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Differentiation of three whitefly-transmitted geminiviruses from the Republic of Yemen

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Abstract. Three viruses collected in southern Yemen in 1990, infecting watermelon, tobacco and tomato were shown to be transmitted by the whitefly *Bemisia tabaci* and to have particle morphologies typical of geminiviruses. Colonies of *B. tabaci* collected from different locations and from different hosts were used in virus transmission tests with the same host range of plants. Colonies established from both watermelon and cotton in the Yemen were identified as the squash silverleaf-inducing 'B' biotype. The culture host of the colony did not influence virus acquisition and transmission efficiencies to and from other hosts. The tobacco and tomato geminiviruses had a similar host range, but differed in their severity in some hosts. Both these viruses differed from the watermelon geminivirus in host range and symptoms. *Datura stramonium*, an alternative host for all three viruses, could be co-infected by the watermelon and tobacco viruses. *B. tabaci* was able to acquire both viruses from the co-infected *D. stramonium* and infect seedlings of either original host plant species with their respective viruses or *D. stramonium* with both. The viruses were identified as watermelon chlorotic stunt virus, tobacco leaf curl virus and tomato yellow leaf curl virus and were distinguished by cross hybridisation.

Introduction

Since the 1970s, whitefly-transmitted viruses have caused increasing economic damage to crops in the Yemen. The most serious of these is watermelon chlorotic stunt virus (WCSV), which causes yellow veining, chlorotic mottling, severe stunting of young leaves and drastically reduced fruit yield. It was first identified in the former Peoples Democratic Republic of Yemen in 1986, where it infected 90–100% of the watermelon plants. It is present wherever watermelons are regularly grown [Jones et al., 1988]. Symptoms typical of whitefly-transmitted viruses were identified in two other crops; leaf curl of tobacco, and yellow leaf curl of tomato.

Tobacco leaf curl virus (TLCV), was first described from *Nicotiana tabacum* in Tanzania where it caused 'cabbaging, leaf curling and severe stunting of tobacco plants' [Storey, 1931]. Diseases with similar symptoms

to TLCV have been reported throughout the tropical and sub-tropical regions of the world causing severe crop losses in tobacco [Pruthi and Samuel, 1940; Osaki and Inouye, 1978; Brown and Bird, 1992].

Throughout the warmer parts of the world tomato crops have increasingly been associated with geminivirus infections causing stunting of plants, chlorotic mottling, curling and chlorosis of the young leaves and low fruit yield; these viruses are usually called tomato yellow leaf curl virus (TYLCV) and tomato leaf curl virus (ToLCV). Geminiviruses have genomes of single stranded DNA and are characterised by their twinned (geminate) particle morphology [Stanley, 1985]. Tomato yellow leaf curl virus has been widely reported as causing serious economic problems in the tomato crops of the Mediterranean region [Cohen and Nitzany, 1966; Makkouk et al., 1979; Al-Musa, 1982; Cherif and Russo, 1983; Mansour and Al-Musa, 1992]. It has spread rapidly throughout the Middle East and is reported from Asia [Rochester et al., 1990], the Americas [Brown and Bird, 1992; Harrison et al., 1991], Africa [Harrison et al., 1991] and more recently, from southern Europe [Macintosh et al., 1992]. Symptoms alone are not sufficient to distinguish strains of tomato geminiviruses which have been grouped serologically [Harrison et al., 1991] and by sequence homology [Dry et al., 1993]. New world and old world tomato-infecting geminiviruses were found to be more distantly related to each other than those within either region.

The vector of the three viruses in the Yemen was assumed to be *Bemisia tabaci* (Genn.), the tobacco or sweet potato whitefly. *B. tabaci* is found on every continent and is indigenous to most of the tropical and sub-tropical regions of the world [Cock, 1986]. It is the known vector of about 50 dicotyledon-infecting geminiviruses [Markham and Bedford, 1993, Bedford et al., 1994]. The aim of this study was to ascertain if *B. tabaci* was the vector of these three viruses from the Yemen and to compare their biological characteristics.

