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Transgenic tobacco expressing geminiviral RNAs are resistant to the serious viral pathogen causing cotton leaf curl disease

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Summary. Cotton, the major cash crop in Pakistan, suffers 30% losses to cotton leaf curl disease, caused by the geminivirus, cotton leaf curl virus DNA A, plus a satellite component, DNA β responsible for symptom development with plants failing to produce cotton bolls. We constructed transgenic tobacco expressing sense and antisense RNAs representing: [i] the 5′ half of the viral DNA replication gene, AC1, [ii] the 3′ half of AC1, [iii] two overlapping genes, AC2, a transcription activator, and AC3, a replication enhancer. In contrast to controls, 25% of 72 transgenic tobacco lines tested showed heritable resistance [$T_1 - T_3$ generations]: symptom-free and no replication of DNA A or DNA β even after 120 days continuous exposure to viruliferous whiteflies. As geminiviral and transgene RNAs are not detected in resistant lines following infection, and selected uninfected resistant tobacco sense lines reveal double-stranded and small interfering RNAs, the most likely mechanism is via post-transcriptional gene silencing.

Introduction

Cotton is a major world crop contributing significantly to agricultural-based economies. In Pakistan, cotton, the main cash crop brings in more than 60% of total foreign exchange earnings. However, over the last ten years cotton production has suffered 30% losses, exceeding US$5 billion, from the whitefly-transmitted cotton leaf curl disease [CLCuD]. The disease is spreading: beginning with an epidemic in the Punjab in Pakistan, in 1988, by 1992 it had spread to all of

Fig. 1. Symptom development in transgenic T_1 tobacco, *Nicotiana tabacum* (cv samsun) plants three weeks following CLCuD infection by viruliferous whiteflies. **A**, left, resistant tobacco transgenic line 3.28 and right susceptible transgenic tobacco line 3.23; **B** shows the susceptible line 5.21 with typical leaf vein thickening and leaf-like enanation

the cotton-growing districts of the Punjab, then spreading to the Indian Punjab in 1995 and in 1997 spreading to the Pakistani province of Sindh. Disease symptoms include leaf curling, vein thickening with leaf-like growths [enations] appearing from these veins and stunted plant growth with plants failing to reach maturity to produce cotton bolls (Fig. 1).

A geminivirus is associated with CLCuD [25]. Geminiviruses [GV] are small circular single-stranded [ss] plant DNA viruses comprising four subgroups: I, mastreviruses; II, curtoviruses; III, begomoviruses; IV, topocuviruses. Of the GVs, the begomoviruses are the most serious plant pathogens in agriculture. Their genomes typically comprise two components, designated DNA A and DNA B. DNA A encodes a coat protein plus proteins required for DNA replication and DNA B encodes movement proteins, for both within and between cells, allowing systemic infection [14]. CLCuD was originally thought to be caused by a bipartite begomovirus, as all such viruses are whitefly-transmitted; however, only one begomoviral component of cotton leaf curl virus [CLCuV], DNA A has been identified [25]. Subsequently four variants of DNA A were identified in Pakistani isolates [33]. However, CLCuV DNA A alone, although infectious, does not yield symptoms of CLCuD [7]. However, a recently discovered satellite ssDNA molecule, DNA β, together with CLCuV DNA A, gives the symptoms typical of CLCuD both in cotton and in tobacco [8]. DNA β requires CLCuV DNA A for replication and encapsidation and encodes putative proteins; but these share no similarity to the DNA B of other begomoviruses.

A variety of strategies have been employed to engineer virus-resistant transgenic plants. One exploits the natural phenomenon of cross-protection [24, 4] but unlike with RNA viruses, has had limited success with DNA viruses. For,

begomoviruses, expression of truncated defective transdominant viral coat protein, replicase and movement proteins has proved more promising [22, 16, 29, 11, 9]. Another approach is to express antisense transgenes complementary to a target mRNA. The original rationale of antisense RNA technology [19] was that by pairing with a complementary target mRNA, antisense would inhibit expression of homologous genes by preventing translation or promoting degradation of the target mRNA. Indeed this technology has been successfully applied to engineering resistance to geminiviruses [10, 5, 6, 2]. However antisense is actually part of complex natural pathways for gene regulation by homology sensing mechanisms where sense transcripts are also able to silence gene expression [21, 31, 26].

