An unusual alphasatellite associated with monopartite begomoviruses attenuates symptoms and reduces betasatellite accumulation

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Received 30 July 2010
Accepted 10 November 2010

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The Oman strain of Tomato yellow leaf curl virus (TYLCV-OM) and its associated betasatellite, an isolate of Tomato leaf curl betasatellite (ToLCB), were previously reported from Oman. Here we report the isolation of a second, previously undescribed, begomovirus [Tomato leaf curl Oman virus (ToLCOMV)] and an alphasatellite from that same plant sample. This alphasatellite is closely related (90% shared nucleotide identity) to an unusual DNA-2-type Ageratum yellow vein Singapore alphasatellite (AYVSGA), thus far identified only in Singapore. ToLCOMV was found to have a recombinant genome comprising sequences derived from two extant parents, TYLCV-OM, which is indigenous to Oman, and Papaya leaf curl virus from the Indian subcontinent. All possible combinations of ToLCOMV, TYLCV-OM, ToLCB and AYVSGA were used to agro-inoculate tomato and Nicotiana benthamiana. Infection with ToLCOMV yielded mild leaf-curl symptoms in both hosts; however, plants inoculated with TYLCV-OM developed more severe symptoms. Plants infected with ToLCB in the presence of either helper begomovirus resulted in more severe symptoms. Surprisingly, symptoms in N. benthamiana infected with the alphasatellite together with either of the helper viruses and the betasatellite were attenuated and betasatellite DNA accumulation was substantially reduced. However, in the latter plants no concomitant reduction in the accumulation of helper virus DNA was observed. This is the first example of an attenuation of begomovirus-betasatellite symptoms by this unusual class of alphasatellites. This observation suggests that some DNA-2 alphasatellites encode a pathogenicity determinant that may modulate begomovirus-betasatellite infection by reducing betasatellite DNA accumulation.

INTRODUCTION

Members of the genus Begomovirus, family Geminiviridae, have small circular, ssDNA genomes and are transmitted by the whitefly vector Bemisia tabaci (Gennadius) (Moffat, 1999; Brown 2007a, b; Mansoor et al., 2003a) sibling species group (Brown, 2010). Some begomoviruses have a bipartite genome consisting of two components, known as DNA-A and DNA-B components (Stanley et al., 2005). In the eastern hemisphere (EH) the vast majority of begomoviruses have a monopartite genome, whereas no such genome type has been identified that is unequivocally native to the western hemisphere (WH). Bipartite begomoviruses are present in both the EH and WH. The begomovirus monopartite genome is essentially homologous to the DNA-A component of the EH bipartite begomoviruses, which encodes six proteins (Rojas et al., 2005).

Monopartite begomoviruses are frequently associated with two classes of satellite molecule, referred to as alphasatellites (Briddon et al., 2004; Mansoor et al., 1999) or betasatellites (Briddon et al., 2008), and thus far are exclusively found in the EH. However, recently alphasatellites have been

The GenBank/EMBL/DDBJ accession numbers for sequences of the following isolates determined in this study are given in parenthesis after the name of the isolate: pAlb1.2 (FJ956701), pAlb1.3 (FJ956702), pAlb2.2 (FJ956700), pAlb2.3 (FJ956703), pAlb2.6 (FJ956704), pAlb3.4 (FJ956705) and pAlb4.3 (FJ956707).

Two supplementary figures and two supplementary tables are available with the online version of this paper.
identified in conjunction with WH bipartite begomoviruses (Paprotka et al., 2010; Romay et al., 2010). Begomovirus-associated satellites are circular ssDNA molecules that are approximately half the size of the helper begomovirus genome. They rely upon the helper virus for encapsidation (transmission by insect) and systemic infection of the plant host (Briddon & Stanley, 2006). Alphasatellites encode a nanovirus-like replication-associated protein and so are capable of autonomous replication in host plants cells (Saunders & Stanley, 1999). In contrast, betasatellites depend upon the helper virus for replication (Cui et al., 2004; Saunders et al., 2004, 2008).

Betasatellites are required for the development of wild-type symptoms in naturally infected hosts and enhance helper begomoviral pathogenicity by modulating host defences (Briddon et al., 2003; Jose & Usha, 2003; Zhou et al., 2003; Saunders et al., 2004; Cui et al., 2005; Idris et al., 2005; Saeed et al., 2005). A major distinction between the two satellite types is that alphasatellites are thought to be dispensable and do not contribute to disease development (Saunders et al., 2000; Briddon et al., 2004; Briddon & Stanley, 2006). A thus-far rare subclass of alphasatellite, referred to as the ‘DNA-2’ type, shares low nucleotide sequence identity with the commonly occurring ‘DNA-1’ type, which has (until this report) been found in association with the monopartite Ageratum yellow vein virus (AYVV) from Singapore (Saunders et al., 2002). DNA-1 and DNA-2 alphasatellites have a similar genomic organization; however, no conclusive contribution to pathogenicity has been demonstrated for either satellite type.