Materials and methods

Origins and maintenance of viruses and insects

The three viruses were collected from field locations in the Yemen. WCSV and TYLCV-Y were collected from the Abyan region and TLCV-Y from the Hadramauvat region. They were maintained initially by grafting infected scions to healthy plants. Once insect transmission had been established, all three viruses were maintained by insect transmission to their respective hosts.

Two *B. tabaci* colonies were established from individuals collected in the Abyan region of Yemen in 1990 from cotton (YC) and watermelon (YW). A further colony, collected in Turkey in 1985, was cultured on

cotton (TC). Non-viruliferous stock populations of these three *B. tabaci* colonies were maintained separately on their respective host plants in perspex cages at 25 °C with a 16h daylength.

Analysis of insects

Morphological studies were made of individual adults and nymphs from each of the *B. tabaci* colonies. In particular the vasiform orifice region of the fourth instar/puparium stage was examined using a Cam Scan Series Four scanning electron microscope [Bedford et al., 1994] with reference to taxonomic keys [Martin, 1987]. The ability of the larval stages of colonies to induce a phytotoxic response in squash plants [Yokomi et al., 1990; Costa and Brown, 1991; Costa et al, 1993; Bedford et al., 1994] was assessed by placing single seedlings of squash (*Cucurbita pepo* cv. Fordhook) into an insect cage with adult whiteflies allowing ovulation followed by the emergence and feeding of the whitefly larvae.

Electron microscopy

Leaf material was ground in a pestle and mortar with distilled water (1:1 w/v). The resulting sap was squeezed through muslin and either mixed with an equal volume of 2% neutral phosphotungstic acid before spraying onto a carbon/colloidion EM grid or stained on the grid with aqueous uranyl acetate. Grids were viewed in a Jeol 1200EX electron microscope. Immunosorbent electron microscopy (ISEM) was performed as described by Adams et al. [1988] using antisera raised to African cassava mosaic virus (kindly provided by M. Swanson, SCRI, Dundee, UK) or squash leaf curl virus (kindly provided by Dr J.K. Brown, Univ. of Arizona, Arizona, USA).

Virus transmission tests

Groups of approximately 200 non-viruliferous adult *B. tabaci* were placed on a virus infected plant in a perspex cage in growth rooms at 25 °C with a 16 h daylength. The insects were allowed to feed on the virus infected source plant for a 24 h acquisition access period (AAP). The infected source plant was then removed after shaking off the insects and a minimum of three healthy test seedlings were added to the cage. The insects were then allowed a 48 h inoculation access period (IAP) to the healthy test seedlings. After this the test seedlings were removed from the insect cage. Control experiments were performed by caging the same numbers of healthy insects on three test seedlings for 48 h. Following exposure to insects all plants were transferred to an insect proof glasshouse and fumigated with a carbamate-based insecticide (Propoxur, Octavius Hunt Ltd.). Plants were checked daily for symptoms. Where symptoms were not conclusive, infection was confirmed by dot blot hybridisation [Maule et al., 1983].

Efficiency of transmission of the viruses was determined by caging groups of non-viruliferous *B. tabaci* on a virus infected plant for a 24 h AAP. Insects were removed and transferred, either singly or in groups of 5 or 10 to healthy seedlings and given a 48 h IAP.

Cloning, DNA sequencing and cross-hybridisation

A full-length clone of WCSV DNA A was obtained by ligating viral supercoiled, replicative form DNA (scDNA; isolated from infected watermelon as described for African cassava mosaic virus [Stanley and Townsend, 1985]), linearised using the restriction endonuclease *Xba*I, into M13mp19 [Norrander, 1983].

A partial clone of TYLCV-Y DNA A was produced by PCR amplification using the 'universal primers' and reaction conditions described by Briddon and Markham [1994]. Restriction endonuclease cleavage sites included in the primers were used to clone the amplification products in to M13mp19. This yielded an approximately 1500 bp *Eco*RI-*Xba*I fragment covering the amino-terminal part of the C1 open reading frame.

