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Use of the cotton leaf curl Multan alphasatellite as a silencing or expression vector

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Summary. – Alphasatellites, formerly known as DNA 1, are a satellite-like components associated with begomoviruses (the family *Geminiviridae*) that require betasatellite for symptom induction but depend on DNA-A for systemic movement. We have converted alphasatellite into gene-silencing vector (modified alphasatellite (ΔDNA 1)) by deleting its A-rich region that does not affect the replication nor the movement of the helper virus. Insertion of a transgene green florescence protein (GFP) into ΔDNA 1 resulted in the silencing g of the cognate gene in *Nicotiana benthamiana*. The silencing persisted for more than one and half month and was associated with the decreased level of mRNA of the target gene. This satellite-like DNA vector induced gene silencing (VIGS) promises to be applicable to other begomovirus/alphasatellite systems, thereby providing the powerful approach to gene discovery and the analysis of gene functions in malvaceous crops.

Keywords: cotton; begomovirus; alphasatellite; RNAi

Introduction

Gene expression in plants, animals and fungi can be suppressed by RNA silencing (also known as RNA interference [RNAi]), which is mediated in these organisms by a mechanism that involves sequence-specific mRNA degradation (Waterhouse and Helliwell, 2003). In plants, a procedure known as virus-induced gene silencing (VIGS) has emerged as a powerful method for studying gene functions (Baulcombe, 1999; Waterhouse and Helliwell, 2003). Typically, VIGS is induced by infecting a plant with a plant virus with its genome modified to include a sequence identical to that of the mRNA transcribed from the host target gene. However, this procedure has some limitations. Most applications of VIGS have been studied in model hosts such as *Nicotiana benthamiana* and *Arabidopsis thaliana*. Virus vectors used in these systems may not have important crop plants as hosts. Also, the efficiency of silencing depends on the virus-host combination or gene silencing may not occur in all hosts of the virus. In some cases, the virus vector may cause symptoms in plants that may mask the phenotype caused by silencing of the target gene.

Viruses of the genus *Begomovirus* (the family *Geminiviridae*) are whitefly-transmitted, plant-infecting pathogens with genomes that consist of either one or two molecules of circular single-stranded (ss)DNA (Brown *et al*., 2012). For bipartite begomoviruses, with components known as DNA-A and DNA-B, both components are required for infectivity of plants. The single component forming the genome of monopartite begomoviruses (which is a homolog of the DNA-A components of bipartite begomoviruses) is sufficient for symptomatic infection of plants, although monopartite begomoviruses are believed to be phloem limited (Rojas *et al*., 2001), due to the lack of additional movement functions encoded by DNA-B whereas bipartite begomoviruses may infect tissues beyond the phloem (Wang *et al*., 1996). In the Old World, most begomoviruses have monopartite genomes and the majority of these are associated with

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Abbreviations: CLCuMA = cotton leaf curl Multan alphasatellite; CLCuMB = cotton leaf curl Multan betasatellite; CLCuMV = cotton leaf curl Multan virus; CP = coat protein; CpCDV = chickpea chlorotic dwarf virus; dpi = days post inoculation; ToLCNDV = tomato leaf curl New Delhi virus; VIGS = vector induced gene silencing

symptom modulating ssDNA satellites known collectively as betasatellites (formerly DNA β) (Briddon and Stanley, 2006; Briddon *et al*., 2008). Betasatellites are approx. 1350 nucleotides long and are, in some cases, required by their helper begomoviruses to systemically infect the hosts from which they were isolated (Briddon *et al.*, 2001; Saunders *et al.*, 2000). The majority of begomovirus-betasatellite complexes are also associated with additional ssDNA components, the alphasatellites (previously known as DNA 1) (Briddon and Stanley, 2006; Briddon *et al*., 2004).

Alphasatellite components are satellite-like, circular ssDNA molecules approx. 1375 nucleotides long (Briddon *et al*., 2004). They encode a single gene, a rolling circle-replication initiator protein (Rep) and are capable of autonomous replication in permissive plant cells, however they require a helper begomovirus for movement within and between plants (Mansoor *et al*., 1999; Saunders and Stanley, 1999). They are closely related to the Rep-encoding components of nanoviruses (a second family of plant-infecting ssDNA viruses (Vetten *et al.*, 2004)), from which they are believed to have evolved (Briddon and Stanley, 2006). Interestingly, all alphasatellites so far investigated are phenotypically silent, playing no part in the pathogencity of the complex with which they are associated. Although no definitive function has been ascribed to alphasatellites, their presence in plants infected with begomovirus-betasatellite complexes may reduce virus and/or betasatellite DNA levels and possibly attenuate symptoms (Wu and Zhou, 2005; Ali *et al.*, 2011). Nawaz-ul-Rehman *et al.* (2010) have shown that at least some, alphasatellites encode Rep proteins with the ability to suppress post-transcriptional gene silencing – meaning that they may be involved in overcoming host defences based upon RNAi.