Previously, we have reported the discovery of a new strain of *Tomato yellow leaf curl virus* [the Oman strain (TYLCV-OM)] and its associated tomato leaf curl betasatellite (ToLCB) from symptomatic tomato plants in Oman (Khan et al., 2008). In this study, we report the discovery of another new monopartite begomovirus and only the second example of a 'DNA-2'-type alphasatellite, in the same field-infected tomato plant from which the TYLCV-OM–betasatellite complex was previously cloned. Until now the DNA-2-type alphasatellite was considered an anomaly, but now it is expected that additional DNA-2-type satellites will be found. We also provide the first demonstration that a DNA-2-type satellite has a role in pathogenicity, and more specifically, its ability to attenuate disease symptoms by modulating the activity of its associated betasatellite, which is expected to encode a suppressor of host-plant gene silencing.

**RESULTS**

**Analysis of clone Alb2.2**

The complete nucleotide sequence of 11 clones carrying a ~2.7 kb insert was determined. DNA sequence alignment revealed the presence of six groups. The sequences in each of the groups shared 100% nucleotide identity, and so only one isolate from each group was included in the analysis. The isolates, pAlb1.2 (FJ956701), pAlb1.3 (FJ956702), from sample Alb1, pAlb2.2 (FJ956700), pAlb2.3 (FJ956703) and pAlb2.6 (FJ956704), from sample Alb2 and pAlb3.4 (FJ956705) from sample Alb3, were deposited in GenBank. Each nucleotide sequence was screened against the GenBank database using BLASTN to identify the most closely related taxa, whose sequences were then used for phylogenetic and other sequence comparisons.

Sequence alignment showed that five clones (pAlb1.2, pAlb1.3, pAlb2.3, pAlb2.6 and pAlb3.4) shared greater than 94% nucleotide sequence identity with the previously reported TYLCV-OM[OM:Alb:05] (DQ644565). Based on the species demarcation cut-off for begomoviruses, >89% nucleotide sequence identity (Fauquet et al., 2003, 2008), they are all isolates of TYLCV-OM. Overall the sequences for the clones showed a greater level of sequence identity, at 91–99%, with previously studied TYLCV strains or isolates from Oman and Iran, than to other TYLCV strains/isolates (84–87%), indicating that they are isolates of TYLCV-OM. They are designated OM[OM: Alb1.2 : 05], OM[OM: Alb1.3 : 05], OM[OM:Alb2.3 : 05], OM[OM:Alb2.6 : 05] and OM[OM:Alb3.4 : 05]. The TYLCV variants were representative of isolates cloned from all six of the field-collected tomato samples. In contrast, the nucleotide sequence of pAlb2.2, which consists of 2763 nt, shared only 86% nucleotide identity with its closest relative, TYLCV-OM[OM:Alb2.6 : 05], both of which were cloned from sample Alb2. This clone thus represents a new species in the genus *Begomovirus* and the name *Tomato leaf curl Oman virus* (ToLCOMV) is proposed.

The genome organization of ToLCOMV is similar to that of other monopartite begomoviruses, with six conserved genes (Table 1). The non-coding sequence that is located between the C1 and V2/V1 ORFs [referred to as the intergenic region (IR)] is 289 nt in length and contains a conserved (between all geminiviruses) stem–loop structure, which contains, within the loop, the geminivirus-conserved nanonucleotide sequence TAATATTAC. This sequence represents a portion of the virion-strand origin of DNA replication (ori), and is nicked by the replication-associated protein (Rep) between the thymine and adenine residues (TAATATT↓AC) (Laufs et al., 1995; Hanley-Bowdoin et al., 2000). The ori also contains the Rep-binding region containing repeated sequence units known as iterons. The iterated sequences, predicted to be involved in ToLCOMV Rep binding, consist of one iteron (5'-GGGGGA-3') located between nucleotide coordinates 2630 and 2634 and two directly repeated units with a 4 nt spacer (5'-GGGGACTCTGGGGA-3') located between nucleotide coordinates 2656 and 2669, just upstream of the Tata box of the Rep promoter. Inspection of the N terminus of the Rep of ToLCOMV revealed the iteron-related domain [IRD; the amino acid sequences of Rep that are predicted to bind the iteron FCVN (Argüello-Astorga & Ruiz-Medrano, 2001)] (Table 1). The iterons and IRD of ToLCOMV were...
found to be identical to those of Papaya leaf curl virus (PaLCuV), a begomovirus that occurs on the Indian subcontinent. Also, the arrangement of the ToLCOMV iterons was similar to other monopartite begomoviruses from the Mediterranean–North Africa region, and consists of three directly repeated sequences upstream of the TATA box of the Rep-gene promoter and one inverted repeat (Arguèello-Astorga et al., 1994). Multiple attempts to detect a begomovirus DNA-B component using degenerate primers (Idris & Brown, 1998) were unsuccessful (results not shown).