Here we provide the first report showing that transgenic tobacco expressing sense and antisense RNAs of CLCuV DNA A inhibit replication of both CLCuV DNA A and DNA β and that such plants are free of symptoms of infection. This provides a promising solution to this serious cotton pathogen in Pakistan.

Materials and methods

Construction of transgenic plants

To construct the plant expression cassettes, a 1.5 kb *Sst I/Xho* I fragment containing the enhanced CaMV 35S promoter and poly A tail, flanking a *Sma* I site was subcloned from plasmid pJIT60 [gift from Dr. P. Mullineaux, John Innes Centre, UK] into the pBluescript II KS+ to yield pSQW1. CLCuV DNA A (CLCuV Pak2/Fsd/1 [72b] [33]) genes were amplified by PCR (cycle 95 °C, 5 min, then 40 cycles of 95 °C, 1 min, 50 °C, 1 min, 72 °C, 1 min, then 72 °C, 10 min) using the primer pairs:

(i) for D1/4, (position 2,600–2,581) 5′-AGTCAACATGCCTCCAAAGC-3′; (position 2,135–2,154) 5′-AGCTAGTTCCTTAATGACTC-3′,
(ii) for D1, (position 2,141–2,121) 5′-ACTAGCTCCTAAAGATTTTG-3′; (position 1,599–1,618) 5′AAGATCGCATTCTTTACTCG-3′,
(iii) for D1/d2/d3, (position 1,606–1,588) 5′-TGCAATCTTCATCAGCCTCG-3′; (position 1,082–1,096) 5′-AAGATGATTGGTCTACAAATAC-3′.

The PCR products were end-filled with T4 DNA polymerase and subsequently cloned in sense and antisense orientations into the *Sma* I site of pSQW1. The 6 expression cassettes, sense and antisense of D1/4, D1 and D1/d2/d3, were then each individually subcloned as *Sst I/Eco* R V fragments into the *Sst I/Hpa* I sites of pGA482 [1]. The plasmid recombinants were transformed into *Agrobacterium tumefaciens* strain LBA4404 by electroporation [27]. Tobacco (*Nicotiana tabacum* cv Samsun) was transformed by leaf disc agroinoculation [17]. T_0 lines were self-pollinated and T_1 seeds germinated on MS medium containing 500 ug/ml of kanamycin and T_1 seedling were transplanted into soil a month after germination.

Plant inoculation and symptom development

Whiteflies were reared on CLCuD-infected cotton plants under containment. Seedlings were exposed to viruliferious whiteflies at the four to five leaf stage for 120 days at 28–30 °C. The presence or absence of symptoms was observed on weekly basis.

Analysis of nucleic acids

Total genomic DNA was isolated from leaf tissue samples [20]. The presence of transgenes was analysed by PCR. The primers used for *npt*II transgenes were as described [22], while specific primers [see above] were used for CLCuV DNA A transgenes. Southern blotting was performed using 15 μg of *Hin*d III digested genomic DNA [30] probing with PCR fragments [labelled with the Rad-primed ^{32}PdCTP-labelling kit, GIBCO-BRL]. Hybridisation, washing and detection were carried as described [3]. Total RNA from tobacco leaves was extracted using TRIzol reagent [GIBCO-BRL], and for isolation of cotton RNA as described [18]. Northern blotting was performed using 20 μg of total RNA in formaldehyde agarose gels [30, 3]. Analysis of dsRNA and small RNA were as described [32]. Multiplex PCR was used to amplify CLCuV DNA A variants clc26 and 72b [33] using the primers:

5′-ATGTCGAAGCGACTCCGATATCGTCATTTCTACG-3′,
5′-TGATGAGTTCCCCTGTGCGTGAATCCATGGTTGT-3′,
5′-GGAATAAATACCGTTCCGCTTTGGAGGCATGTTG-3′.

Southern blotting, as above, of 5 μg of total undigested plant DNA was used to detect CLCuD DNA β, using a full length DNA β probe [8].