The components and satellites of begomoviruses form an ideal basis for the construction of VIGS vectors. Lessons learnt from the construction of VIGS vectors from non-malvaceous begomoviruses point to the construction of successful vectors for use in the family *Malvaceae*. Thus, vectors based on the DNA-A component of the bipartite viruses utilize the coat protein (CP) gene replacement strategy, while for a betasatellite the βC1 gene replacement strategies. The use of an alphasatellite transcription fusion vector, using a New World bipartite (viruses with mild symptoms) helper virus (such as cotton leaf crumple virus), is a promising system. The ability of VIGS vectors based on geminiviruses to induce gene silencing even in fruit and the meristem offer great advantages over VIGS vectors based on RNA viruses. The ultimate aim with any VIGS vector is an infectious unit which causes no (or only mild) symptoms in plants; so as not to mask the phenotypic effect of silencing of the target gene. Efforts to adapt the cotton leaf curl disease-associated alphasatellite component for use as a VIGS and expression vector are reported here.

Methods and Materials

Production of alphasatellite-based vectors. Three constructs were produced based upon the cotton leaf curl Multan alphasatellite (CLCuMA-[PK:Fai1:98]; Acc. No. AJ132344; Mansoor, 1999). Modifications were introduced into the CLCuMA sequence by PCR-mediated mutation using the oligonucleotide primers (Table 1). The primers were designed to introduce a restriction endonuclease recognition sequence, either at the 5'end of the Rep coding sequence (yielding CLCuMA^{N} with an *Mlu*I site; using primers DNA1^{N}F and DNA1^{N}R) or at the 3' end of the Rep coding sequence (yielding CLCuMA^{C} with an *Mlu*I site; using primers DNA1^{C}F and DNA1^{C}R). A further mutant, CLCuMA$^{\Delta A}$, was produced with the A-rich sequence of the alphasatellite, replacing it with a *Cla*I recognition sequence (using primers DNA1^{mut}F and DNA1^{mut}R). PCR (*Taq* DNA Polymerase, Thermo Fisher, USA) reactions used standard conditions as, an initial denaturation for 3 min at 95°C; followed by 35 cycles of denaturation at 95°C for 45 s, annealing at 55°C for 35 s, and extension at 72°C for 45 s; and final extension was at 72 °C for 10 min.) and the recircularised (*Bam*H1 restriction site) insert of the alphasatellite clone as the template. Resultant PCR products were cloned into pTZ57R/T (Fermentas, USA) and the clones were sequenced to ensure that there were no additional mutations introduced by the mutagenesis procedure (Fig. 1).

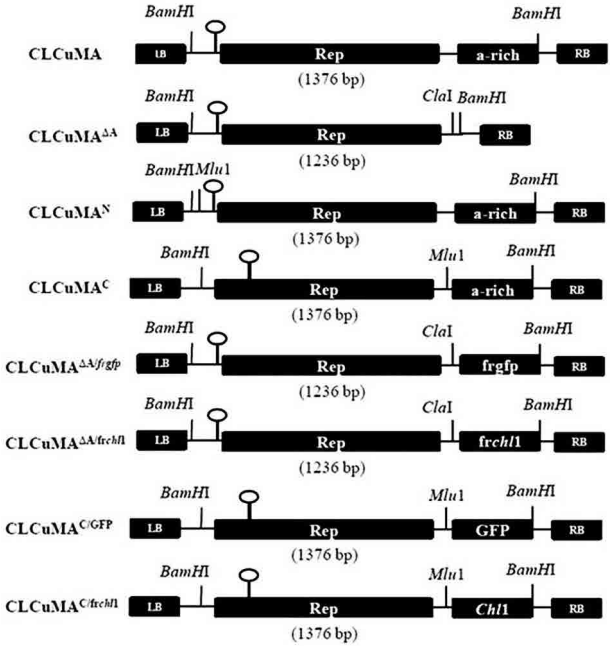

Fig. 1

Alphasatellite constructs

For the CLCuMA$^{\Delta A}$ vector, the A-rich region was replaced with *Cla*I restriction site. CLCuMA^{N} was produced by inserting an *Mlu*I restriction site at the 5' end of the start codon of the Rep gene whereas for CLCuMA^{C} an *Mlu*I site was introduced in to the 3' end of the Rep gene. GFP and *Chl*1 constructs were produced and shown accordingly.

Table 1. Primers used for production of CLCuMA constructs and amplification of *GFP* and *Chl*1

Primer name	Primer sequence
DNA1^{mut}F	ATATCGATAAAAAATCAGGCCCCGCAG
DNA1^{mut}R	ATCATCGATAGGTCCTTCAGCAAAGTGA
DNA1^{N}F	TTCACGCGTTCTCTCTCTTCTGTGTTC
DNA1^{N}R	AGAACGCGTGAAAGGCACGAGGGAAC
DNA1^{C}F	GGTACGCGTTGTAATTTTAAAAGACT
DNA1^{C}R	CAACGCGTACCCCAAAACTTTCAATAT
GFPF	ATCGATGGTGATGTTAATGGGCAC
GFPR	ATCGATGTCATGCCGTTTCATATG
*Chl*182F	ATCGATCTGAGGGTGTCAAGGCATTT
*Chl*I427R	ATCGATTTCCGAATCGATCAAGAAGC
*Chl*MF	ACGCGTCTGAGGGTGTCAAGGCATTT
*Chl*MR	ACGCGTTTCCGAATCGATCAAGAAGC
GFP56F	ACGCGTTCAAGGAGGACGGAAACATC
GFP785R	ACGCGTAAAGGGCAGATTGTGTGGAC