Partial nucleotide sequences of both the full-length clone of WCSV DNA A and the fragment of TYLCV-Y DNA A were determined by dideoxynucleotide chain termination sequencing [Sanger et al., 1980] using Sequenase Version II (USB) and [α-^{35}S]dATP (New England Nuclear). Sequencing products were resolved by electrophoresis in denaturing buffer gradient gels [Biggin et al., 1983] using acrylamide solutions from Severn Biotech. Sequence information was stored, assembled and analyzed using the Program Library (version 7) of the Genetics Computer Group (Madison, Wisconsin [Devereux et al., 1984]). Phylogenetic analyses were conducted using the Fitch program in the PHYLIP (version 3.5) package of programmes (J. Felsenstein, Dept. of Genetics, University of Seattle, Oregon, USA). Sequence alignments were produced using the GAPALIGN program (GCG) and sequence distance matrices were produced using DNADIST program (PHYLIP).

Relative levels of cross-hybridisation were determined by dot blot hybridisation [Maule et al., 1983]. Samples for analysis consisted of caesium chloride gradient purified scDNAs of ACMV, WCSV, TYLCV-Y and TLCV-Y. Nitrocellulose filters were prepared in triplicate by spotting approximately equal amounts (determined spectrophotometrically) of scDNA, diluted in an equal volume of 1 M NaOH, for each virus. Filters were then neutralised for 5 min in 0.5 M Tris-HCl (pH 7.5), 1.5 M NaCl and baked for 2 h at 80 °C under vacuum. Probes were produced from full-length clones of ACMV DNA A (Kenyan isolate [Stanley and Gay, 1983]), WCSV DNA A and the partial clone of TYLCV-Y DNA A. These were labelled with [α-^{32}P]dCTP (New England Nuclear) by nick translation [Rigby et al. 1977]. Following hybridisation, filters were washed at low stringency (2 × SSC, 0.1% SDS at room temperature), medium stringency

(2 × SSC, 0.1% SDS at 50 °C) and high stringency (0.1 × SSC, 0.1% SDS at 65 °C) to determine levels of hybridisation.

Results

Analysis of whiteflies and viruses

Electron microscopic analysis of the fourth instar/puparium stage of individuals of the whitefly colonies, and in particular the vasiform orifice [Bedford et al., 1994], showed all three colonies to have typical *B. tabaci* characteristics [Martin, 1987]. In culture the YC and YW colonies both showed a tendency to swarm at regular intervals particularly at high population densities. In contrast, the TC colony never swarmed during eight years of culturing. Both the YC and YW insects were also able to induced the silverleaf phytotoxic response in squash seedlings whereas the TC colony did not. Squash 'silverleaf' (SSL) phytotoxicity has been attributed to the newly identified 'B' biotype of *B. tabaci* [Costa and Brown, 1991; Cohen et al., 1992; Bedford et al., 1993, 1994; Costa et al., 1993]. Previous work has shown both the YC and YW colonies belong to the 'B' biotype by esterase pattern analysis [Bedford et al., 1992, 1994].

Plants infected with the three viruses, either experimentally or collected from the field, were shown to contain virus particles with a twinned morphology typical of geminiviruses [Stanley, 1985] by either the leaf dip or ISEM methods (results not shown). No other virus particles were detected in leaf dips.

The results of cross-hybridisation of the three viruses to probes of ACMV DNA A, WCSV DNA A and a fragment of TYLCV-Y are shown in Table 1. All three viruses cross-hybridised to the ACMV probe with TYLCV-Y showing the strongest response. TLCV-Y showed low levels of hybridisation to all three probes with the strongest response to TYLCV-Y. Partial sequences spanning 393 bp of the C1 open reading frames of WCSV DNA A and TYLCV-Y DNA A were determined. These sequences are available in the EMBL, GenBank and DDJB nucleotide sequence databases under accession numbers 79429 and 79430 for TYLCV-Y and WCSV respectively. The similarity of these sequences to the equivalent sequences of African cassava mosaic virus (ACMV [Stanley and Gay, 1983]), Indian cassava mosaic virus (ICMV [Hong et al. 1993]), tomato golden mosaic virus (TGMV [Hamilton et al. 1984]), tomato leaf curl virus (ToLCV [Dry et al. 1993]) and isolates of TYLCV originating from Israel (TYLCV-I [Navot et al. 1991]), Sardinia (TYLCV-S [Kheyr-Pour et al. 1992) and Spain (TYLCV-E; EMBL accession number Z25751) were determined using the GAP program (GCG package) and are summarised in Table 2. Phylogenetic analysis of the sequences is shown in Fig. 1. TYLCV-Y segregates with the previously characterised TYLCV isolates

Table 1. Relative levels of cross-hybridisation

Virus	Probe		
	ACMV	WCSV	TYLCV-Y*
ACMV	4	2	2
WCSV	2	4	2
TYLCV-Y	3	2	4
TLCV-Y	1	1	2

* The clone of TYLCV-Y was not full-length.
Scores range from 1 (very weak hybridisation detectable only at low stringency) to 4 (homologous reaction).