Phylogenetic analysis

Phylogenetic relationships were reconstructed using the maximum-parsimony (MP) option of PAUP* and placed the newly cloned TYLCV variants from Oman with other TYLCV isolates originating from the Middle East, representing the Arabian peninsula and North Africa, and the Mediterranean region (Fig. 1). The analysis placed ToLCOMV in an intermediate position between the clade containing TYLCV-OM and that containing PaLCuV, indicating that ToLCOMV has diverged from ancestors (see recombination analysis) that are also shared by TYLCV, which is endemic to Iran. All TYLCV isolates from Oman formed a subclade containing taxa whose overall nucleotide variability was low (94–99% identity). In contrast, the Oman isolates of TYLCV shared 88–94% nucleotide identity with the two strains of TYLCV (AJ132711 and EU635776) from Iran. Comparative sequence analysis of the TYLCV ORFs revealed that the phylogenetic relatedness of V1, V2, C2, C3 and the 3' end of the C1 ORF, differed from that of the 5’ end sequence of the C1 and C4 ORFs, suggesting that this virus has undergone at least one recombination event in its evolutionary history (Table 1).

The close evolutionary relationship predicted between ToLCOMV and TYLCV-OM was corroborated by the results

<table>
<thead>
<tr>
<th>Virus Full-length genome</th>
<th>C1</th>
<th>C4</th>
<th>C2</th>
<th>C3</th>
<th>V1</th>
<th>V2</th>
<th>Rep binding iteron</th>
<th>Iteron-related domain</th>
</tr>
</thead>
<tbody>
<tr>
<td>TYLCV-OM[Alb2.6]</td>
<td>86.1</td>
<td>77.5</td>
<td>58.8</td>
<td>99.8</td>
<td>100.0</td>
<td>98.8</td>
<td>94.5</td>
<td>GGTTATTGGAGT</td>
</tr>
<tr>
<td>TYLCV-OM[Alb05]</td>
<td>83.4</td>
<td>76.2</td>
<td>60.4</td>
<td>97.5</td>
<td>96.8</td>
<td>96.6</td>
<td>93.7</td>
<td>GGTTATCGGAGT</td>
</tr>
<tr>
<td>PaLCuV-PK</td>
<td>74.6</td>
<td>84.3</td>
<td>94.1</td>
<td>68.9</td>
<td>71.6</td>
<td>71.6</td>
<td>71.6</td>
<td>GGGGACTCGGGGA</td>
</tr>
<tr>
<td>ToLCSDV-YE</td>
<td>75.8</td>
<td>76.1</td>
<td>64.3</td>
<td>88.4</td>
<td>89.8</td>
<td>72.4</td>
<td>95.7</td>
<td>TGTATATCGTAC</td>
</tr>
<tr>
<td>TYLCV-IL</td>
<td>82.1</td>
<td>72.5</td>
<td>55.7</td>
<td>86.7</td>
<td>91.8</td>
<td>97.0</td>
<td>95.4</td>
<td>GGTTATACGTGTG</td>
</tr>
<tr>
<td>TYLCV-Gez</td>
<td>81.4</td>
<td>76.7</td>
<td>62.0</td>
<td>87.4</td>
<td>90.0</td>
<td>95.6</td>
<td>94.0</td>
<td>GGTTATACGTGTG</td>
</tr>
<tr>
<td>TYLCV-Mld</td>
<td>83.4</td>
<td>77.1</td>
<td>62.7</td>
<td>86.7</td>
<td>91.8</td>
<td>98.6</td>
<td>95.4</td>
<td>GGTTATACGTGTG</td>
</tr>
</tbody>
</table>

Fig. 1. Phylogenetic relationships of Tomato leaf curl Oman virus (ToLCOMV) and Tomato yellow leaf curl virus sequences obtained here with selected begomoviruses. The begomoviruses included in the analysis were selected from the GenBank BLAST results. The sequences used were Papaya leaf curl virus (PaLCuV), Tobacco leaf curl Zimbabwe virus (TbLCZV), Tomato curly stunt virus (ToCSV), Tomato leaf curl Bangladesh virus (ToLCBV), Tomato leaf curl Malaysia virus (ToLCMYV), Tomato leaf curl Mayotte virus (ToLCYTV), Tomato leaf curl Sudan virus (ToLCSDV), Tobacco yellow leaf curl Malvi virus (TylCMVL), Tobacco yellow leaf curl Sardinia virus (TylCSV) and Tomato yellow leaf curl virus (TYLCV). The strain and isolate descriptors are as given in Fauquet et al. (2008). In each case the database accession number is given. The number placed at each major node indicates the percentage bootstrap confidence score for 1000 replicates.