Production of constructs for Agrobacterium-mediated inoculation. A partial direct repeat construct of CLCuMA$^{\Delta A}$ was prepared by digesting the clone with *Cla*I and *Bam*HI to release an approx. 1120 bp fragment which was ligated into the binary vector pGreen0029 (Hellens *et al*., 2000). The pGreen0029 clone was then digested with *Cla*I and the full length CLCuMA$^{\Delta A}$ insert was ligated as a *Cla*I fragment. A similar strategy was used to produce partial direct repeat constructs of CLCuMA^{N} and CLCuMA^{C} using *Mlu*I and *Bam*HI restriction sites.

Origin and cloning of gene/gene fragments for silencing/expression. An approximately 200 bp fragment of the GFP gene was PCR amplified from *Nicotiana benthamiana* line 16c (Ruiz, 1998) and inserted into the partial direct repeat construct of CLCuMA$^{\Delta A}$, to yield CLCuMA$^{\Delta A/frgfp}$, using primers GFP56F and GFP785R (Fig. 1; Table 1). A similar strategy and size of a magnesium chelatase subunit I (*Chl*I) gene fragment (amplified using primers *Chl*IF182F and *Chl*I427R; based upon the sequence of the gene of *Gossypium hirsutum* [Acc. No. EU541445]) was amplified from *G. hirsutum* to produce CLCuMA$^{\Delta A/frchl1}$ and CLCuMA$^{C/frchl1}$ at the *Mlu*1 site (Fig. 1). For the construction of the GFP expression vector based on CLCuMA, a 729 bp fragment from pSMRSGFP (Acc. No. U70496; Davis SJ, 1998) was amplified using primers GFPF and GFPR and inserted into the partial direct repeat construct of CLCuMA^{C} to yield CLCuMA$^{C/GFP}$.

Origins of geminivirus clones used for infectivity studies. Constructs for the *Agrobacterium*-mediated inoculation of the monopartite begomovirus cotton leaf curl Multan virus (CLCuMV), the betasatellite cotton leaf curl Multan betasatellite (CLCuMB), the bipartite begomovirus tomato leaf curl New Delhi virus (ToLCNDV) and the mastervirus chickpea chlorotic dwarf virus strain C (CpCDV; previously known as Chickpea chlorotic dwarf Pakistan virus) have been described previously (Hussain *et al*., 2007; Nahid *et al*., 2008; Amin *et al*., 2011). Plants were inoculated by infiltration as previously described (Amin *et al*., 2011).

Results

Production and testing of constructs

To investigate the potential for using an alsphasatellite as the basis for a VIGS vector, three distinct mutants were produced using CLCuMA as the template. The first involved the insertion of an *Mlu*I restriction site upstream of the ATG codon of the Rep gene of the alphasatellite (yielding CLCuMA^{N}; Fig. 1). The second involved the introduction of an *Mlu*I restriction site downstream of the Rep gene (CLCuMA^{C}). Both constructs were designed to allow the insertion of foreign gene sequences which would be expressed as a transcription fusion gene of the Rep gene. The third construct, CLCuMA$^{\Delta A}$, involved replacement of the A-rich region with a *Cla*I restriction site (Fig. 1), to investigate whether foreign sequences at this distance from the end of the Rep gene could be incorporated in the Rep mRNA, and thus induce silencing, and to investigate whether an alphasatellite is infectious to plants even without the A-rich sequence (thereby potentially increasing the size of a foreign insertion that could be tolerated, see later discussion).

The three constructs were agroinoculated to *N. benthamiana* with either CpCDV, ToLCNDV or CLCuMV (in both the presence and absence of CLCuMB) (Table 2). For these inoculations the wild-type CLCuMA was inoculated as a control. Neither CLCuMA nor any of the constructs based on CLCuMA were maintained in *N. benthamiana* by CpCDV. CLCuMA^{N} was not maintained by any of the helper viruses.

Table 2. Results of the inoculation of *N. benthamiana* with CLCuMA (and its derivatives) in the presence of distinct helper viruses

Inoculum*	No. of plants containing alphasatellite / No. of plants infected / No. of plants inoculated	
	Exp.1	Exp.2
CLCuMV + CLCuMB + CLCuMA	6/6/6	5/5/6
CLCuMV + CLCuMB + CLCuMA$^{\Delta A}$	6/6/6	5/5/6
CLCuMV + CLCuMB + CLCuMA^{N}	0/5/6	0/6/6
CLCuMV + CLCuMB + CLCuMA^{C}	6/5/6	3/4/6
CLCuMV + CLCuMA	3/4/6	5/5/6
CLCuMV + CLCuMA$^{\Delta A}$	5/5/6	4/5/6
CLCuMV + CLCuMA^{N}	0/4/6	0/4/6
CLCuMV + CLCuMA^{C}	3/5/6	4/4/6
ToLCNDV* + CLCuMA	3/5/6	2/6/6
ToLCNDV*+ CLCuMA$^{\Delta A}$	4/5/6	4/6/6
ToLCNDV*+ CLCuMA^{N}	0/6/6	0/5/6
ToLCNDV*+ CLCuMA^{C}	3/5/6	2/5/6
CpCDPKV + CLCuMA	0/3/6	0/4/6
CpCDPKV + CLCuMA^{N}	0/5/6	0/4/6
CpCDPKV + CLCuMA$^{\Delta A}$	0/3/6	0/4/6
CpCDPKV + CLCuMA^{C}	0/4/6	0/5/6