Results and discussion

Construction of tobacco carrying CLCuV DNA A transgenes

CLCuV DNA A encodes six proteins [33] (Fig. 2), which, given their high similarity to those of other begomoviruses, presumably have similar functions: AV1 and AV2 encode the coat-protein and pre-coat protein, respectively; AC1 encodes an essential replication protein, Rep, a sequence- and strand-specific endonuclease/helicase/ATPase/ligase that generates the circular ss viral DNA monomers, by

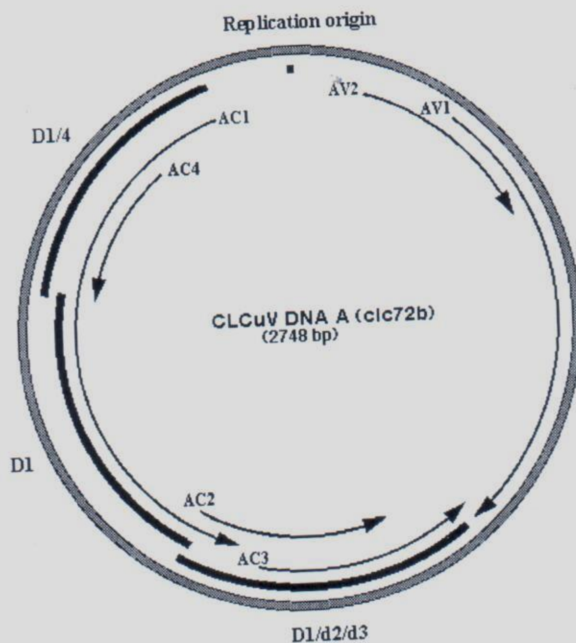


Fig. 2. Genetic map of CLCuV DNA A variant clc72b [■] showing, as arrows, the location of the ORFs for AC1-4 and AV1-2. The regions selected for PCR amplification, D1/4, D1, D1/d2/d3 are shown as thick lines. The black box marks the highly conserved nonanucleotide sequence that is the replication origin of geminiviruses

rolling circle replication from a double-stranded [ds] replicative form intermediate at a *cis*-essential origin mapping next to the AC1 gene [14]. AC2, overlapping AC1, encodes a transcription activator, TrAP [15]; AC3, overlapping AC2 encodes a replication enhancer protein, REn [14]. AC4, embedded within AC1 in another reading frame is of unknown function in begomoviruses.

We constructed *Agrobacterium* binary vectors with expression cassettes driving, in the sense and antisense orientations, three different regions of the AC1-4 genes encoding (Fig. 2): [i] D1/4, the 5′ half of the viral replication gene, AC1 and all of the AC4 ORF; [ii] D1, the 3′ half of AC1 (excluding overlap with AC2 and AC4); and [iii] D1/d2/d3, two overlapping genes, AC2, a transcription activator, and AC3, a replication enhancer (plus the last 97 bases of the AC1, and excluding the initiation codon of AC2 and the 3′ 23 bases of AC3).

