



The function of the a-rich region of the alphasatellite associated with the cotton leaf curl disease in Pakistan

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Abstract

A novel type of circular single stranded satellite-like DNA, known as alphasatellite (formerly known as DNA 1, was recently characterized and demonstrated to be associated with the monopartite Begomoviruses. Alphasatellite components are satellite like single stranded DNA (ssDNA) molecules associated with Begomoviruses (Geminiviridae) that require the betasatellite molecule to induce authentic disease symptoms in some hosts. Betasatellite is essential for induction of characteristic symptoms in plants. The function of alphasatellite in Begomovirus betasatellite infections remains unclear. It has been suggested that alphasatellite components may act to down regulate the virus infection by competing for cellular resources. Interestingly, they are closely related to the helper dependent Rep-encoding components of nanoviruses (a second family of single stranded, plant infecting DNA viruses), from which they are presumed to have been evolved. Alphasatellite molecules have two major sequence features. Firstly the component encodes a replication-associated protein (Rep), which is required to initiate the rolling circle replication. Consequently alphasatellite components are capable of self replication in host cells but require the helper Begomovirus to spread both within and between host plants. The second feature is a region of sequence rich in adenine (a-rich). To investigate the function of the a-rich sequence, this was deleted from the CLCuD alphasatellite by PCR mediated amplification. The a-rich deleted mutant of the alphasatellite remained capable of replication and systemic infection in plants, in the presence of a helper begomovirus. This indicates that the a-rich region is not required for replication or maintenance in plants.

Keywords: Alphasatellite, Begomovirus, Geminivirus, Pakistan, whitefly.

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INTRODUCTION

Geminiviruses are a group of single stranded circular DNA viruses that cause severe diseases in several economically important crops worldwide (Zhou et al.1998, Harrison and Robinson 1999). According to the number of viral genomes, Geminiviruses are divided into bipartite or monopartite viruses (Lazarowitz 1992). Begomoviruses are plant-infecting viruses with genomes that consist of either one or two molecules of circular ssDNA (Stanley et al. 2004). In the Old World, most Begomoviruses have

monopartite genomes and the majority of them are associated with the symptom modulating ssDNA satellites known collectively as betasatellite. Betasatellites are approx. 1360 nucleotides in length and are required by their helper begomoviruses to systemically infect the hosts from which they were isolated (Saunders et al. 2000, Bridson et al. 2001). The majority of Begomovirus-betasatellite complexes are also associated

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with an additional molecule. Alphasatellite components are satellite-like, circular ssDNA molecules approx. 1375 nucleotides in length. They encode a single gene, a rolling circle replication initiator protein, and are capable of autonomous replication in plant cells. Closely related to the replication associated protein encoding components of nanoviruses (a second family of plant infecting ssDNA viruses (Vetten et al. 2004)), from which they are believed to have evolved, they require a helper Begomovirus for movement within and between plants (Mansoor et al. 1999, Saunders and Stanley 1999). Interestingly, alphasatellite components are phenotypically silent, playing no part in the symptoms of the complex and their precise function remains unclear.

Cotton is an important source of fibre, feed, and edible oil. It is the fourth largest crop in terms of economic value in the USA and is grown in more than eighty other countries. Foreign exchange earnings from cotton are the mainstay of the Pakistan economy. The major problems faced by the cotton industry in Pakistan are abiotic and biotic stresses, insect resistance against pesticides, low quality of fibre and contamination in fibre. The future of Pakistan's share in the International cotton trade depends on utilization of emerging technologies and information generated from the cotton genome initiative. It is expected that utilization of cotton genome information will play a major role in improving resistance against major pathogens and in improving the yield and quality of fibre, feed, and oil. These technologies will enhance the production efficiency and will reduce both the monetary cost and environmental impact of cotton production.

MATERIAL AND METHODS

The plasmid construction

The a-rich region of the alphasatellite was

deleted by PCR. The primers used (forward primer, 5'AAAATATCGATGTTACCTTGCGG AAGG-3': reverse primer, 5'-TATTAATCG-ATTTATTCCATATATTCGCC-3') introduced a unique *Cla*I restriction site (underlined) to delete the a-rich region. The mutated amplification of alphasatellite was obtained by PCR (Fig. 2., lane 2). The reaction mixture consisted of a template DNA (diluted) 5 μ L, dNTPs (2 mM) 5 μ L, a PCR reaction buffer (10X) 5 μ L, MgCl₂ (1.5 mM) 3 μ L, primers (5 mM) 1 μ L of each (Forward and Reverse), a Taq DNA polymerase 0.5 μ L, and finally to make the volume 50 μ L nuclease free water was added. Denaturation, annealing, and extension temperatures were set at 94°C, 50°C, and 72°C each for 1 min. Thirty five cycles were repeated for each PCR reaction.