Table 2. Sequence similarity between published data and the Yemen viruses over 393 nucleotides

	ACMV	TGMV	ToLCV	TYLCV-S	TYLCV-I	TYLCV-Y	TYLCV-E	ICMV	WCSV
ACMV	100								
TGMV	64	100							
ToLCV	76	61	100						
TYLCV-S	77	63	77	100					
TYLCV-I	76	63	78	76	100				
TYLCV-Y	80	63	78	83	80	100			
TYLCV-E	77	64	78	95	78	84	100		
ICMV	73	57	73	68	69	70	69	100	
WCSV	68	58	63	67	65	68	68	67	100

showing the closest relationship to TYLCV-E (84% homology). In contrast, WCSV appears unrelated to any of the viruses to which it was compared, showing the highest sequence similarity (68%) to ACMV, TYLCV-E and TYLCV-Y. In the phylogenetic comparisons WCSV is distantly related to ICMV as indicated by the high mutation distance. Phylogenetic relationships determined using the partial sequences of the C1 open reading frame are comparable to those produced for whole C1 sequences with the exception of ACMV. In phylogenetic trees produced from whole C1 sequences ACMV segregates with ICMV.

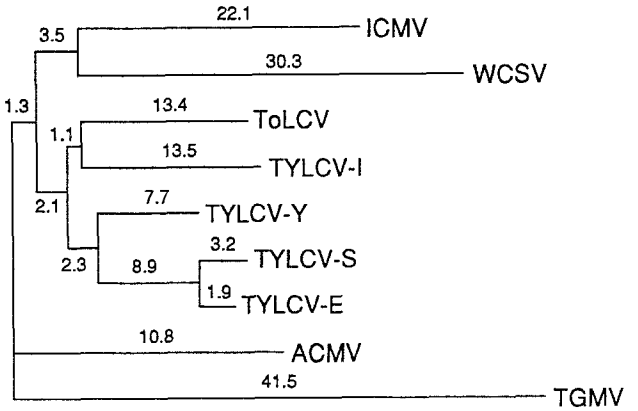

Fig. 1. Unrooted phylogenetic tree calculated from pairwise nucleotide sequence distance data. The shortest tree (tree with smallest sum of squares) calculated using Fitch is shown. Vertical distances are arbitrary. Horizontal branches are proportional to mutation distances as indicated by numbers. Sum of squares was 0.25 and average percent standard deviation was 5.94%. A total of 6807 trees were examined.

Virus transmission

The results in Table 3 summarise the transmission efficiency of the viruses by the three whitefly colonies. All three whitefly colonies were able to transmit TYLCV-Y and TLCV-Y with similar efficiencies. However, the efficiency of transmission of WCSV by the *B. tabaci* colonies was significantly lower in the single and five insect assays than the other two viruses.

Table 3. Transmission efficiency of Yemen viruses

Virus	Insect N°s	*Bemisia tabaci* colony		
		YC	YW	TC
WCSV	1	1/30	3/60	1/30
(in watermelon)	5	17/30	19/30	14/30
	10	30/30	30/30	30/30
TYLCV-Y	1	6/30	6/30	4/30
(in tomato)	5	28/30	30/30	29/30
	10	30/30	30/30	30/30
TLCV-Y	1	5/30	10/60	4/30
(in tobacco)	5	46/60	42/60	21/30
	10	59/60	59/60	30/30

Figures given show numbers of plants infected/number of plants inoculated. Insects were given a 24 h acquisition access period on an infected plant followed by a 48 h inoculation access period on 30 or 60 seedlings of the respective host plants of the three viruses.