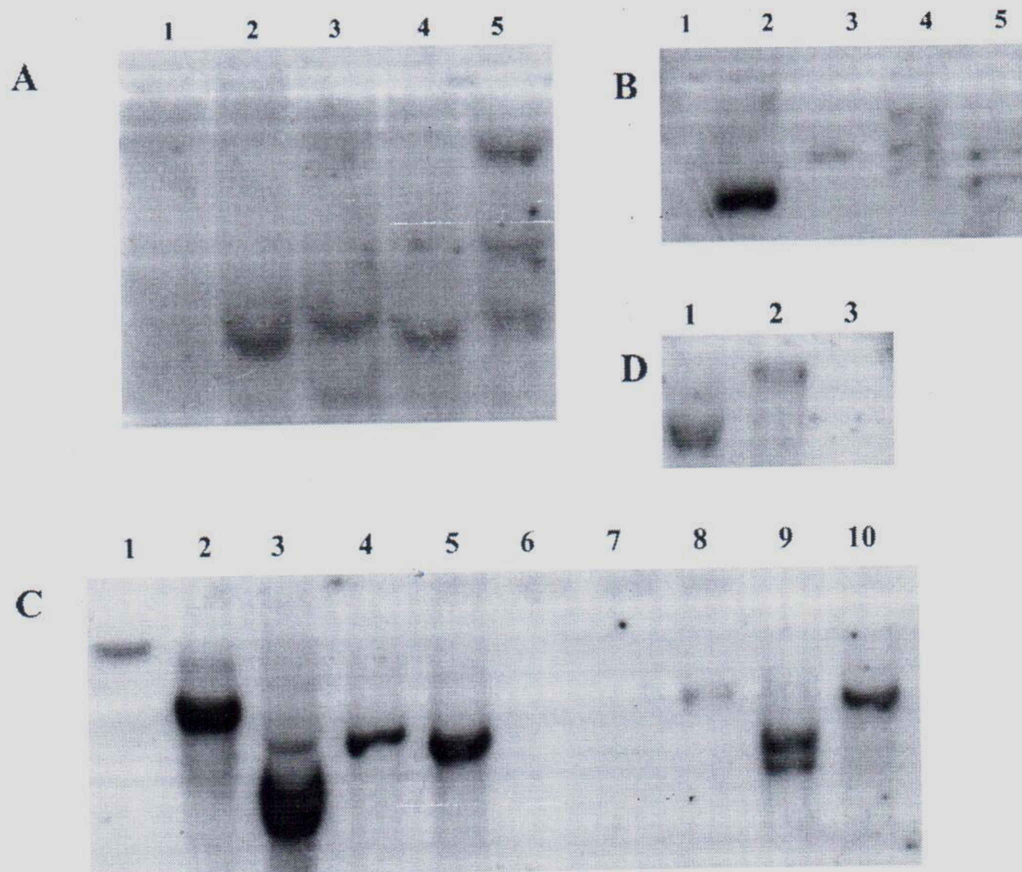


Fig. 3. *Hin*d III-digested genomic Southerns of tobacco transgenic lines. **A**, D1/4 antisense tobacco, *1*, untransformed control, *2–5*, lines 1.7, 1.15, 1.23, 1.50 respectively; **B**, D1/4 sense tobacco, *1*, untransformed control, *2–5*, lines, 2.20, 2.60, 2.75, 2.500; **C**, tobacco D1 antisense and sense, *1*, plasmid positive control carrying D1, *2–5*, antisense lines, 3.7, 3.18, 3.20 and 3.28 respectively, *6*, untransformed control, *7–10*, sense lines, 4.21, 4.45, 4.75 and 4.90 respectively; **D**, D1/d2/d3 tobacco antisense, *1–2*, lines 5.6, 5.22, respectively, *3*, untransformed control; The analysis reveals low copy number of integrated transgenes, transgenes carry no *Hin*d III sites

About 20–25 independent transgenic plant lines were produced in three independent experiments for each of the six constructs in tobacco [cv. samsun]. Twenty selected T_0 lines for each construct, were selfed to produce T_1 plants. A subset of these T_0 lines, 12 lines/construct, all phenotypically normal, were verified by PCR to contain both transgene and the *npt*II gene encoding kanamycin resistance [the selectable marker] and similarly selfed to make T_2 and T_3 lines. Southern blots of selected transgenic lines show transgenes present in one to three copies per genome (Fig. 3).

Transgenic tobacco lines expressing viral RNA are resistant to CLCuD and inhibit viral DNA A and DNA β replication