*Both the DNA-A and DNA-B components of ToLCNDV were inoculated.

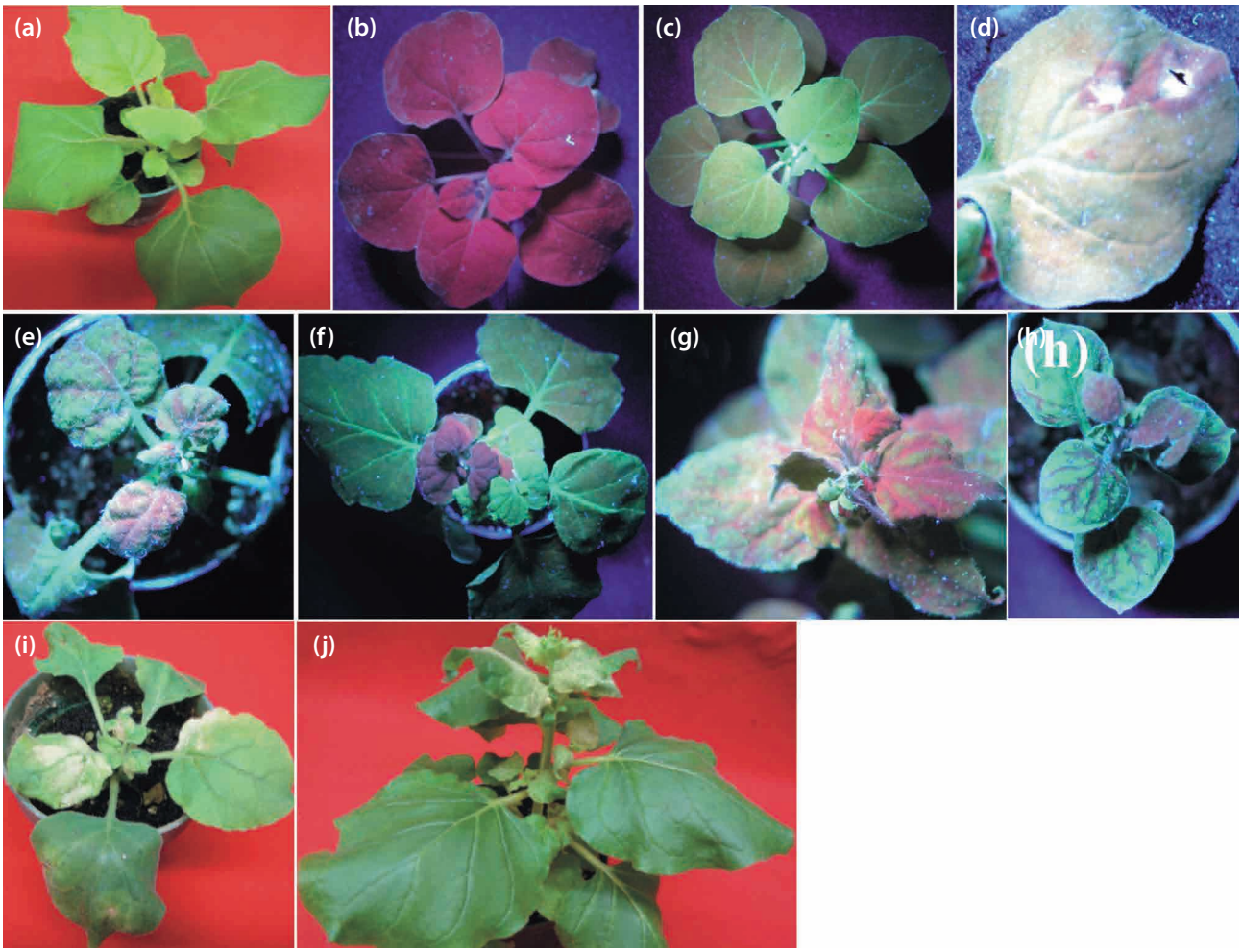

Fig. 2

Transgene GFP and endogenous gene *Chl*I silencing in 16c and wild type *N. benthamiana* plants