Table 1. Percentage nucleotide sequence identities for the full-length genome and ORFs of Tomato leaf curl Oman virus (ToLCOMV) and selected monopartite begomoviruses

Values in bold highlight the highest nucleotide sequence identities with ToLCOMV for each gene compared. Other begomoviruses used in this comparative analysis and their accession numbers are: PaLCuV-PK, Papaya leaf curl virus-PK (PK:Cot:02), (AJ436992); Tomato leaf curl Sudan virus-YE, ToLCSDV-YE, (YE:Tih:06), (EF110890); TYLCV-IL, Tomato yellow leaf curl virus-IL (X15656); TYLCV-Gez, TYLCV-Gez (SD:96), (AY044138); TYLCV-Mld, TYLCV-Mld (IL:93), (X76319); and TYLCV-OM [OM:Alb05] (DQ644565). The iteron-related domain for ToLCOMV is FCVN.
of recombination analysis. Mapping of the aligned sequences for break points indicative of recombination with the multiple applications available in RDP-V2 Beta 08 (Martin et al., 2005) and PhylPro version 0.8.1.0 (Weiller, 1998) (each at the default settings) resulted in a similar result for all types of analyses. CHIMAERA (Posada & Crandall, 2001), GENECOIN (Sawyer, 1989), MAXCHI (Smith, 1992), RDP (Martin et al., 2005) and PhylPro consistently identified fragments of approximately 1768 nt, between nucleotide coordinates 253 and 2020 (fragment I), and between coordinates 2021 and 2742, about 522 nt, (fragment II) to be of interspecific origin. Because multiple analyses yielded a concordant conclusion only the PhylPro results are presented (Fig. 2b).

A detailed analysis of the alignment indicated that ToLCOMV probably evolved as the result of a sequence exchange between TYLCV-OM (fragment I) and PaLCuV (fragment II). Furthermore, a sequence comparison involving fragment I showed that ToLCOMV and TYLCV-OM[OM:Alb2.6:05] shared 98.0 % identity, whereas the two viruses shared only 86.1 % nucleotide identity across the entire sequence, and only 64.1 % identity across fragment II of ToLCOMV, which contains the cognate cis-acting motif in the IR and the 5’ end of the Rep gene from PaLCuV-PK[PK:Cot:02] (AJ436992) (Fig. 2b). Maintenance of the cognate motif in the IR and at the Rep 5’ end (see Hanley-Bowdoin et al., 2000), respectively, appears to have been essential for selection of this recombinant begomovirus. The nucleotide sequence comparison of fragment II, which is less than one-fifth unit size in relation to the begomovirus genome, showed that it shared 93.0 % nucleotide identity with ToLCOMV and PaLCuV, even though the latter two viral species share only 74.6 % nucleotide sequence identity across the entire genome sequence, and only 71 % for fragment I.

### Alphasatellite sequence analysis

The complete nucleotide sequence for six clones carrying 1379 bp was determined. Sequence comparisons revealed that they all shared 100 % nucleotide sequence identity, and for this reason a single representative sequence, pAlb4.3 (FJ956707), was deposited in GenBank. The nucleotide sequence comparisons revealed that this clone shared 90 % nucleotide sequence identity with *Ageratum* yellow vein Singapore alphasatellite (AYVSGA-[SG:97]) (AJ416153), an unusual alphasatellite that is distinct from all other alphasatellites characterized thus far (referred to by the authors as ‘DNA-2’; Saunders et al. 2004). Therefore, pAlb4.3 is considered a strain of AYVSGA and herein named AYVSGA-[OM:Alb:05]. A search of the genome for ORFs (encoding proteins >10 kDa) revealed the presence of a Rep-associated protein gene on the positive strand, located between nucleotide coordinates 75 and 941. The phylogenetic analysis grouped the two AYVSGA strains in a clade distinct from all other alphasatellites (Fig. 3).
Infectivity of cloned helper begomoviruses and satellites

Tomato seedlings that were agro-infiltrated with pG–Alb2.2 and/or pG–Alb2.3, harbouring the cloned ToLCOMV and TYLCV-OM genomes, respectively, developed systemic symptoms beginning 7–8 days post-inoculation (p.i.). ToLCOMV induced mild leaf curling and stunting, whereas TYLCV-OM-infected plants developed moderately severe leaf curling and chlorotic margins on the leaflets. Tomato seedlings co-inoculated with both viruses developed more severe yellow leaf curling and stunting than with ToLCOMV alone (Fig. 4). Symptoms in tomato seedlings that were co-inoculated with the dimeric betasatellite ToLCB and with either of the helper viruses, or a mixture of the two helper viruses, developed more severe symptoms compared with the symptoms observed in tomato plants inoculated with either of the helper viruses alone (not shown), or with a mixture of the two viruses (Fig. 4).