The agroinoculation of plants

The alphasatellite with the a-rich region deleted was cloned as a partial dimeric repeat constructs in the binary vector pGreen (Hellens et al. 2000). The constructs were then transformed into the *Agrobacterium tumefaciens* strain GV 3101. The production of constructs for *Agrobacterium*-mediated inoculation of Cotton leaf curl Multan virus and its associated betasatellite (Briddon et al. 2001) as well as the DNA A and DNA B components of Tomato leaf curl New Delhi virus (ToLCNDV) were described previously (Hussain et al. 2005). Plants were inoculated with viruses and alphasatellite constructs using *Agrobacterium* as described previously (Briddon et al. 2001). All plants were grown in controlled conditions in growth rooms at 25°C with a 16 h dark period/8 h light period with 65% humidity in small 5 inch diameter plastic pots containing clay, silt, sand, and compost in equal proportions. All plants were watered daily and with a Hoagland solution by Merck™ (60% Foliar Lettice, 10% Linear Sulphate, 0.37% w/w Iron Chelate, 9.57% w/w Nitrogen, 0.01% Catalytic Enzyme Glyosides, and Artificial colouring.).

RESULTS AND DISCUSSION

The mutated alphasatellite is stably maintained in plants by a helper Begomovirus

Nicotiana benthamiana plants were coagroinoculated with CLCuMV and CLCuMA Δ and maintained at 25°C in an insect free glasshouse. The typical symptoms of CLCuMV consisting of alphasatellite appeared after 3-4 w. These symptoms were indistinguishable from symptoms induced by the virus in the absence of CLCuMA Δ . *N. benthamiana* plants inoculated with CLCuMV, CLCuMB and or CLCuMA Δ does not have any effect on the symptoms severity of the plants (Fig. 3., panel B, C and D). Analysis of these plants by PCR showed the presence of CLCuMA Δ in the systemic tissues (Fig. 4, lane 2, 5 and 6). This finding was confirmed by Southern blot analysis (data not shown). These findings indicate that sequences can be deleted from the alphasatellite without affecting its ability to replicate autonomously and move, in trans, by a helper Begomovirus.

The adaptation of CLCuD alphasatellite as a vector

Alphasatellite has some properties which make it a potentially a useful vector. It is capable of autonomous replication. This ability to amplify itself is useful in a vector since it will increase the copy number (and thus also expression) of inserted sequences. The molecule is small and easy to manipulate. The alphasatellite appears to have a wide host range and can apparently be maintained by a large number of distinct Begomovirus species. This means that the potential host range of a vector derived from an alphasatellite is also large.

A map of the structure of CLCuD alphasatellite (Fig. 1) shows that there are sequences which can, potentially, be removed from alphasatellite to increase its capacity to accept and maintain foreign gene sequences. The construct CLCuMA Δ was produced by replacing the a-rich sequences with a *Cla*I restriction site, providing a site suitable for insertion of foreign sequences.

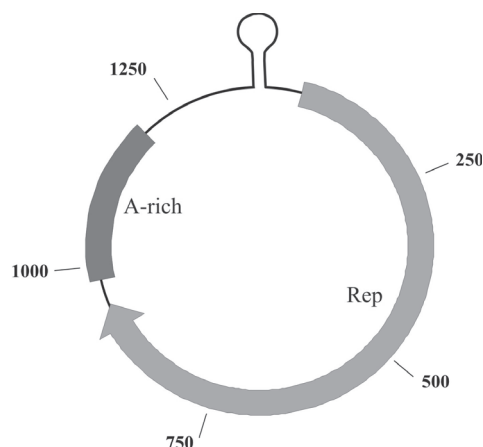


Fig. 1. The CLCuD associated alphasatellite is 1375bp in length, encodes a single gene in the positive orientation (a replication initiator protein [Rep]) and a region of sequence rich in adenine (a-rich).

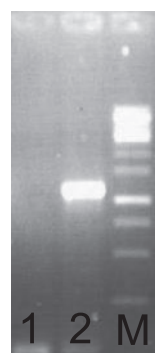


Fig. 2. The PCR-mediated amplification of an alphasatellite with the a-rich region deletion mutagenic primers in lane 2 with lane 1 used as a negative control and with a DNA size marker run in lane M.

The CLCuMA Δ can be used as a flexible vector

The CLCuMA Δ has been co-inoculated into the plants with several distinct Begomoviruses including the CLCuMV and ToLCNDV. The ability of both of these viruses to maintain the CLCuMA Δ and spread it systemically in plants demonstrates that CLCuMA Δ has little, if any, helper virus specificity and that a vector derived from it will be flexible and useable with a number of different helper Begomoviruses.