(a) *N. benthamiana* plant under visible light; **(b)** *N. benthamiana* plant under UV light; **(c)** GFP transgenic *N. benthamiana* plant (16c) under UV light; **(d)** 16c inoculated with CLCuMA$^{\Delta A/frgfp}$ and CLCuMV showing local silencing; **(e)** 16c inoculated with CLCuMA$^{\Delta A/frgfp}$ and CLCuMV showing systemic leaf silencing; **(f)** 16c inoculated with CLCuMV, CLCuMB and CLCuMA$^{\Delta A/frgfp}$; **(g)** GFP silencing with CLCuMV, CLCuMB and CLCuMA$^{\Delta A/frgfp}$; **(h)** GFP silencing with ToLCNDV DNA-A+ DNA-B and CLCuMA$^{\Delta A/frgfp}$; **(i)** *Chl*1 silencing with CLCuMA$^{\Delta A/frchl1}$ and CLCuMV in *N. benthamiana* and **(j)** *Chl*1 silencing with CLCuM$^{AC/frchl1}$, CLCuMV and CLCuMB in *N. benthamiana*. Photographs were taken at 24 and 32 dpi.

Upon these findings CLCuMA^{N} was not used for the further studies (Table 2).

CLCuMA constructs were efficiently maintained by CLCuMV, both in the presence and absence of CLCuMB, and by ToLCNDV (Table 2). This indicates that the introduced mutations, in each case, have not affected the ability of the molecule to replicate autonomously and be moved *in trans*, by the respective viruses.

The symptoms induced in each case (16 to 18 days for CpCDV, 22 to 25 days for ToLCNDV, after three weeks for CLCuMV and 14 to 16 for CLCuMV in the presence of CLCuMB) were indistinguishable from the symptoms in the absence of the aphasatellite (or mutated alphasatellite). This is in agreement with earlier reports which demonstrated that the presence of an alphasatellite does not significantly affect symptoms, although possibly a slight reduction in virus DNA levels was detected (personal observation).

Alphasatellite vector-mediated silencing of GFP

Initial investigation of the possibility of adapting CLCuMA for use as a silencing vector used the well established GFP silencing system that is based upon *N. benthamiana* line 16c (Ruiz, 1998). This contains a stable GFP transgene expressed under the control of the constitutive promoter 35S derived from cauliflower mosaic virus (CaMV). 16c plants appear

green under UV illumination due to GFP fluorescence (Fig. 2, panel c). In contrast, (non-transgenic) *N. benthamiana* emits red light under UV illumination due to chlorophyll autofluorescence (Fig. 2, panel b).

Inoculation of CLCuMA$^{\Delta A/frgfp}$, in the presence of CLCuMV, to 16c led to typical symptoms of infection at approximately 24 days. Maintenance of the alphasatellite construct was shown by PCR amplification in 13 plants of 18 inoculated. Under UV illumination, silencing at the site of inoculation was evident at 13 days post inoculation (dpi). This appeared as a red area around the infiltration site (Fig. 2, panel d). Within 18 dpi, some silencing was present on leaves that were developing at the time of inoculation. This appeared as silencing of the GFP along the veins. Silencing in these leaves was not complete. The leaves above this, developing after inoculation, initially showed silencing along the veins continuing to spread to the whole leaf surface (Fig. 2, panel e). All subsequently developing tissues were entirely silenced; thus, appearing red under UV illumination. The silencing was persistent, remaining until the plants senesced.

A similar result was obtained when CLCuMA$^{\Delta A/frgfp}$ was inoculated in the presence of CLCuMV and CLCuMB. In this case the symptoms of infection appeared after 14 to 16 days. Symptoms were more severe, as is typical for the infections in the presence of a betasatellite (Briddon *et al.*, 2001). In this case maintenance of the alphasatellite vector was detected in 5 plants of 6 inoculated. Silencing progressed more rapidly and was more complete than in the plants inoculated only with CLCuMV. Tissues at the top of the plant showed initial silencing at 13 days post inoculation, in comparison to 18 days for the infection in the absence of CLCuMB (Fig. 2, panels f, g).

In contrast, inoculation of CLCuMA$^{\Delta A/frgfp}$ with ToLCNDV DNA-A and DNA-B yielded relatively mild infection with less pronounced leaf curling. Also, the silencing induced was significantly delayed over infection involving CLCuMV. For ToLCNDV, initial symptoms of infection appeared 21 to 24 dpi. Again, there was a halo of silencing visible around the site of inoculation at approx. 17 dpi. This spread to non-inoculated tissues by 20 dpi (Fig. 2, panel h). However, the plants did not show as bright red as those inoculated with CLCuMV, suggesting that silencing was not complete (some GFP fluorescence remained). Maintenance of the alphsatellite vector was detected in 10 of 18 inoculated plants by PCR.

Alphasatellite vector-mediated silencing of an endogenous gene

As the transgenes are the more favorable to gene silencing than the endogenous genes (Fagard and Vaucheret, 2000; Ruiz, 1998), we have decided to test the ability of alphasatellite base vector CLCuMA$^{\Delta A/frchl1}$ to silence the endogenous gene magnesium chelatase subunit I (*chl*1).