Nicotiana benthamiana plants inoculated with ToLCOMV alone developed upward leaf curling, stunted growth, vein thickening and yellowing, foliar epinasty and narrowed margins on the developing leaves, whereas plants inoculated with only TYLCV-OM developed severe downward leaf curling symptoms and the newest developing leaves were reduced in size (Fig. 5). Similar to the observations for inoculated tomato plants, the N. benthamiana plants co-inoculated with the betasatellite and one or both helper viruses showed more severe symptoms than those

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**Fig. 3.** Phylogenetic relationships for *Ageratum* yellow vein Singapore alphasatellite (AYVSGA-OM[OM:Alb:05]) cloned from symptomatic tomato plants collected in Oman. Begomovirus-associated alphasatellites included in the analysis were selected from the GenBank BLAST results. Alphasatelites used in the analysis are *Ageratum* yellow vein Kenya alphasatellite (AYVKA), Cotton leaf curl Dabwali alphasatellite (CLCuDaA), Cotton leaf curl Gezira alphasatellite (CLCuGA), *Coconut foliar decay virus* (CFDV) (out-group), Hollyhock leaf crimple alphsatellite (HLCrA), Malavstrum yellow mosaic alphasatellite (MalYMA), Okra leaf curl alphsatellite (OLCuA) and Sida yellow vein Vietnam alphsatellite (SiYVA). In each case the database accession number is given. The number at each major node indicates the percentage bootstrap confidence score for 1000 replicates.

**Fig. 4.** Symptoms observed in young tomato *Solanum lycopersicum* plants 4 weeks p.i. with the different possible combinations of helper viruses and satellites, and mock-inoculated tomato plants.
inoculated with either helper virus alone. However, disease symptoms were milder overall for the combinations that included AYVSGA (Fig. 5).

The presence of the helper viruses and their associated satellite molecules in total DNA extracted from newly developing leaves was confirmed by PCR amplification using primers specific for each of the components used to inoculate plants. Results indicated that when the helper virus(es) was present alone and/or in combination with one or both satellites, all components that were expected to be present (based on their inclusion in the inoculum) were detected by PCR in systemically infected tomato and \textit{N. benthamiana} plants (Supplementary data, available in JGV Online). However, neither satellite was detectable by PCR in plants inoculated with either of the satellites alone, or when they were co-inoculated into plants in the absence of a helper virus (data not shown). Seedlings infiltrated with cultures of \textit{Agrobacterium} harbouring the binary vector that lacked an insert (mock-inoculated control) did not develop symptoms (Fig. 5) and were negative for the respective virus genomes or satellites when tested by PCR. These experiments therefore demonstrated that the clones pAlb2.2 and pAlb2.3 each harboured a full-length, infectious begomovirus genome, and furthermore that the AYVSGA (pAlb4.3) and the betasatellite ToLCB (pAlb01) were maintained in plants only when they were co-inoculated with at least one of the helper begomoviruses. This observation confirmed previous findings that betasatellites and alphasatellites are helper virus dependent for systemic movement (Briddon \textit{et al.}, 2003; Dry \textit{et al.}, 1997; Saunders \textit{et al.}, 2000; Saunders & Stanley, 1999).

\textbf{Fig. 5.} Symptoms observed in \textit{N. benthamiana} plants 4 weeks p.i. with the different possible combinations of helper viruses and satellites, and mock-inoculated \textit{N. benthamiana} plants.
Effect of alphasatellite on virus and betasatellite replication

To demonstrate further that begomoviruses and the associated satellites were capable of systemically infecting test plants, total DNA was isolated from *N. benthamiana* and subjected to Southern analysis. The analysis showed that both of the helper viruses, ToLCOMV and TYLCV-OM, were present in the newly developing leaves (Fig. 6a). Also AYVSGA (Fig. 6b) and ToLCB (Fig. 6c) were readily detectable in the newly developing leaves of plants co-inoculated with the helper virus clones, ToLCOMV and/or TYLCV-OM. Co-infection of plants with AYVSGA or ToLCB alone or in a mixed infection resulted in the reduced accumulation of betasatellite molecules; however, no significant difference in the accumulation of either of the helper viruses was similarly observed (Fig. 6). This result revealed for the first time that AYVGSA, a DNA-2-type alphasatellite, has the ability to interfere with the accumulation of betasatellite DNA.

**DISCUSSION**

The combined results from this and a previous study (Khan et al., 2008) provide substantial evidence that the yellow leaf curl and leaf curl diseases of tomato in the Sultanate of Oman are caused by a complex of monopartite begomoviruses and one or two different types of associated DNA satellite that each contribute uniquely to virulence. This is based on the variation in symptom severity. Here, we have cloned and determined the complete DNA sequence of a second monopartite begomivirus present in field-infected tomato plants, whose genome shares less than 89% nucleotide identity with its closest relative, TYLCV-OM, with which it was found co-infecting tomato plants in Oman (Fig. 1). Based on the close phylogenetic relatedness of ToLCOMV with other begomoviruses identified previously from the region, this species is probably endemic to Oman. In accordance with the International Committee on Taxonomy of Viruses guidelines for begomovirus species demarcation (Fauquet et al., 2003, 2008) this new species is herein designated ToLCOMV. These results demonstrate the collective
involvement of at least four ssDNA molecules in leaf curl and/or yellow leaf curl disease aetiology in Oman, with each contributing distinctively to the varied symptom phenotypes observed in field-infected tomato plants.