The colony adapted to watermelon (YW) showed a marginally higher efficiency of transmission of WCSV than the YC and TC colonies which were adapted to cotton. The whitefly colonies originating from the Yemen were also marginally more efficient at transmitting TLCV-Y and TYLCV-Y than the colony originating from Turkey. In each case, however, the use of 10 insects per assay gave efficiencies of approximately 100%. The lower transmission efficiencies of the WCSV compared to the TYLCV-Y and TLCV-Y could not be attributed to survival rates of the insects on the different plant species since significantly more insects were found to survive after 24 h on watermelon than on tomato or tobacco (results not shown). The lowest mortality levels were found in transmission tests with WCSV to watermelon using the YW colony which was already adapted to watermelon.

The ability of the three viruses to infect alternative hosts by insect transmission is shown in Table 4. None of the viruses were able to infect *Portulaca oleracea* and *Amaranthus graecizans*, common field weeds in the Yemen. TLCV-Y and TYLCV-Y showed the same host range in these experiments. Common hosts to all three viruses were *N. benthamiana* and *D. stramonium*. The symptoms of TLCV-Y and TYLCV-Y were typically, stunting, leaf curling and chlorosis when infecting alternative host plants (Table 4). The symptoms of TYLCV-Y were in all cases less severe than TLCV-Y (Figs. 2, 3 and 4), being almost symptomless in *N. tabacum* where only a slight twisting of the main stem was seen (Fig. 3). *B. tabaci* could transmit the viruses back to the original plant hosts from all the

Table 4. The transmission of three geminiviruses to alternative hosts

Test host plant	Virus		
	WCSV	TLCV-Y	TYLCV-Y
Citrullus vulgaris cv. Charleston gray	+	–	–
Cucumis melo cv. Cantaloupe	+	–	–
Cucumis sativus cv. Telegraph	–	–	–
Nicotiana tabacum cv. Samsun	–	+	+
Nicotiana benthamiana	+	+	+
Phaseolus vulgaris cv. Top Crop	+	–	–
Vicia faba cv. Sutton	–	–	–
Lycopersicon esculentum cv. Kondine Red	–	+	+
Capsicum annum cv. Annaheim	–	–	–
Gossypium hirsutum cv. Delta Pine 70	–	–	–
Datura stramonium	+	+	+
*Portulaca oleracea**	–	–	–
*Amaranthus graecizans**	–	–	–

* Yemen field weeds. + = infected / – = not infected.
Experiments used a minimum of three test seedlings with 200 adult *Bemisia tabaci* and were repeated three times.

Fig. 2. Nicotiana benthamiana plant; healthy (left), infected with TYLCV-Y (middle), infected with TLCV-Y (right).

Fig. 3. Tobacco (left) and tomato (right) infected with TYLCV-Y.

Fig. 4. Tobacco (left) and tomato (right) infected with TLCV-Y.

alternative hosts, except from *N. benthamiana* which proved lethal to *B. tabaci* after the 4-leaf stage (data not shown).

Typical symptoms induced by WCSV were marked mosaics and mottling in watermelons, and the production of mild symptoms in alternative hosts. All alternative hosts investigated showed mild chlorotic mottling and, in *N. benthamiana* and *D. stramonium*, a downwards cupping of the leaves. All alternative hosts, with the exception of *N. benthamiana*, could be used as a source to infect healthy watermelon seedlings by insect transmission resulting in typical WCSV symptoms. For *D. stramonium* and *P. vulgaris* transmission was only achieved in the early stages of infection.

Dual infections of *D. stramonium* with WCSV and TLCV-Y were achieved by sequentially exposing viruliferous insects to the test plants. Dual infection was confirmed by feeding non-viruliferous whiteflies on the dual infected plants and then transferring these to watermelon and tobacco; differential hosts for the viruses (Table 4). Both viruses could be transmitted to further *D. stramonium* test plants and dual infection shown by means of the differential hosts. The symptoms of dual infected *D. stramonium* were those of the most severe virus of the pair, TLCV-Y (Fig. 5).

Fig. 5. Datura (centre) infected with both WCSV and TLCV-Y. *B. tabaci* could recover both viruses from the infected Datura and infect the original virus hosts plant species; watermelon (right) and tobacco (left). Further transmissions to Datura again resulted in a dual infection.