Table 3. *N. benthamiana* plants showing silencing and expression using *GFP* and *Chl*I genes

Constructs	No. of plants silenced / No. of plants inoculated		
	Exp.1	Exp.2	Exp.3
CLCuMV+CLCuMA$^{\Delta A/frgfp}$	4/6	5/6	4/6
CLCuMV+ CLCuMB+ CLCuMA$^{\Delta A/frgfp}$	5/6	6/6	4/6
CLCuMV+ CLCuMB+ CLCuMA$^{\Delta A/frChl1}$	5/6	4/6	6/6
CLCuMV+ CLCuMB+ CLCuMA$^{C/frChl1}$	5/6	4/6	4/6
ToLCNDV^{A+B} + CLCuMA$^{\Delta A/frgfp}$	3/6	2/6	5/6
*CLCuMV+ CLCuMB+ CLCuMA$^{C/GFP}$	3/5	2/5	2/5

*CLCuMA$^{C/GFP}$ used as an expression vector.

Inoculation of CLCuMA$^{\Delta A/frchl1}$, in the presence of CLCuMV and CLCuMB, to *N. benthamiana* led to typical symptoms of infection at approximately 14 to 16 days. Maintenance of the alphasatellite construct was shown by PCR amplification in 15 plants of 18 inoculated (Table 3). Silencing at the site of inoculation was evident at 10 dpi. This appeared as a white area around the infiltration site (Fig. 2, panel i). Within 13 dpi, some silencing was present on leaves that were developing at the time of inoculation. This appeared as bleaching along the veins. Silencing in these leaves was not complete. The leaves above this, developing after inoculation, initially showed silencing along the veins but this spread to the whole leaf surface. All subsequently developing tissues were entirely silenced; thus, appearing white. The silencing was persistent, remaining until the plants senesced.

In comparison, in the presence of CLCuMV and CLCuMB inoculation of CLCuMA$^{C/frchl1}$, to *N. benthamiana* a similar result was obtained. Also, the silencing induced was delayed a few days over infections involving CLCuMA$^{C/frchl1}$. For CLCuMA$^{C/frchl1}$, initial silencing appeared 11 dpi. There was a halo of silencing visible around the site of inoculation at approx. 13 dpi. This spread to non- inoculated tissues by 16 dpi (Fig. 2, panel j). However, the plants did not show much silencing in case of CLCuMA$^{C/frchl1}$ than CLCuMA$^{\Delta A/frchl1}$, suggesting that silencing was not complete (some *Chl*1 remaining). Maintenance of the alphasatellite vector was detected in 13 of 18 inoculated plants by PCR.

Alphasatellite mediated expression of GFP in plants

To investigate the potential of CLCuMA for using as an expression vector, the full length GFP gene was inserted in sense orientation into CLCuMA^{C} to yield CLCuMA$^{C/GFP}$. This construct was agroinoculated to *N. benthamiana* in the presence of CLCuMV and CLCuMB. Typical symptoms of CLCuMV infection in the presence of CLCuMB appeared at approximately 14 to 16 dpi. Control plants inoculated with only CLCuMV and CLCuMB, when viewed under UV