The infectivity studies and positive detection of viral progeny by Southern hybridization confirmed that both monopartite helper virus genomes are infectious and capable of systemic infection of tomato and *N. benthamiana* in the absence of either satellite. *N. benthamiana* plants infected with ToLCOMV and TYLCV-OM exhibited symptoms most like those observed in tomato experimentally infected with TYLCV-OM, although several plants also developed upward leaf rolling, a hallmark of ToLCOMV infection of tomato (Fig. 5).

The most severe symptom phenotype was observed in tomato or *N. benthamiana* plants co-inoculated with TYLCV-OM and ToLCB, and was reminiscent of the most frequently observed symptom in field-infected tomato plants in Oman. When AYVSGA was co-inoculated to tomato and *N. benthamiana* plants with TOLCOMV/TLVC and/or ToLCB, disease symptoms were attenuated and betasatellite molecules accumulated to a lower relative level compared with plants inoculated with the helper virus(es) and betasatellite alone (Fig. 6c). However, AYVSGA did not have a discernible effect on the accumulation of either of the helper begomoviruses (Fig. 6a). Previous studies have shown that several other alphsatellites are capable of replicating and systemically infecting their plant host in the presence of a helper begomovirus without a discernible effect on symptom development or virulence (Briddon et al., 2003; Kon et al., 2009). This result therefore provides the first example of a ‘DNA-2’-class alphsatellite that ameliorates symptom severity in an infected host. Betasatellite have been shown not only to enhance symptom severity in plants co-infected with the helper begomovirus, but also to be required for development of bona fide disease symptoms in some hosts (Idris et al., 2005). Furthermore, betasatellites have also been shown to increase the accumulation of the helper virus (Saunders et al., 2000; Briddon et al., 2003), thereby increasing viral virulence.

Although unprecedented among alphsatellites until now, this DNA-2-type satellite from Oman, AYVSGA, is capable of reducing symptom severity (virulence) and also reduces the relative accumulation of its associated betasatellite, ToLCB. It has been hypothesized that alphsatellites have been acquired by helper begomoviruses to modulate virulence to achieve increased viral fitness (Saunders et al., 2000; Wu & Zhou, 2005). Most recently, two ‘DNA-1-type’ alphsatellites that are phylogenetically divergent from the DNA-2-type alphsatellite described here have each been shown to attenuate symptoms caused by their helper begomovirus and to encode a Rep protein that is a strong suppressor of silencing (Nawaz-ul-Rehman et al., 2010). The specific molecular and cellular mechanisms underlying the interactions between the Oman helper begomoviruses, and the associated betasatellite and type-2 alphsatellite (DNA-2 type) remains to be elucidated. However, we hypothesize that symptom attenuation and a relative reduction in betasatellite accumulation might result from DNA-2-mediated modulation of betasatellite activity.

Clearly, whether the DNA-2 alphsatellite is directly or indirectly involved in suppression of betasatellite C1 and/or in non-coding betasatellite functions needs to be determined.

The results of this study have demonstrated that a previously undiscovered recombinant begomoviral species infects tomato plants in Oman. The recombinant virus systemically infects *Datura stramonium*, a solanaceous weed common in the region, ‘red kidney’ bean *Phaseolus vulgaris* and *Petunia hybridae*, a highly appreciated ornamental, in addition to the naturally infected field host, tomato *Solanum lycopersicon* and the virus-permissive experimental host *N. benthamiana* (Figs 4 and 5; Supplementary data). The cloned viral genomes are independently capable of causing mild symptoms in both tomato and *N. benthamiana* but TYLCV-OM is the more virulent of the two viruses. One explanation for the enhanced virulence of TYLCV-OM over ToLCOMV could be because of the acquisition through recombination of its ‘divergent’ C4 ORF from a PaLCuV-like parent from Asia. This may contribute a unique function to ToLCOMV virulence, as compared with the C4 ORF sequence of TYLCV-OM and the other regional TYLCV strains, which are relatively highly conserved among these endemics. Also, under experimental conditions several TYLCV strains from the region were found to cause more moderate symptoms in tomato than does ToLCOMV (not shown). Indeed, studies have implicated the C4 proteins of monopartite begomoviruses as being determinants of both symptom development (Krake et al., 1998; Rigden et al., 1993, 1994) and systemic movement (Jupin et al., 1994; Rojas et al., 2001).

