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Post-transcriptional gene silencing suppressor activity of two non-pathogenic alphasatellites associated with a begomovirus

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ABSTRACT

Alphasatellites and betasatellites are begomovirus-associated single-stranded circular DNA molecules. Two distinct alphasatellites, *Gossypium darwinii* symptomless alphasatellite and *Gossypium mustelinium* symptomless alphasatellite, were previously isolated from *Gossypium davidsonii* and *G. mustelinium*. Here we show that the replication-associated proteins (Rep: a rolling-circle replication initiator protein) encoded by these alphasatellites interact with the Rep and C4 proteins encoded by their helper begomovirus, Cotton leaf curl Rajasthan virus (CLCuRaV), in a yeast two-hybrid assay. Both the alphasatellite-encoded Reps were found to have strong gene silencing suppressor activity, in contrast to the betasatellite-encoded βC1 and CLCuRaV-encoded C2, C4 and V2 proteins. The presence of alphasatellites maintained suppression of gene silencing in the youngest, actively growing tissue of CLCuRaV-betasatellite-infected plants. This is the first demonstration of a rolling-circle replication initiator protein with suppressor of gene silencing activity and provides a possible explanation for the selective advantage provided by the association of alphasatellites with begomovirus–betasatellite complexes.

Introduction

Monopartite begomoviruses (family Geminiviridae) that are associated with single-stranded DNA satellites are causing increasing problems for agriculture in Indo-China, a region that is the center of diversity of geminiviruses (Nawaz-ul-Rehman and Fauquet, 2009). Two classes of satellites associated with begomoviruses have been identified, which are known as alphasatellites and betasatellites (Briddon and Stanley, 2006). Betasatellites are, in most cases, essential for the helper begomovirus to induce typical disease symptoms in the host from which they were isolated and encode the major pathogenicity determinant of the complex (Briddon and Stanley, 2006; Saunders et al., 2004). In contrast, alphasatellites are not essential and appear to make no significant contribution to the pathogenicity of begomovirus–betasatellite disease complexes (Mansoor et al., 1999, Saunders and Stanley, 1999; Briddon et al., 2004).

Begomoviruses are transmitted by the whitefly *Bemisia tabaci* and occur in both the Old World (OW) and New World (NW). Only bipartite begomoviruses, with components known as DNA-A and DNA-B, are native to the NW. However, in the OW, the bipartite begomoviruses are out-numbered by the monopartite begomoviruses (Nawaz-ul-Rehman et al., 2009) whose genomes consist of a homolog of the DNA-A components of the bipartite viruses. The DNA-A components of bipartite begomoviruses encode all virus factors required for replication, control of gene expression and encapsidation/insect transmission, whereas the DNA-B component encodes two proteins involved in virus movement within and between plants. Monopartite begomoviruses lack the DNA-B component, yet their genomes are genetically indistinguishable from the DNA-A components of OW bipartite viruses. One consequence of this may be that monopartite begomoviruses have greater phloem limitation than their bipartite cousins; the implication being that the DNA-B-encoded movement proteins may provide more efficient escape from phloem-associated tissues (Rojas et al., 2001). The proteins encoded by the genomes of monopartite begomoviruses are the coat protein (CP; required for encapsidation and interaction with the whitefly for transmission), the V2 protein (possibly involved in virus movement), the replication-associated protein (Rep; a rolling-circle replication initiator protein—the only viral protein absolutely required for virus replication), the C2 protein (a transcription factor that up-regulates host gene expression and, for some viruses, up-regulates virion-sense [late] gene expression and for these viruses is known as the transcriptional activator protein [TrAP]), the replication enhancer protein (REn; a protein that up-regulates virus replication, possibly by interacting with Rep and host factors) and the C4 protein (which

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may be a pathogenicity determinant, involved in virus movement and involved in overcoming host defenses).

Betasatellites occur only in the Old World (Briddon et al., 2008; Briddon and Stanley, 2006). These satellites are ~1350 bp in size, approximately half the size of the genomes of their helper begomoviruses, and require the helper begomovirus for replication and movement in host plants as well as for insect transmission between plants. They encode a single gene in the complementary sense, known as βC1, the product of which has been shown to be a pathogenicity determinant, possibly involved in virus movement in plants, to bind DNA and to be a suppressor of post-transcriptional gene silencing (PTGS; a host defense mechanism, that is also known as RNA interference [RNAi]) (Saunders et al., 2004; Cui et al., 2005; Saeed et al., 2007). The vast majority of betasatellites have been identified in association with monopartite begomoviruses.

The begomovirus-associated alphasatellites (previously known as DNA-1) have until recently only been identified with monopartite begomoviruses that are associated with betasatellites in the Old World, although they were not found associated with all begomovirus–betasatellite complexes (Briddon et al., 2004). Significantly, Paprotka et al. (2010) recently showed the presence of alphasatellites in association with bipartite begomoviruses occurring in the New World. Alphasatellites are also approximately half the size of their helper begomoviruses (~1380 bp). They have a single large gene encoding a replication-associated protein (Alpha-Rep), which is a rolling-circle replication initiator protein. Consequently alphasatellites are capable of autonomous replication in the cells of host plants (and hence are described as satellite-like since, by definition, satellites rely on their helper virus for replication), although they require the helper begomovirus for movement within and insect transmission between plants. Although no definitive function has been ascribed to alphasatellites, their presence in plants infected with begomovirus–betasatellite complexes may reduce virus and/or betasatellite titres and possibly attenuate symptoms (Wu and Zhou, 2005).