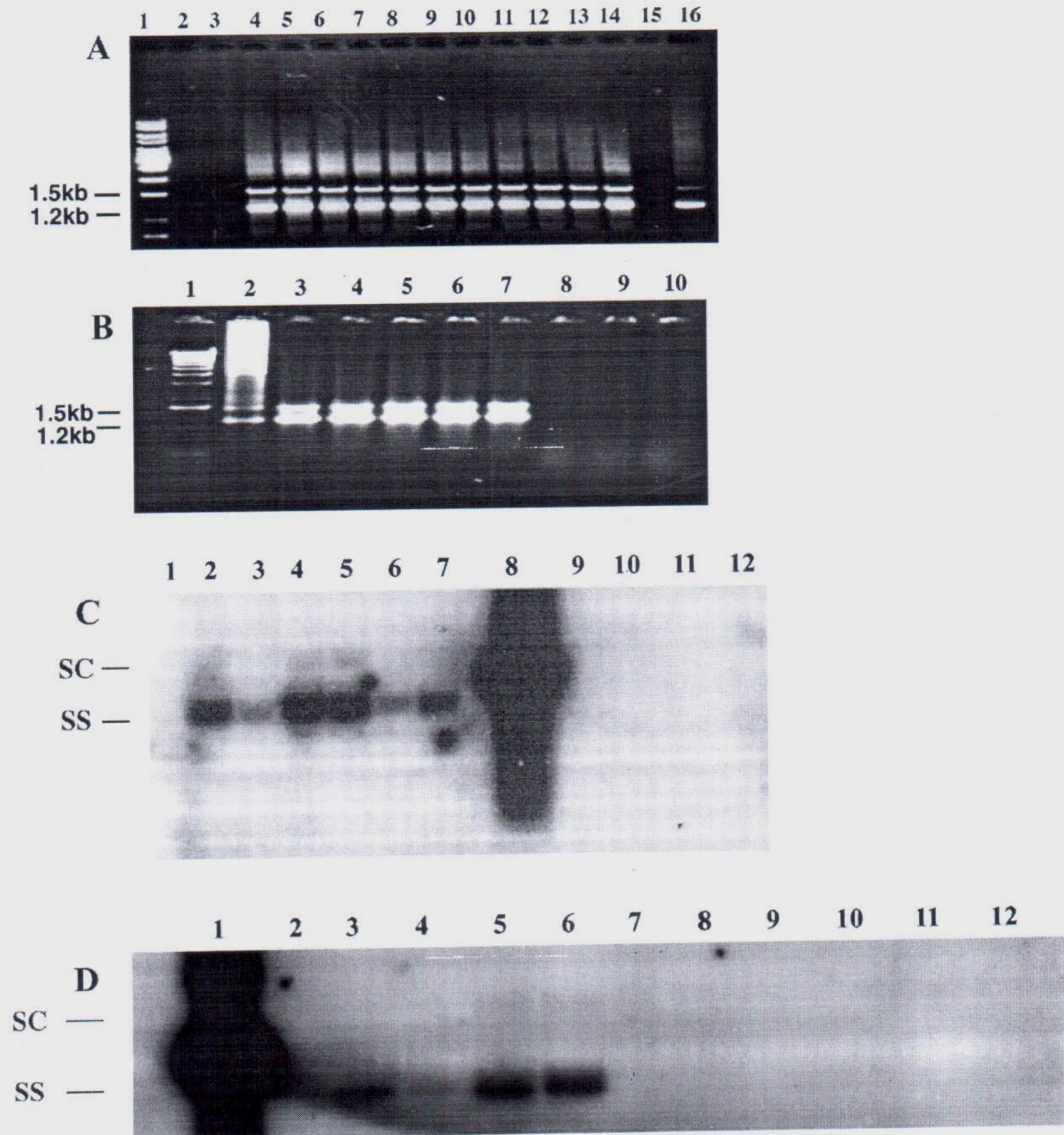
We performed four independent inoculation experiments with viruliferous whiteflies carrying CLCuD components with a mixture of two different but closely related DNA A components CLCuV-26 and CLCuV-72b prevailing at NIBGE, Faisalabad, Pakistan [33]. Comparing CLCuV-26 and CLCuV-72b in the regions encompassing the transgenes show overall homologies of: D1/4 84%, D1 85% and D1/d2/d3 77% with more significant stretches of homology within. We tested for virus resistance in untransformed controls and kanamycin resistant T_1 plants carrying the transgenes, where lines were scored as resistant if greater than 70% of plants showed no symptoms; accumulation of viral DNAs were analysed by multiplex PCR to discriminate between CLCuV-26 and CLCuV-72b and by Southern blot analysis to detect the DNA β component.

Of the 12 lines per construct tested many were still fully susceptible to infection and some showed mild and delayed symptoms [data not shown]. No recovery phenomenon was observed in any of these susceptible transgenic lines. But six T_1 antisense transgenic tobacco lines [line 1.50 from D1/4; lines 3.18, 3.20