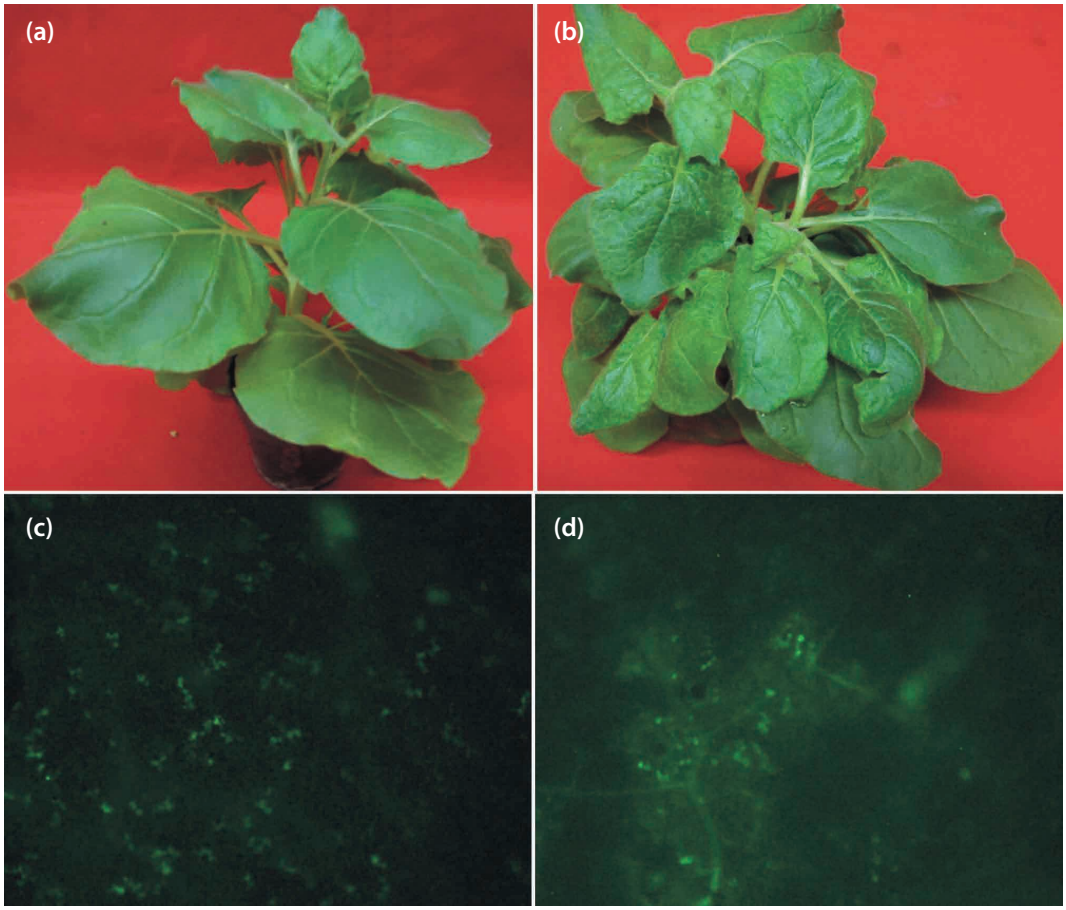

Fig. 3

Detection of GFP fluorescence in *N. benthamiana* inoculated with CLCuMV, CLCuMB and CLCuMA$^{C/gfp}$
(a) mock-inoculated *N. benthamiana* control; **(b)** *N. benthamiana* inoculated with CLCuMV, CLCuMB and CLCuMA$^{C/gfp}$; **(c)** green fluorescence in inoculated leaf and **(d)** GFP detection in systemic leafs after 4 weeks of agroinoculation.

illumination, showed red fluorescence due to chlorophyll autofluorescence. In contrast, *N. benthamiana* inoculated with CLCuMV, CLCuMB and CLCuMA$^{C/gfp}$ showed green fluorescence under UV illumination at the site of inoculation. This fluorescence was first visible approx. 13 days post inoculation and at that time symptoms of infection were/were not visible on the plants. However, under the confocal microscope GFP fluorescence was clearly visible in tissues developing after inoculation. In a leaf which was just expanding at the time of inoculation (Fig. 3, panel c) faint GFP fluorescence was visible. In subsequently developing leaves clear GFP fluorescence was evident (Fig. 3, panel d). This was closely associated with the veins and did not extend away from vascular tissues. This is likely due to the fact that monopartite begomoviruses are phloem-limited (Rojas *et al.*, 2001). In case of CLCuMV in the presence of the CLCuMB, the symptoms induced (enations and leaf-like outgrowths on the major veins on the undersides of leaves, Briddon *et al.*, 2001) would suggest that this is probably the case that GFP did not extend away to the veins.

Discussion

The results presented here indicate that an alphasatellite can be used as the basis for a vector for VIGS. Both CLCuMA$^{\Delta A/frgfp}$ and CLCuMA$^{C/frgfp}$, carrying fragments of the *gfp* gene in antisense orientation, were capable of silencing GFP expression in *N. benthamiana* 16c when maintained by begomoviruses. To further validate the vectors, a fragment of *Chl*I was introduced into CLCuMA^{C} and inoculated to *N. benthamiana*. This yielded bleaching in systemic leaves characteristic of the loss of chlorophyll, providing a visible marker for gene silencing. The results confirmed that the vector is capable of initiating silencing of an endogenous gene in *N. benthamiana*.

The reason for the inability of CLCuMA^{N} to systemically infect plants, in the presence of a begomovirus, is unclear. Although only minimal changes were introduced into the alphasatellite sequence (*Mlu*I restrictions site) it is possible that this adversely affected the transcription or translation of the Rep gene. Although little is yet known about the maintenance of alphasatellites by begomoviruses, it is likely that high-level replication of these molecules is required for their maintenance, which depends upon the Rep (Mansoor *et al*., 1999; Zhou, 2013). There is no evidence for a (strong) selection mechanism for alphasatellite maintenance. For betasatellites the βC1 gene is an essential factor for infection of the helper begomoviruses; it up-regulates virus titers and is a host range determinant (Saeed, 2005; Zhou, 2013). There is thus a strong selection pressure for maintenance of betasatellites. Maintenance of alphasatellites thus possibly is a "numbers game", plants containing such high levels of the satellite that it is almost always transmitted to the next cell by the virus-encoded movement proteins or to the next plant by the vector of the helper begomovirus. Thus, it is likely that the changes introduced into CLCuMA^{N} affected Rep expression such that there was no longer sufficient alphasatellite replication for it to be maintained during systemic infection.