Our results indicate that ToLCOMV appears to have arisen from interspecific recombination between TYLCV that is native to the Middle East, and PaLCuV, which is native to the Indian subcontinent. The high degree of nucleotide identity detected in fragment I (Fig. 2b), a sequence that is shared between ToLCOMV and TYLCV-OM[OM;Alb2.6:05] suggests that this recombination event probably occurred rather recently. Recombination is a major mechanism of begomovirus evolution (Garcia-Arenal et al., 2001), and the results presented here for ToLCOMV support the contribution of recombination to diversification and speciation among yet another begomovirus. Indeed, several recombinants have emerged within the last few years in the Mediterranean region, namely *Tomato yellow leaf curl Axarquia virus* (Garcia-Andres et al. 2006) and *Tomato yellow leaf curl Malaga virus* (Monci et al., 2002); both involved genetic exchanges between TYLCV and *Tomato yellow leaf curl Sardinia virus*. For the latter recombinants the natural host range(s) of each of the parental viruses is relatively certain, and
probably comprises tomato and solanaceous weeds. In the case of ToLCOMV, tomato is the only known host of one parent virus, TYLCV-OM. Because the sequence has been determined for only two cloned PaLCuV genomes, one from papaya, PaLCuV-IN[IN:Luc](Y15934), and another from cotton plants exhibiting leaf curl symptoms, PaLCuV-P[K:PK:Cos:OM2] (AJ436992), the exact origin of the second ToLCOMV parent virus remains uncertain but would feasibly seem to be southern Asia. Furthermore, PaLCuV, the predicted donor of ToLCOMV C4 (according to recombination analysis of fragment II), is known to cause leaf curl disease in cotton in the presence of the Cotton leaf curl Multan betasatellite (Mansoor et al., 2003b). However, infectivity of the cloned PaLCuV genome in papaya has not been demonstrated (Saxena et al., 1998), and it is not known whether PaLCuV infects solanaceous species.

Owing to the close proximity and the extensive trade that occurs between southern Asia and the Middle East, PaLCuV could feasibly have been introduced from the Indian subcontinent, so it may be possible to locate the unidentified parent virus there in wild or cultivated species. The close sequence relatedness of the two AYVSGA isolates, one each from Singapore and Oman, suggests that they have a common origin. The phylogenetic evidence suggests that the ‘founder complex’, comprising PaLCuV and the associated alphasatellite AYVSGA, originated in southern Asia and were co-introduced into Oman. An endemic parent TYLCV already associated with ToLCB recombined with PaLCuV to create ToLCOMV. Of the original introduced complex, the DNA-2 satellite was the only known extant survivor. Although there is extensive trade between Oman and South-east Asia, which would provide a possible means for the introduction of this DNA-2-type alphasatellite, it would seem unlikely that one component of the complex (helper virus or alphasatellite) would have been introduced without the other. However, to date there is no evidence that the putative ‘second parent’ is extant, leading to the hypothesis that the Asian helper-alphasatellite DNA-2 were not overwhelmingly fit in the ‘local Oman context’. Thus the DNA-2 alphasatellite has instead adopted new helper viruses, rendering the complex highly fit by down-modulating the betasatellite activity required for virulence by the endemic TYLCV virus.

In summary, evidence provided here and elsewhere indicates that several types of alphasatellite are associated with begomovirus–betasatellite complexes (Briddon et al., 2004; Mansoor et al., 1999). However, a role for alphasatellites in disease aetiology or virulence has not been shown. Here we have demonstrated for the first time that combinations of one or two helper begomoviruses, with a typical betasatellite and DNA-2-type alphasatellite can systemically infect tomato and N. benthamiana plants, and cause variable symptoms in tomato that are reminiscent of those observed in field-infected tomato plants. Owing to the decreased symptom severity observed when the two satellites were present in a mixed infection, compared with a single infection, with either or both helper begomoviruses, it is tempting to speculate that the alphasatellite modulates begomovirus–betasatellite pathogenicity by interfering with βC1, a key virulence factor (Briddon et al., 2003).

METHODS

Viruses. Leaves from six symptomatic tomato plants were collected from fields in the Al-Batinah (Alb) region, Oman, during the 2005 tomato-growing season. The symptomatic plants exhibited moderate to severe symptoms that included leaf curling, and/or chlorosis, reduced leaflet size and stunting. Total DNA was isolated from tomato leaf samples using the CTAB method (Doyle & Doyle, 1987).

Cloning of begomoviral genomes and satellite DNAs. Total DNA isolated from the symptomatic tomato plants was used as a template to amplify begomovirus genome and satellite DNA molecules by rolling circle amplification technology (RCA; Inoue-Nagata et al., 2004) using a TempliPhi 100 amplification kit (Amersham Biosciences) as previously described (Khan et al., 2008; Idris et al., 2007). The RCA product was digested with restriction endonucleases to identify unique sites for cloning unit length molecules (~2.7 or ~1.4 kb; not shown). Restriction products of ~2.7 and ~1.4 kb were ligated into pGEM5Zf+(Promega) as NcoI fragments. The inserts of recombinant plasmids were completely sequenced using a primer walk strategy. The complete nucleotide sequences were blasted against GenBank (NCBI) and nucleotide sequences for the top hits were included in a comparative analysis. Using MEGAALIGN (DNAstar) sequences were aligned with the CLUSTAL W option for the calculation of nucleotide identities and with the CLUSTAL V option for the phylogenetic analysis. Sequence alignments were used to reconstruct phylogenetic trees using the MP option in PAUP* (Swofford, 2002). Simplex sequences were used to identify all putative recombinatorial breakpoints attributable to possible recombination, using RDP2 (Martin et al., 2005), and PhylPro (Weiller, 1998). The default search parameters for scanning the aligned sequences for recombination were implemented, and the highest acceptable probability (P value) was set for RDP2 at 0.001.