Fig. 4. Analysis of replication of CLCuD components DNA A and DNA β in transgenic T_1 tobacco and cotton plants following infection by viruliferous whiteflies. **A**, **B**, multiplex PCR analysis of replication of DNA A viral variants 72b, 1.5 kb and clc26, 1.2 kb [33]. **A**, tobacco antisense lines: *1*, DNA marker ladder; *2*, resistant D1 line 3.28, *3*, resistant D1/d2/d3 line 5.22, *4–14*, susceptible lines, 1.7, 1.15, 1.23, 3.0, 3.3, 3.7, 3.13, 3.23, 3.27 and 5.21, 5.51, respectively, *15*, uninfected control, *16*, positive control PCR of cloned templates. **B**, tobacco sense lines, *1*, DNA marker ladder, *2*, positive control PCR of cloned templates, *3*, untransformed control, *4–7*, susceptible lines, 2.6, 2.500, 4.21 and 6.7 respectively, *8–10* resistant lines 2.20, 4.45 and 6.60, carrying D1/4, D1, D/d2/d3, respectively. **C–D**, Southern blot analysis of replication of the CLCuV DNA β component (1350 bp). **C**, *1*, uninfected tobacco, *2–7*, susceptible tobacco antisense lines, 1.15, 1.23, 3.0, 3.23, 5.21, 5.51 respectively, *8*, positive control PCR of cloned templates, *9–12*, resistant lines 1.50, 3.20, 3.28, 5.22, of D1/4, D1, D1 and D1/d2/d3 respectively. **D**, tobacco sense lines, *1*, positive control PCR of cloned templates, *2*, negative control, *3*, untransformed control, *4–6*, susceptible sense lines, 2.500, 4.21 and 6.7, of D1/4, D1 and D1/d2/d3 respectively, *7*, uninfected control, *8–12*, resistant sense lines, 2.20, 2.60, 4.45, 4.47 and 6.60 of D1/4, D1/4, D1, D1 and D1/d2/d3 respectively

and 3.28 from D1; lines 5.6 and 5.22 from D1/d2/d3], 12 tobacco sense lines, [lines 2.7, 2.20, 2.60 and 2.75 from D1/4; lines 4.2, 4.45, 4.75 and 4.90 from D1; lines 6.10, 6.60, 6.61 and 6.80 from D1/d2/d3], neither developed symptoms (Fig. 1) nor contained detectable amounts of DNAs of either CLCuD components (Fig. 4) so indicating inhibition of viral DNA replication.

In contrast, control plants, like susceptible transgenic plants, all showed the typical symptoms of CLCuD -vein thickening, enation and leaf curling; multiplex