The inoculations studies with CLCuMA$^{\Delta A}$ indicate that the A-rich sequence is not required for the infectivity or maintenance of CLCuMA. The deleted alphasatellite was maintained in plants in the presence of a begomovirus. This is in agreement with earlier studies that deleted the A-rich sequences from a betasatellite. Betasatellites have a similar A-rich sequence and this was shown not to be required for trans-replication or maintenance (Tao, 2004). The function of the A-rich sequence remains unclear. Upon their first identification it was suggested that the A-rich sequence is merely a "stuffer" required to raise the size of a nanovirus component (from which alphasatellites are believed to have evolved) to half (~1400 bp) of a begomovirus component (~2800 bp) (Mansoor *et al*., 1999; Briddon and Stanley, 2006). Geminiviruses have a strict genome size control mechanism which is believed to act during both movement in plants (mediated by the movement associated proteins; Gilbertson *et al*., 2003) and for encapsidation – monomeric particles containing half size molecules (Frischmuth *et al*., 2001). Etessami *et al.* (1989) showed that an African cassava mosaic virus (ACMV) isolate with deletions across the coat protein gene reverted to wild-type size during infection, likely due to strict size requirements for cell-to-cell spread of the virus. It will be interesting, in the future, to investigate this phenomenon to determine what mechanism is responsible for the size increase. This may provide information on the movement of these sub-genomic molecules in plants and their interactions with their helper begomoviruses.

The reason for the lack of maintenance of CLCuMA and the CLCuMA-derived vectors by CpCDV is unclear. Although alphasatellites have been shown to be maintained in plants by viruses with which they are not usually associated, such as the bipartite begomovirus ACMV and the curtovirus beet curly top virus (Saunders *et al*., 1999, 2002). There are no reports of either the association of alphasatellites with mastreviruses in the field or of the ability of mastreviruses to maintain alphasatellites in plants experimentally. Earlier attempts to see whether the mastrevirus tobacco yellow dwarf virus could maintain CLCuMA were unsuccessful (R.W. Briddon, unpublished results). One possible constraint to the maintenance of alphasatellites by mastreviruses is size. Mastreviruses are typically smaller (~2600 bp) than begomoviruses (~2800 bp) and alphasatellites could be too large to be encapsidated, being greater than half the size of the genome. However, this would not explain why the A-rich deletion mutant, which is ~140 bp, was not maintained by CpCDV. Further studies are required to investigate this finding.

One of the advantages of an alphasatellite vector, over many of the other vectors, is that it can, at least in theory, be used with any begomovirus or even curtovirus (Saunders *et al*., 2002). Thus, it can possibly be used to investigate gene expression (or as an expression vector) on the entire plant host range of the begomoviruses/curtoviruses. The experiments conducted here illustrate this possibility by showing that GFP can be used for silencing as helper virus for both a bipartite begomovirus (ToLCNDV) and a monopartite begomovirus (CLCuMV; in both the presence or absence of a betasatellite) in *N. benthamiana*. The ability of a modified alphasatellite to induce gene silencing using several different begomoviruses has also been shown by Huang *et al.* (2009). In the context of Pakistan, cotton is the main crop species of interest and it is hoped that the vectors produced here will be useful for silencing/expression in this species. However, at this time, we have no inoculation system for this host.

Although the work conducted here has shown the potential usefulness of an alphasatellite vector for gene silencing and expression, one major hurdle remains to be overcome before their full potential can be realized. As mentioned earlier, one of the main requirements for a useful gene silencing vector is that it does not induce appreciable symptoms, which could interfere with interpretation of the silenced phenotype. All the viruses used here induce significant symptoms (Padidam *et al*., 1995; Amin *et al*., 2011). Betasatellite-associated begomoviruses, not including the betasatellite (as was shown) can reduce the symptom severity, but they do not entirely abolish symptoms. The next step is thus, to identify means of either abolishing, or at least diminishing, the symptoms induced by potential helper viruses, without significantly affecting their infectivity and ability to systemically move in plants. Ongoing studies into the pathogenicity determinants of begomoviruses may provide possible means to consistently down-regulate symptoms induced by begomoviruses which would make it possible to modify potential helper begomo-

viruses for silencing studies using the alphasatellite vectors (Amin *et al.*, 2011; Hanley-Bowdoin *et al.*, 2013).

GFP has been used extensively to study the movement of viruses in plants. Coat protein (CP) replacement vectors have, for example, been used to study the movement of the bipartite begomovirus bean dwarf mosaic virus (Sudarshana *et al.*, 1998). These studies made use of the fact that bipartite begomoviruses do not require the CP for systemic infection of host plants (Stanley and Townsend, 1986; Gardiner *et al.*, 1988; Azzam *et al.*, 1994). However, for monopartite geminiviruses, including monopartite begomoviruses, the CP is essential for infectivity and it likely is providing some essential movement functions – movement of these viruses is either as virions or as CP-DNA complexes (Briddon *et al.*, 1989). This thus precludes the CP replacement strategy for expressing GFP for virus movement studies. An alternative strategy, which may be useful for monopartite begomoviruses and curtoviruses, is to use an alphasatellite vector expressing the GFP. This would overcome any effects of mutation of the virus to facilitate GFP expression. Even for bipartite begomoviruses mutation of the CP has deleterious effects which may interfere with the interpretation of the results; for example, the time to symptom appearance is extended for bipartite begomoviruses with mutated CP (Klinkenberg *et al.*, 1989). It is thus anticipated that the vector produced here will be useful for studying the movement of monopartite begomoviruses and curtoviruses *in planta*. Possibly the first use of the vector will be to study the difference between monopartite begomoviruses in the presence and absence of betasatellites, since the βC1 gene encoded by betasatellites has been shown to have possible virus movement functions (Saeed *et al.*, 2007).

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