ABSTRACT

Viruses of the family *Geminiviridae* have single-stranded DNA genomes encapsidated in characteristic geminate particles. Economically the most important amongst these are the begomoviruses (genus *Begomovirus*) that are transmitted by the whitefly *Bemisia tabaci*. Cotton production in Pakistan and India has been severely affected by cotton leaf curl disease since the late 1980s. The disease was associated with multiple monopartite begomoviruses, including *Cotton leaf curl Multan virus* (CLCuMuV) and *Cotton leaf curl Kokhran virus* (CLCuKoV), and a single disease specific DNA satellite known as Cotton leaf curl Multan betasatellite (CLCuMB). Following the introduction of CLCuD resistant cotton lines during the late 1990s a resistance breaking strain of CLCuD evolved, known as the Burewala strain. This is now endemic across most cotton growing areas of Pakistan and is associatted with a single monopartite begomovirus, *Cotton leaf curl Burewala virus* (CLCuBuV), and a variant of CLCuMB (referred to as the Burewala strain [CLCuMB^{Bur}]). Both CLCuBuV and CLCuMB^{Bur} have recombinant origins. The study presented here was designed to map the major transcripts of CLCuBuV and CLCuMB^{Bur} for comparison to the transcript maps of other begomoviruses/betasatellites and to investigate whether the recombination/mutation events involved in their evolution have affected gene expression at the level of transcription. Additionally the pathogenicity of the virus and its betasatellite and their interactions with the host were examined.

Northern blotting and RNA ligase-mediated rapid amplification of cDNA ends were used to map the major transcripts of CLCuBuV, CLCuKoV (one of the parents of CLCuBuV) and their associated betasatellites from infected *Nicotiana benthamiana* plants and described the upstream and downstream regulatory regions. Two complementary-sense transcripts of ~1.7 and ~0.7 kb were identified for both viruses. The ~1.7 kb transcript is similar in position and size to that of several begomoviruses and likely directs the translation of C1 and C4 proteins. Both complementary-sense transcripts can potentially direct the translation of C2 and C3 proteins. A single virion-sense transcript of ~1 kb, suitable for translation of the *V1* and *V2* genes was identified. A single complementary-sense transcript was also identified for the betasatellites.

All characterised begomoviruses encode a C2 protein (known as the transcriptional-activator protein for some) with the potential to encode a protein of ~ 134 amino acids (aa). Surprisingly, CLCuBuV encodes a truncated *C2* gene which is capable of encoding only 35aa. The role of this truncated C2 in symptom induction/pathogenicity, silencing suppression and also its effect on thirteen developmental microRNAs (miRNAs) was assessed and compared with the full length C2 encoded by CLCuMuV (the second parent of CLCuBuV)) and its N-terminal 35aa fragment. The *C2* gene of CLCuMuV, the naturally truncated *C2* gene of CLCuBuV (encoding 35aa) and a gene fragment of the CLCuMuV *C2* gene (encoding the first 35aa) were expressed in *N. benthamiana* using a *Potato virus X* (PVX) vector. All the three constructs induced leaf curling, vein thickening and leaf crumpling. However the C2 of CLCuMuV induced more severe symptoms that additionally included a severe necrotic response in both inoculated leaves and leaves developing subsequent to inoculation that was reminiscent of a hypersensitive response and the plants remained stunted.

Plants use RNA interference (RNAi) as a mechanism to counter viruses. In the arms race between plant and viruses, viruses have evolved proteins, referred to as suppressors of gene silencing that can interfere with the RNAi pathway and thus overcome the plant defense response. The C2 proteins of begomoviruses are known suppressors. Using conventional northern and western blot analysis the suppressor activity of CLCuMuV was confirmed. The analysis also showed that the 35aa peptides of both CLCuMuV and CLCuBuV exhibit suppressor activity, at least under the conditions of the analyses conducted here.

miRNAs are a family of endogenous, non-coding, small (21-24nt) RNAs that play an important role in regulating gene expression in both animals and plants. In plants, miRNAs are involved in a variety of activities, including development, signal transduction, protein degradation, responses to a wide array of stresses, maintaining genome integrity and defense against pathogens. Numerous studies have shown that interfering in miRNA-directed processes might be a general feature of virus pathogenicity. CLCuBuV, CLCuKoV and their cognate betasatellites were assessed for their effects in *N. benthamiana* on ten miRNAs known to be important in plant development. Additionally, the effects of the expression of C2 and both 35aa peptides from a PVX vector on the levels of thirteen miRNAs were studied. Northern blot

analysis using specific oligonucleotide probes for miRNAs showed that, in general, virus infection increases the accumulation of miRNAs. This increase was significantly more in association with their cognate betasatellites. However, each begomovirus induced a distinct pattern of response. The naturally truncated C2 of CLCuBuV increased the accumulation of miRNAs while C2 encoded by CLCuMuV decreased the accumulation. However, the 35aa fragment of CLCuMuV did not have a significant affect on miRNA levels.

Overall the results obtained show that, despite having a truncated C2, CLCuBuV is a “fitter” virus than either of its parents. The study provides further insight into the possible mechanism of resistance breaking by CLCuBuV/CLCuMB^{Bur}, which at this time remains unclear, and may allow novel mechanism(s) of resistance to be developed, as well as strategies to prevent future resistance breaking.