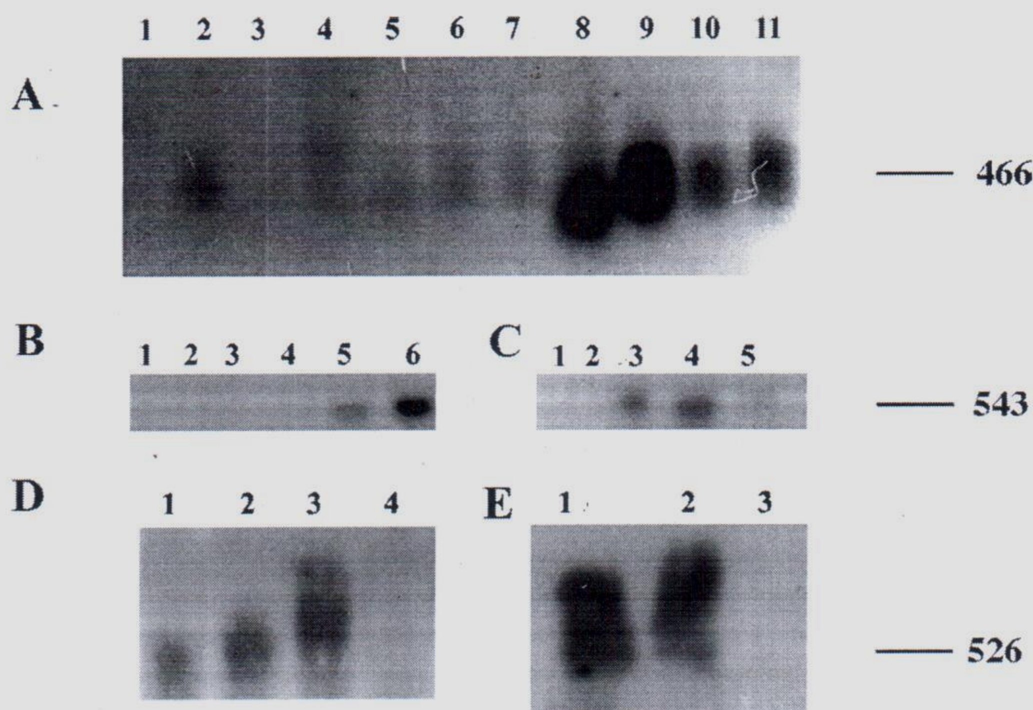


Fig. 5. Northern blot analysis of transgenic T_1 tobacco [Panels A–E]. **A**, *1*, untransformed control, *2–5*, susceptible D1/4 antisense lines 1.1, 1.7, 1.15 and 1.23, *6*, resistant D1/4 antisense line 1.50, *7–10*, resistant D1/4 sense lines, 2.1, 2.20, 2.60 and 2.75, *11*, susceptible D1/4 sense line 2.500. **B**, *1*, untransformed control, *2–3*, susceptible D1 antisense lines, 3.3, 3.7, *4–6*, resistant D1 antisense lines 3.18, 3.20 and 3.28. **C**, *1*, untransformed control, *2*, susceptible D1 sense line, 4.21, *3–5*, resistant D1 sense lines 4.45, 4.75 and 4.90. **D**, *1* and *3*, resistant D1/d2/d3 antisense lines, 5.6, and 5.22, *2*, susceptible D1/d2/d3 antisense line 5.21, *4*, untransformed control. **E**, *1–2*, resistant D1/d2/d3 sense lines 6.60, 6.80, *3*, untransformed control

PCR revealed the presence of both CLCuV-26 and CLCuV-72b in amplifying 1.2 and 1.5 kb diagnostic fragments respectively; and Southern blots revealed DNA β replication.

To ask whether resistance is a stably inherited trait, we challenged T_2 and T_3 lines derived from all resistant lines with viruliferous whiteflies and found 90–100% resistance, so showing stable inheritance over three generations.

Northern blot analysis, performed using tissue from the same plants chosen for the Southern analysis, shown in Fig. 3, yielded transcripts of the following sizes in lines transformed with the contructs: D1/4, 466nts; D1, 543nts; D1/d2/d3 526nts (Fig. 5). No transcripts were detected by northern analysis of selected resistant lines after infection in contrast to susceptible lines and controls [data not shown].

Analysis of resistance mechanism in tobacco sense lines

The sense transgenes had originally been designed as negative controls to the antisense lines but, as they also showed resistance to CLCuD, we investigated whether

resistance involved homology sensing mechanisms leading to post-transcriptional gene silencing, PTGS. Here mRNA is degraded by 21–23 nt long so-called guide RNAs or small interfering RNAs, siRNAs which prime synthesis of dsRNA from an mRNA template by an RNA-dependent RNA polymerase; the dsRNA is then processed by a dsRNA-specific RNase, Dicer, to more siRNAs to repeat the cycle of dsRNA synthesis and degradation [13, 23].

To look for dsRNA, we treated total RNA from two resistant, but uninfected sense lines, for each construct with RNase I to remove ssRNA and saw discrete size classes of dsRNA at 400 nt, 280 nt, 155 nt and smaller (Fig. 6). We also were able to detect faintly, in low MW RNA preparations, approx. 22 nt siRNAs hybridising with strand-specific RNA probes (data not shown).

