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Effects of the mutation of selected genes of *Cotton leaf curl Kokhran virus* on infectivity, symptoms and the maintenance of Cotton leaf curl Multan betasatellite

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A B S T R A C T

Cotton leaf curl Kokhran virus (CLCuKoV) is a cotton-infecting monopartite begomovirus (family *Geminiviridae*). The effects of mutation of the coat protein (CP), V2, C2 and C4 genes of CLCuKoV on infectivity and symptoms in *Nicotiana benthamiana* were investigated. Each mutation introduced a premature stop codon which would lead to premature termination of translation of the gene. Mutation of the CP gene abolished infectivity. However, transient expression of the CLCuKoV CP gene under the control of the *Cauliflower mosaic virus* 35S promoter (35S-Ko^{CP}), at the point of inoculation, led to a small number of plants in which viral DNA could be detected by PCR in tissues distal to the inoculation site. Mutations of the V2, C2 and C4 genes reduced infectivity. The V2 and C2 mutants did not induce symptoms, whereas the C4 mutation was associated with attenuated symptoms. Infections of plants with the C4 mutant were associated with viral DNA levels equivalent to the wild-type virus, whereas viral DNA levels for the V2 mutant were low, detectable by Southern blot hybridisation, and for the C2 mutant were detectable only by PCR. Significantly, transient expression of the CLCuKoV C2 gene at the point of inoculation, raised virus DNA levels in tissues distal to the inoculation site such that they could be detected by Southern hybridisation, although they remained at well below the levels seen for the wild-type virus, but reduced the infectivity of the virus. These findings are consistent with earlier mutation studies of monopartite begomoviruses and our present knowledge concerning the functions of the four genes suggesting that the CP is essential for long distance spread of the virus in plants, the C4 is involved in modulating symptoms, the C2 interferes with host defence and the V2 is involved in virus movement. The results also suggest that the V2, C2 and C4 may be pathogenicity determinants. Additionally the effects of the mutations of CLCuKoV genes on infections of the virus in the presence of its cognate betasatellite, Cotton leaf curl Multan betasatellite (CLCuMuB), were investigated. Mutation of the C4 gene had no effect on maintenance of the betasatellite, although the betasatellite enhanced symptoms. Inoculation of the C2 mutant with CLCuMuB raised the infectivity of the virus to near wild-type levels, although the numbers of plants in which the betasatellite was maintained was reduced, in comparison to wild-type virus infections with CLCuMuB, and viral DNA could not be detected by Southern hybridisation. Transient expression of the C2 gene at the point of inoculation raised virus DNA levels in tissues distal to the inoculation site but also reduced the infectivity of the virus and the numbers of plants in which the betasatellite was maintained. CLCuMuB restored the infectivity of the V2 mutant to wild-type levels but only in a small number of plants was the satellite maintained and infections were non-symptomatic. Although inoculation of the CP mutant with CLCuMuB did not restore infectivity, co-inoculation with 35S-Ko^{CP} increased the number of plants in which the virus could be detected, in comparison to plants inoculated with the mutant and 35S-Ko^{CP}, and also resulted in two plants (out of 15 inoculated) in which the betasatellite could be detected by PCR. This indicates that the V2, C2 and almost certainly the CP are important for the maintenance of betasatellites by monopartite begomoviruses. The significance of these findings is discussed.

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1. Introduction

Viruses of the family *Geminiviridae* have small, single-stranded (ss)DNA genomes that are encapsidated in characteristic twinned quasi-icosahedral particles and are ascribed to one of the four

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genera (*Topocuvirus*, *Curtovirus*, *Mastrevirus* or *Begomovirus*) based upon genome arrangement, insect vector and sequence relatedness (Brown et al., 2012). The genus *Begomovirus* encompasses the economically most destructive geminiviruses that are transmitted by the ubiquitous whitefly *Bemisia tabaci*. All begomoviruses originating from the New World (NW) typically have genomes consisting of two components, referred to as DNA A and DNA B, both of which are required for virus infectivity. In the Old World (OW), although a small number of bipartite begomoviruses are known, the majority have only a single genomic component, a homolog of the DNA A of the bipartite viruses. A small number of these are truly monopartite, their single component genomes inducing disease in plants in the field, such as *Tomato leaf curl virus* (ToLCV) in Australia (Dry et al., 1993). Most monopartite begomoviruses instead associate with ssDNA satellites known as betasatellites (earlier referred to as DNA β) and satellite-like components known as alphasatellites (earlier referred to as DNA 1; Briddon and Stanley, 2006).

The genomes of monopartite (and DNA A components of bipartite) begomoviruses are typically ~2800 nucleotides in length and transcribe genes bi-directionally from a non-coding intergenic region which contains promoter elements and the origin of virion-strand DNA replication. The virion-sense strand encodes the coat protein (CP; required for insect transmission and movement in plants) and V2 protein (believed to be involved in virus movement in plants; Rojas et al., 2001). The complementary-sense strand genes encode the replication-associated protein (Rep; the only virus-encoded gene product required for viral DNA replication, which is a rolling circle replication initiator protein; Hanley-Bowdoin et al., 2004), the C2 protein (which for some begomoviruses up-regulates the late, virion-sense genes [and is then known as the transcriptional activator protein; TrAP], is a suppressor of post-transcriptional gene silencing [PTGS; Yang et al., 2007] and also overcomes virus induced hypersensitive cell death [Hussain et al., 2007; Mubin et al., 2010]), the replication enhancer protein (that is involved in establishing an environment conducive for virus replication; Settlage et al., 2005) and the C4 protein (the function of which remains unclear but for some viruses is a pathogenicity determinant and a suppressor of PTGS; Gopal et al., 2007; Saeed et al., 2008; Vanitharani et al., 2004).