Discussion

The results presented confirm that all three viruses belong to the whitefly-transmitted subgroup III of the geminiviruses [Hull et al., 1991]. Both the nucleotide sequence homologies and host range analyses show WCSV to be distinct from the other viruses to which it was compared, having relatively low levels of homology to TYLCV-Y, TYLCV-E and ACMV (a virus originating from Africa). In contrast, TYLCV-Y shows the highest levels of homology to the Spanish isolate of TYLCV (84%) followed by the Sardinian isolate (83%). Cross-hybridisation tests using sulphonated DNA probes have previously shown that TYLCV isolates originating from Lebanon and Israel share more homology with each other than either does to an isolate from Thailand [Swanson et al., 1992]. Similar conclusions were reached by Harrison et al. [1991] for different TYLCV isolates by analysis of epitope profiles using monoclonal antibodies. Our results also show the TYLCV-Y may be similar to a Jordanian isolate of TYLCV which was recently reported to give symptomless infections in different *Nicotiana spp.* and not to infect *P. vulgaris* [Mansour and Al-Musa, 1992], unlike an Israeli isolate [Cohen and Nitzany, 1966] and a Cyprus isolate which did not infect *Nicotiana spp.* [Ioannou, 1985]. We therefore provi-

sionally classify TYLCV-Y as an isolate belonging to the complex of yellow leaf curl geminiviruses prevalent in the Middle East and Mediterranean being most closely related to the TYLCV isolate recently identified in Spain [Moriones et al., 1993].

In the absence of any sequence data (despite numerous attempts to isolate clones), the determination of the relationship of TLCV-Y to the other viruses is more speculative. The fact that TLCV-Y and TYLCV-Y have the same host range and induce the same symptoms, differing only in severity, would suggest that these two viruses are isolates/strains of the same virus. However, in cross-hybridisation only relatively weak reactions are detected to all three probes with the highest levels of hybridisation to TYLCV-Y. It would appear, therefore, that TYLCV-Y and TLCV-Y are related but their exact relationship will require further analysis.

Analysis of the transmission efficiencies of the three viruses using single insect assays showed TLCV-Y and TYLCV-Y transmitted far more efficiently than WCSV despite, in most cases, the higher mortality rates of insects on tobacco and tomato compared to watermelon. The basis for this phenomenon remains unclear but may relate to the amounts of virus available to the whiteflies on feeding (WCSV possibly having a lower virus titre) or the distribution of the virus in plants (WCSV not being in suitable cells for acquisition). No significant difference was found in the efficiencies of transmission of the viruses by the different insects, indicating that a *B. tabaci* population from a geographically isolated region is able to transmit any whitefly-transmitted geminivirus with equal efficiency. Similarly, host adaptation of the insect population appears to play little part in acquisition and transmission efficiencies, although it has a significant effect on survival rate.

The ability of geminiviruses to co-infect has been reported previously [Brown and Nelson, 1989] but has also involved two strains of the same virus [Giddings, 1950]. In this study two distinct viruses have been introduced into a single seedling with no evidence of cross-protection. Strains of most plant RNA viruses cross protect against superinfection by related strains, which is the basis for coat protein-mediated protection [Beachy, 1988]. For geminiviruses, interference with replication by defective-interfering viral genomes may be a likely method for controlling them [Stanley et al., 1990], and since subgenomics occur naturally they may account for the appearance of milder than normal symptoms or attenuation of symptoms in certain hosts.

Geminiviruses are of increasing importance to agriculture in the Yemen and the results shown here indicate that relatively low levels of *B. tabaci* would be sufficient to give rise to levels of virus infection which would have a devastating economic effect on a susceptible crop. The indiscriminate use of insecticides has undoubtably led to resistant *B. tabaci* populations [Byrne and Devonshire, 1993]. This has been compounded by the provision in many areas of irrigation, allowing crops such as water-

melons to be grown all year round so providing a continuous crop infecting cycle. The need now is for more suitable means of controlling *B. tabaci* and the identification of potential weed hosts which harbour the viruses between crops particularly in the case of tobacco and tomato crops which are not grown continuously throughout the year.

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