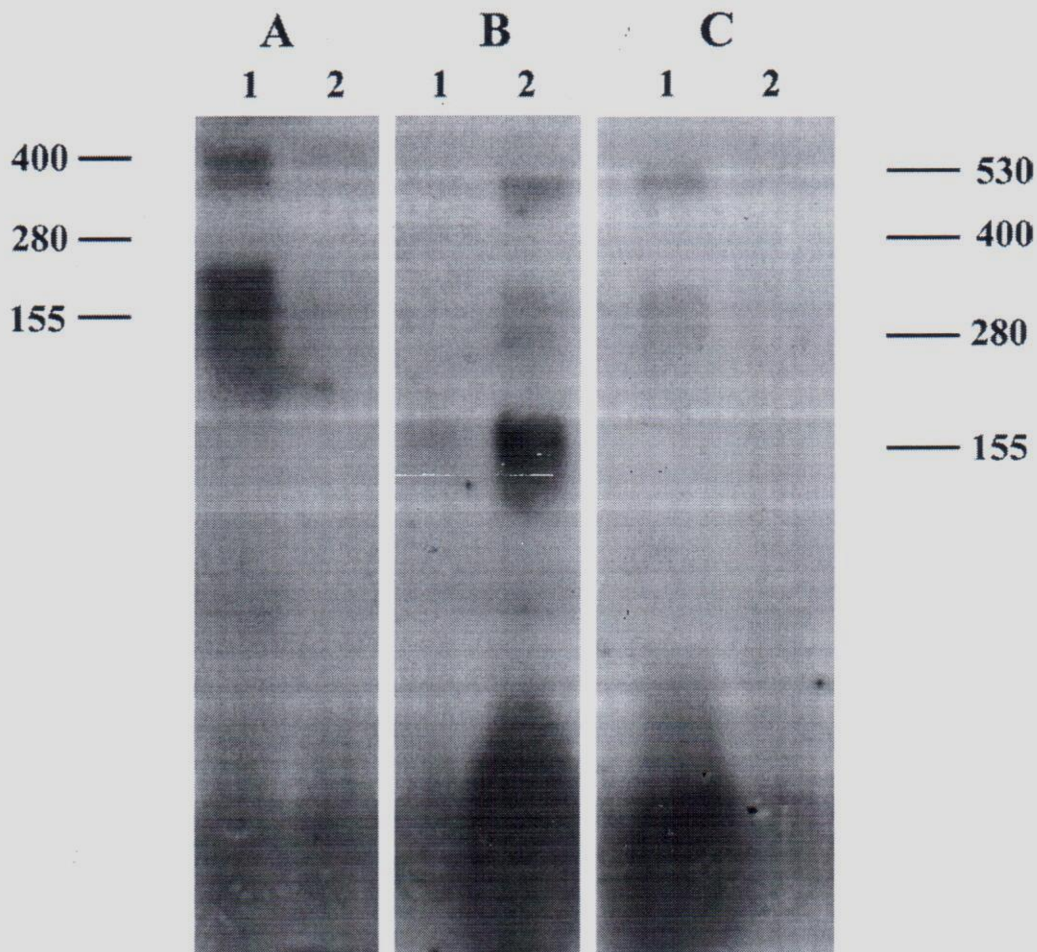


Fig. 6. Analysis of double-stranded RNA in resistant transgenic tobacco sense lines. 100 μg of total RNA, treated with RNase I to remove single-stranded RNA, was fractionated on 1.5% agarose formaldehyde gel and probed with PCR products oligolabelled with ^{32}PdCTP of D1/4, D1 and D1/d2/d3 in **A**, **B** and **C** respectively. **A** *1–2*, lines 2.20 and 2.60 respectively; **B** *1–2*, lines 4.45 and 4.75 respectively; **C** *1–2*, lines 6.60 and 6.80 respectively

Analysis of the sense transgenic lines suggest that a PTGS mechanism is primed to attack geminiviral mRNA following infection as no steady state levels of geminiviral mRNA nor transgene RNA are detected in resistant lines following infection. siRNAs can also promote transcriptional gene silencing, TGS, by promoter methylation [28, 32]. As uninfected resistant lines express transgene RNA, TGS cannot be operating prior to infection; but we cannot rule out any such epigenetic gene silencing by methylation of geminiviral and transgene promoters following infection.

With regard to the sense lines another possible mechanism of resistance is via production of trans-dominant defective geminiviral proteins encoded by the transgenes. The D1/4 construct encodes the amino-acids 1–213 of the 360 long Rep protein (encoded by AC1); D1 encodes amino-acids 212–330 of Rep, but with an internal methionine residue, for translational initiation, only at position 274; D1/d2/d3 encodes amino-acids 331–360 of Rep [no methionine], amino-acids 2–118 of the 134 long TrAP with methionine at position 112 (encoded by AC2) and amino-acids 1–127 of the 134 long REn (encoded by AC3). As antibodies were not available, we were unable to look for production of such truncated geminiviral transgenes cannot rule this out as an additional possible mechanism. Note however that the D1 construct is as effective at giving resistance yet may not yield a translation product as the internal methionine may be too far into the transcript to serve as an efficient initiation codon for only 56 residues of Rep.

Our data show that CLCuV DNA A transgene expression confers stable heritable resistance to CLCuD in tobacco, as a model system. This resistance is likely to be due to RNA silencing by the geminiviral transgenes rather than by expression of trans-dominant proteins as both antisense and sense constructs lack the signal expected for protein synthesis. The transformation of local elite cultivars of cotton with such geminiviral transgenes should be a solution to this serious plant pathogen in Pakistan.

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