Betasatellites are small (~1350 nucleotides in length) ssDNA satellites that occur exclusively in the OW and associate with monopartite begomoviruses (Briddon et al., 2008). Since they were first identified in 1999 (Saunders et al., 2000), more than 400 full-length betasatellite sequences have been deposited with the databases. Although considerable advances have been made in determining the contributions made by betasatellites to begomovirus–betasatellite complexes, little is known about the contributions made by the virus for maintenance of betasatellites. All betasatellite functions have been shown to be mediated by the single product they encode, βC1, which include suppression of PTGS and extending virus host range (reviewed by Amin et al., 2010). Betasatellites are not capable of autonomous replication and thus rely on the virus-encoded Rep. However, the precise interactions between Rep and the betasatellite DNA to initiate replication remain far from clear at this time (Saunders et al., 2008).

Several studies have examined the effects of mutagenesis of genes on the infectivity and symptoms of monopartite begomoviruses. These studies concerned the monopartite begomoviruses *Tomato yellow leaf curl virus* (TYLCV) and ToLCV which are adapted to plants of the family *Solanaceae* and do not associate with betasatellites (Rigden et al., 1993, 1994; Wartig et al., 1997). The study described here examined for the first time a monopartite, *Malvaceae*-adapted, betasatellite-associated begomovirus – *Cotton leaf curl Kokhran virus* (CLCuKoV). In addition to assessing the effects of mutagenesis of genes on infectivity and symptoms, the effects of the mutations on maintenance of the cognate betasatellite, Cotton leaf curl Multan betasatellite (CLCuMuB), was assessed.

2. Materials and methods

2.1. PCR-mediated mutagenesis, production of constructs for infectivity and transient gene expression

Specific gene mutations were introduced into a CLCuKoV clone (acc. no. AJ496286) which has previously been shown to be infectious (Mansoor et al., 2003). Mutants were produced by designing back-to-back oligonucleotide primers (Supplementary Table 1), containing the desired sequence changes, and using these to PCR-amplify the complete viral genome. The mutations of the C2 gene were introduced into the area of the gene which does not overlap either the C1 or C3 genes. Mutation of the C4 gene consisted of a change from C to G at position 2297 introducing a stop codon in the C4 but only a silent change (no change in amino acid sequence) for the overlapping C1 gene. Resulting full-length virus clones were sequenced in their entirety to ensure no additional sequence changes were introduced.

A partial direct repeat construct for the CLCuKoV clone bearing a mutation in the V2 gene ($Ko^{\Delta V2}$) was produced by cloning an approx. 250 bp *Bam*HI–*Not*I fragment in pGreen0029 (Hellens et al., 2000). The full length genome, released as a *Bam*HI fragment, was then cloned into the unique *Bam*HI site of the pGreen0029 construct containing the 250 bp fragment. A similar strategy was followed for clones harbouring mutations of the CP gene ($Ko^{\Delta CP}$; ~650 bp *Kpn*I–*Not*I fragment), the C2 gene ($Ko^{\Delta C2}$; ~1400 bp *Xho*I–*Bam*HI) and the C4 gene ($Ko^{\Delta C4}$; ~550 bp *Xho*I–*Bam*HI fragment). The production of a construct for the *Agrobacterium*-mediated inoculation of CLCuMuB (acc. no. AJ298903; Briddon et al., 2001), in the binary vector pGreen0029, has been described previously (Saeed et al., 2005).

Constructs for the expression of CLCuKoV genes under the control of the *Cauliflower mosaic virus* (CaMV) 35S promoter were produced by PCR-mediated amplification of the coding sequences using specific oligonucleotide primers (Supplementary Table 1). Restriction endonuclease recognition sites for *Sal*I and *Bam*HI were included in the forward and reverse primers, respectively, to allow directional cloning in the expression vector pJIT163 (Guerineau et al., 1992). Resulting pJIT163 expression cassettes were transferred into the binary vector pGreen0029 as *Kpn*I–*Eco*RV fragments. PCR-mediated amplifications, restriction endonuclease digestion, and cloning were conducted by standard methods with enzymes obtained from Fermentas (Arlington, Canada).

2.2. Agrobacterium-mediated inoculation

Binary vector constructs were electroporated into *Agrobacterium tumefaciens* strain GV3101. *Agrobacterium* inocula were prepared and inoculated to plants, by infiltration, as described previously (Hussain et al., 2005, 2007). Plants were kept in an insect-free glasshouse at 25 °C with supplementary lighting to give a 16 h photoperiod or a growth chamber set for an 18 h photoperiod (200 μ/m²/s light intensity), at 25 °C day/22 °C night temperature and a relative humidity of 65%. Following inoculation plants were observed daily for the appearance of symptoms. At 25–30 days post-inoculation (dpi) the plants were photographed and leaf samples were harvested to isolate DNA for PCR and Southern blot analysis.

2.3. PCR-mediated diagnostics and Southern blot hybridisation

Total genomic DNA was isolated from plants as described previously (Dellaporta et al., 1983). PCR-mediated diagnostics was

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