Production of constructs for Agrobacterium-mediated inoculation. Two recombinant plasmids carrying helper begomoviruses, pAlb2.2 and pAlb2.3, were selected for the construction of agroclones into a binary vector, pGreen0029 (Hellens et al., 2000). pAlb2.2 was digested with NcoI/XhoI to obtain a 2481 bp fragment, and with NcoI/Xhol to obtain a 2500 bp fragment. pG0029 was digested with XbaI/Xhol to obtain a 4569 bp fragment. The three fragments were ligated. The recombinant plasmid pG–Alb2.2 was selected for biological characterization. pAlb2.3 was digested with Ncol/BamHI to obtain a 923 bp fragment and with Ncol/PstI to obtain a 2259 bp fragment. pG0029 was digested with BamHI/PstI to obtain a 4624 bp fragment and finally, the three fragments were ligated into the plasmid vector. The recombinant plasmid pG–Alb2.3 was selected for biological characterization. The clones pAlb4.3, harbouring AYVSGA, and pAlb01, carrying ToLCB (Khan et al., 2008), were selected for the production of dimeric constructs using the partial digestion approach (Stenger et al., 1991). The resulting tandemly repeated copies for AYVSGA and ToLCB were released with Apal/Sacl and Ncol/Sacl digestion, respectively, and individually cloned into the binary vector pGSA1403, which had been linearized with either Apal/Sacl or Ncol/Sacl. The recombinant clones pGSA–Alb4.3 and pGSA–Alb01 were selected for biological characterization experiments. The recombinant plasmids pG–Alb2.2
and pG–Alb2.3 were introduced separately into aliquots of Agrobacterium tumefaciens strain GV3101, while pGSA–Alb4.3 and pGSA–Alb01, were transformed separately into LBA4404, all by electroporation. The unaltered plasmids pGreen0029 and pGSA1403 were introduced into the A. tumefaciens strains GV3101 and LBA4404, respectively, and used as mock-inoculation negative controls.

Agrobacterium-mediated inoculation and assessment of inoculated plants. Agrobacterium GV3101 cultures harbouring the constructs pG–Alb2.2 or pG–Alb2.3 were grown overnight at 28 °C in 50 ml Luria–Bertani broth supplemented with 50 mg ml−1 kanamycin. The slower-growing strain LBA4404 that was transformed with pGSA–Alb4.3 or pGSA–Alb01and was cultured for 48 h with 3 mg ml−1 chloramphenicol. The test plants were agro-infiltrated, as described by Hussain et al. (2005). The inoculation studies were conducted a minimum of three times. The agro-infiltrated plants were monitored for the appearance of symptoms in insect-free and secured growth rooms at 28 °C, with a daily cycle of 14 h light and 10 h dark for 4 weeks.

Southern hybridization. Total DNA was extracted from leaf samples using the method of Doyle & Doyle (1987). Approximately 7 µg of total DNA per sample was loaded in each lane and resolved by electrophoresis in a 1 % agarose gel in Tris/acetate-EDTA (TAE) buffer, pH 8.0, transferred by capillary action to Hybond-N+ nylon membranes (GE Healthcare) and UV cross-linked. The component-specific primers that were used for molecule detection in field samples (Supplementary data, available in JGV Online) were also used to amplify DNA probes. The PCR amplicons were cloned and the identification of the insert was confirmed by DNA sequencing. Probes were radioactively labelled using a Ready-To-Go kit (GE Healthcare) and α-32P-dCTP (PerkinElmer). Hybridization was carried out using virus-specific probes, obtained as described above, at 60 °C overnight, followed by washing the membrane at 60 °C twice in 2 x SSC (3.7 M sodium chloride, 0.375 M sodium citrate pH 7.0, 0.1% SDS) and twice at 60 °C in 0.2 x SSC, 0.1%SDS for 30 min each. The membranes were subjected to autoradiography by exposing an X-ray film for 1 h at −80 °C.

ACKNOWLEDGEMENTS

M.S.S. was supported by a PhD fellowship from the Higher Education Commission (HEC), Government of Pakistan. R.W. B. is supported by the HEC under the ‘Foreign Faculty Program’.

REFERENCES


Virology, a novel pathogenic phenotype and is becoming prevalent in Spain

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