruses, the cotton leaf crumple virus and cotton leaf curl virus (CLCuV) were recognized as distinct WFT geminiviruses of cotton, worldwide. The CLCV was previously described from the southwestern US and Mexico (Brown and Nelson, 1984; 1987; Dickson et al.; 1954), whereas, CLCuV has been documented previously on the Indian subcontinent, and in some African countries (Brown, 1992; Hameed and Khalid, 1994; Idris, 1990; Mansoor et al., 1993). These data suggest that there are many new, previously undescribed WFT geminivirus pathogens of cotton and/or in related (Malvaceous) crop and weed species throughout the world. Although it is likely that cotton-infecting geminiviruses also infect malvaceous weed and cultivated hosts, the importance of related host species to these new cotton diseases have not yet been assessed. Most emerging geminiviruses have not been identified, and the virus biology and molecular epidemiology of the diseases have not been studied. The economic importance and extent of damage to cotton production efforts, are likewise unknown.

Based on genetic analysis and computer-assisted alignment of viral DNA sequences, essentially, three distinct groups of subgroup III viruses are well-supported: (1) one consisting of a virus from Sudan and an uncharacterized isolate from Texas cotton (1994), plus the Old World outgroup virus, African cassava mosaic virus (ACMV), (2) another from Guatemala cotton (1994) containing a single representative that appears to form a bridge via a node intersection with groups 1 and 3, and (3) a third that contains isolates obtained only from the Americas and Caribbean Basin.

As expected, the outgroup virus, ACMV, clustered with the Sudan virus, and is part of the putative Eastern Hemisphere group; however, the validity of the positions of the Texas cotton (1994) and Guatemala cotton (1994) isolates are under further investigation, In light of the present hypothesis that the target region is informative about geographic origin (Brown et al., 1994), it is not possible to explain Eastern Hemisphere origins for these latter isolates, unless an exotic virus has been introduced. Interestingly, both isolates in question were collected in New World sites in 1994, one from a site in Guatemala devoted to extensive breeding of native cottons, and the second, in an area near experimental plots of kenaf in Texas.

The New World cluster further defines several subclusters of isolates. One cluster contains two isolates: the original CLCV collected in Arizona by the author in 1982, and a 1992 isolate from Texas exhibiting a very different symptom phenotype.

A second major subcluster contains two subdivisions, one which is further divided. The first major subcluster contains a geminivirus from 1993 cultivated nursery hibiscus plants (*Althaea* spp.) plus a geminivirus from the Texas weed *Malvastrum corromendelianum* (also referred to by some as *Sida* spp), both hosts being members of the Malvaceae. The larger subcluster contains two subdivisions: the first consisting of isolates that cause leaf mosaic and crumpling and variations of this symptom phenotype WFT geminiviruses from Arizona and Guatemala (putative strains of CLCV, and thus, the first report of CLCV outside of North America), and the second, consisting of a cluster of isolates from cotton in the Dominican Republic, a cultivated hibiscus plant sold as nursery stock in 1994, and one each from okra in Mexico, a Guatemalan weed (*Abutilon* spp), the cultivated *Abutilon* spp. infected with abutilon mosaic geminivirus (historically transmitted through vegetative propagation of the plant because of the valued virus symptoms produced by the infection, and now, no longer transmissible by the whitefly vector (Brown et al., in preparation).

Here, is reported the first evidence for many diverse symptom phenotypes among geminiviruses of cotton, that can be corroborated, in many cases, with evidence of genetic variability based on the DNA sequence of the core region of the coat protein gene. As such, it is clear that there are many more geminiviruses and/or strains of viruses infecting cotton, than previously recognized. Only one virus from this study, CLCV, has ever been investigated in detail. Prior to this report, most of these isolates were not identified or characterized in any manner.

These findings suggest that WFT geminiviruses are emerging as important virus pathogens of cotton on a regional, and likely, a global basis. They further demonstrate a need for identification and routine tracking of emerging geminiviruses to identify those against which disease control measures are warranted. It is important to consider that, following the emergence of a new group of pathogens, only certain strains or quasi-species will prevail under status quo conditions; thus tracking is essential for determining the identity and distribution of the most important and widespread viruses, ultimately providing a focus for plant breeding and genetic engineering efforts devoted to the development of disease resistant cultivars. A second important advantage is the ability to detect new, virulent strains or viruses prior to the development of epidemics.