Cotton leaf curl disease (CLCuD) is a serious constraint to cotton production in Pakistan and northwestern India. The disease first came to prominence during the mid-1980s and was epidemic during the 1990s, spreading to almost all cotton growing areas of Pakistan as well as into western India (reviewed by Briddon and Markham, 2001). The epidemic disease during this time was shown to consist of at least seven begomoviruses (occurring singly or as mixed infections), a disease-specific betasatellite (Cotton leaf curl Multan betasatellite [CLCuMuB]) and an alphasatellite. During the late 1990s, losses due to CLCuD diminished following the introduction of resistant cotton varieties. However, in 2001, CLCuD symptoms became evident on resistant cotton varieties and a resistance breaking form of the disease (known as the "Burewala" strain) has since spread to affect most cotton growing areas of Pakistan and western India (Mansoor et al., 2003). The disease in this case is associated with a newly identified begomovirus (cotton leaf curl Burewala virus [CLCuBuV]; Amrao et al., 2010) and a recombinant derivative of the earlier CLCuMuB (Amin et al., 2006).

During an analysis of the diversity of begomoviruses and associated satellites affecting a collection of exotic cotton species growing in Multan (Pakistan) (Nawaz-ul-Rehman, manuscript in preparation), we identified two unusual alphasatellites, *Gossypium darwinii* symptomless alphasatellite (GDarSLA; which is similar to previously identified alphasatellites but has not been identified elsewhere) and *Gossypium mustelinium* symptomless alphasatellite (GMusSLA; an unusual alphasatellite that is distinct from all other alphasatellites so far identified), as well as Cotton leaf curl Rajasthan virus (CLCuRaV; a species associated with the "Multan" strain of CLCuD), CLCuBuV and the recombinant form of CLCuMuB. Recently we have shown that, in contrast to earlier CLCuD-associated begomoviruses, CLCuRaV is dependent on the betasatellite CLCuMuB to systemically infect *Nicotiana benthamiana* (Nawaz-ul-Rehman et al., 2009). Here we have assessed the effects of the presence of alphasatellites on CLCuRaV/CLCuBuV infection of *N. benthamiana* and investigated the possible basis for these effects.

Table 1
Infectivity of viruses and satellites to *Nicotiana benthamiana*.

Inoculum	Symptom severity[c]	Infectivity (no. of plants infected/no. of plants inoculated)
CLCuRaV	0	0/45
CLCuRaV + CLCuMuB	5	44/45
CLCuRaV + GDarSLA	0	0/45
CLCuRaV + GMusSLA	0	0/45
CLCuRaV + GDarSLA + CLCuMuB	5 (3)[a]	45(30)/45[b]
CLCuRaV + GMusSLA + CLCuMuB	5 (3)[a]	43(26)/45[b]
CLCuBuV	1	40/45
CLCuBuV + CLCuMuB	4	42/45
CLCuBuV + GDarSLA	1	40/45
CLCuBuV + GMusSLA	1	42/45
CLCuBuV + GDarSLA + CLCuMuB	3	44/45
CLCuBuV + GMusSLA + CLCuMuB	3	43/45

The results are the cumulative totals for three independent experiments.
[a] The values in parentheses are the symptom severity rating for plants showing attenuated symptoms.
[b] The values in parentheses are the number of plants showing attenuated symptoms.
[c] The symptoms exhibited are given a rating on a 0–5 scale, as described in Materials and methods.

Results

High level replication and symptom attenuation of begomovirus infection by alphasatellites in plants during the early stages of infection

To investigate the requirements for the infectivity of the alphasatellites GDarSLA and GMusSLA, these were inoculated to *N. benthamiana* plants as dimeric constructs in combination with partial direct repeat constructs of CLCuRaV and CLCuBuV as well as a dimer construct of CLCuMuB, using *Agrobacterium tumefaciens*. Plants infiltrated with only GDarSLA or GMusSLA did not develop symptoms and no evidence of the presence of satellite-specific DNAs could be detected in systemic leaves, distal to the inoculation site, at 12 days post-inoculation (dpi) by PCR with specific primers (results not shown). Analysis of nucleic acids extracted from the inoculated patch using rolling-circle amplification (RCA; Haible et al., 2006) and digestion of the resulting product with specific restriction enzymes showed the presence of only the alphasatellite, with no evidence of the binary vector, indicative of replication.

Similarly, inoculation of plants with CLCuRaV alone did not result in symptoms or the presence of viral DNA in systemic tissues. However, when co-inoculated with CLCuMuB, symptoms of infection appeared at or around 12 dpi. The symptoms were a tight downward rolling of the leaf margins and a reduction in leaf size of newly developing leaves. When inoculated with CLCuRaV, CLCuMuB and either of the alphasatellites, the symptoms appeared at or around 12 dpi and were significantly milder (symptom severity rating 3), in the majority of *N. benthamiana* plants (Table 1), than for plants inoculated with only the virus and betasatellite (severity rating 5). Symptoms for plants infected with CLCuRaV, CLCuMuB and either of the alphasatellites were some mild crumpling of newly developing leaves that were similar in size to non-inoculated plants. Southern blot analysis showed that the accumulation of the betasatellite (CLCuMuB) was reduced in plants infected with CLCuRaV and betasatellite in the presence of either of the alphasatellites (Fig. 1B and C) in comparison to plants infected with only CLCuRaV and betasatellite (Fig. 1A). In contrast the DNA levels of the helper virus, CLCuRaV, were not significantly affected by the presence of the alphasatellites (Fig. 1D–F). The reduction in betasatellite mRNA levels in the presence of the alphasatellites was shown to be consistent in repeated northern blot analyses for three additional infected plants in each case.