Future prospects

In the short term we shall be utilizing the a-rich deleted alphasatellite to silence a

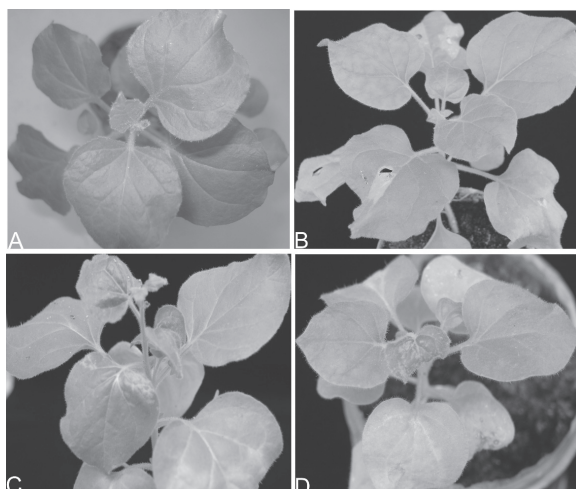


Fig. 3. The symptoms exhibited by *N. benthamiana* plants infected with CLCuMV, CLCuMB and Δ alphasatellite (D), CLCuMV and Δ alphasatellite (C), CLCuMB and CLCuMA Δ (B) healthy *N. benthamiana* plant (A).

transgene green fluorescence protein (GFP) and later on some other endogenous plant genes (such as phytoene desaturase and magnesium chelatase) in *N. benthamiana*, to verify the results obtained with GFP. Once this has been completed the system can be moved into a crop plant and assess the ability of an α -rich deleted alphasatellite for the use of a virus induced gene silencing vector in the tomato (for example). Ultimately the system will be tested in cotton, although there are a few technical problems yet to be overcome before this can be achieved, the major one

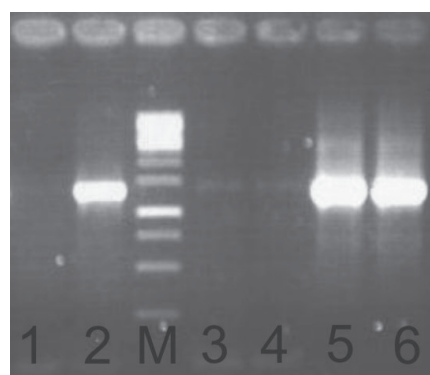


Fig. 4. The detection of Δ alphasatellite in the systemic leaves of *N. benthamiana* by PCR. The samples run resulted from the PCR reactions containing DNA extracted from plants inoculated with CLCuMV and CLCuMA Δ (lane 2), CLCuMV inoculated plant only (lane 3), CLCuMB only Δ alphasatellite (lane 4), CLCuMV and CLCuMA Δ (lane 5), CLCuMV, CLCuMB, and CLCuMA Δ (lane 6). The PCR reaction in lane 1 used DNA extracted from a non-inoculated *N. benthamiana* plant. A DNA size marker was run in lane M.

being the problem of inoculating Begomoviruses successfully to plants of the family Malvaceae.

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Pakistan'daki Pamuk Yaprak Kivircik Hastaligi ile Iliskili Alfasatellitin A-Zengini Bölgesinin Islevi

Özet

Önceleri DNA1 olarak bilinen ve adı alfasatellit olan, dairesel tek iplikli satellit benzeri yeni bir DNA tipi, son zamanlarda karakterize edildi ve monopartit Begomoviruslerle ilgili olduğu gösterildi. Alfasatellit bileşenleri, bazı konukcu canlılarda gerçek hastalık belirtilerini tetiklemek için betasatellit molekülüne ihtiyaç duyan Begomoviruslerle (Geminiviridae) ile ilişkili uydu benzeri tek iplikli DNA molekülleridir. Bu nedenle alfasatellit bileşenleri, konukcu hücrelerde öz-replikasyonu başlatabilir, ancak konukcu bitki içinde ve bitkiler arasında yayılmak için yardımcı Begomoviruse ihtiyaç duyarlar. İkinci özellik adenince zengin (a-zengini) bir dizin bölgesidir. A-zengini dizinlerin işlevini araştırmak için, bu bölge PCR'la gerçekleştirilen bir amplifikasyonla CLCuD alfasatellitinden silindi. Alfasatellitin a-zengini bölgesi kesilmiş mutanti, yardımcı Begomovirus varlığında, replikasyon yapabilme ve bitkilerde sistemik olarak enfeksiyon oluşturabilme yeteneğini korudu. Bu durum, a-zengini bölgesinin bitkilerde replikasyon veya bakım için gerekli olmadığını göstermektedir.

Anahtar Kelimeler: Alfasatellit, Begomovirus, Geminivirus, pamuk yaprak kivircik hastaligi, Pakistan.