Continued evaluation of geminivirus isolates over the next several years will yield invaluable information toward the long term goal of developing geminivirus disease resistant cotton, customized with respect to cotton species and cultivar, to the specific locale(s) in which the cultivar will be grown, ultimately, taking into account knowledge of the identity of virus strains/quasi-species that predominate where the cultivars will be grown.

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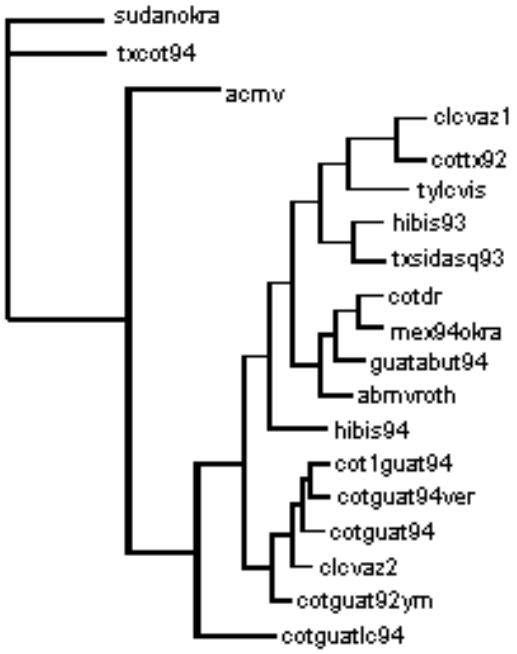
Table 1. Subgroup III (whitefly-transmitted) geminivirus isolates under study; * denotes new disease report

Isolate Designation	Symptom in Cotton	Geographic Source	Source Plant	Year
sudan okra (cot leaf curl)	vein thickening	Sudan	okra	1995
txcot94*	mosaic, stunting	TX, USA	cotton	1994
clcvaz1	leaf crumple	AZ, USA	cotton	1982
cottex92*	foliar stunting	TX, USA	cotton	1992
hibis93*	N/A	AZ, USA	hibiscus	1993
txsida93	N/A	TX, USA	*Malvastrum*	1993
cot1guat*94	leaf crumple	Guatemala	cotton	1994
cotguat94ver*	mosaic	Guatemala	cotton	1994
cot2guat94*	leaf crumple	Guatemala	cotton	1994
clcvaz2	leaf crumple	AZ, USA	cotton	1993
cotguat92ym*	yellow mosaic	Guatemala	cotton	1992
cotdr*	mosaic	Dom Rep	cotton	1992
mx94okra*	yellow mosaic	Mexico	okra	1995
guatabut94	N/A	Guatemala	*Abutilon*	1994
abmvroth	N/A	West Indies	*Abutilon* (ornamental)	1800's
hibis94*	N/A	AZ, USA	hibiscus	1994
cotguatlc94*	leaf curl	Guatemala	cotton	1994

A



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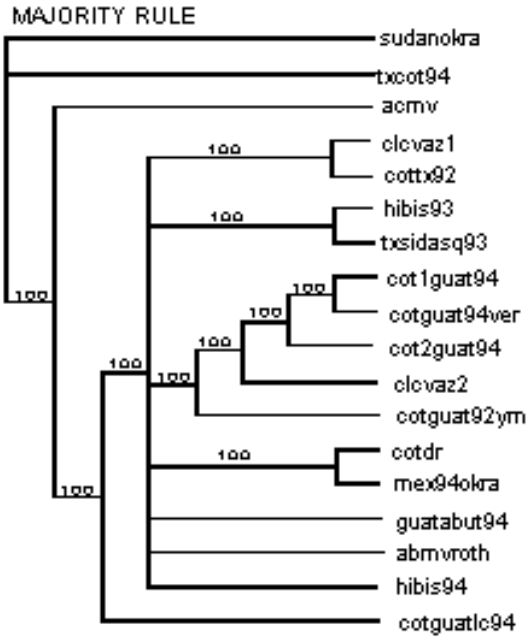

Fig. 1. Phylogram showing relationships predicted by parsimony analysis of viral coat protein gene sequences, based on analysis of the a 550 basepair fragment: (A) single tree generated by a heuristic search with tree bisection-reconnection, random branch swapping (100 replicates), and (B) bootstrap test to estimate robustness of the consensus tree (100 replicates) with application of 50% majority rule