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Association of Satellites with a Mastrevirus in Natural Infection: Complexity of *Wheat Dwarf India Virus* Disease

Jitendra Kumar, Jitesh Kumar, Sudhir P. Singh, Rakesh Tuli

National Agri-Food Biotechnology Institute, Mohali, Punjab, India

ABSTRACT

In contrast to begomoviruses, mastreviruses have not previously been shown to interact with satellites. This study reports the first identification of the association of satellites with a mastrevirus in field-grown plants. Two alphasatellite species were detected in different field samples of wheat infected with *Wheat dwarf India virus* (WDIV), a Cotton leaf curl Multan alphasatellite (CLCuMA) and a Guar leaf curl alphasatellite (GLCuA). In addition to the alphasatellites, a betasatellite, Ageratum yellow leaf curl betasatellite (AYLCB), was also identified in the wheat samples. No begomovirus was detected in the wheat samples, thus establishing association of the above-named satellites with WDIV. Agrobacterium-mediated inoculation of WDIV in wheat, in the presence of either of the alphasatellites or the betasatellite, resulted in infections inducing more severe symptoms. WDIV efficiently maintained each of the alphasatellites and the betasatellite in wheat. The satellites enhanced the level of WDIV DNA in wheat. Inoculation of the satellites isolated from wheat with various begomoviruses into *Nicotiana tabacum* demonstrated that these remain capable of interacting with the viruses with which they were first identified. Virus-specific small RNAs accumulated in wheat upon infection with WDIV but were lower in abundance in plants coinfected with the satellites, suggesting that both the alphasatellites and the betasatellite suppress RNA silencing. These results suggest that the selective advantage for the maintenance of the alphasatellites and the betasatellite by WDIV in the field is in overcoming RNA silencing-mediated host defense.

IMPORTANCE

Wheat is the most widely cultivated cereal crop in the world. A number of viruses are important pathogens of wheat, including the viruses of the genus *Mastrevirus*, family *Geminiviridae*. This study reports the association of subgenomic components, called satellites (alpha- and betasatellites), with a mastrevirus, *Wheat dwarf India virus* (WDIV), isolated from two distant locations in India. This study reports the first identification of the satellites in a monocot plant. The satellites enhanced accumulation of WDIV and severity of disease symptoms. The satellites lowered the concentration of virus-specific small RNAs in wheat plants, indicating their silencing suppressor activity. The involvement of the satellites in symptom severity of the mastrevirus can have implications in the form of economic impact of the virus on crop yield. Understanding the role of the satellites in disease severity is important for developing disease management strategies.

Geminiviruses (family *Geminiviridae*) are transmitted by insect vectors and constitute a large group of plant viruses responsible for causing crop losses worldwide. Their genomes consist of single-stranded circular DNA, encapsidated in twinned particles formed by joining two incomplete icosahedra. The family *Geminiviridae* comprises seven genera: *Begomovirus*, *Becurtovirus*, *Curtovirus*, *Eragrovirus*, *Mastrevirus*, *Topocuvirus*, and *Turncurtovirus* (1). With the exception of begomoviruses, geminiviruses have monopartite genomes. The genomes of begomoviruses are either bipartite (two components known as DNA-A and DNA-B, each approximately 2.6 kb) or monopartite (homologous to DNA-A, approximately 2.8 kb). With a few exceptions (2–5), the monopartite begomoviruses are associated with betasatellites or alphasatellites or both (6–9). In select cases, satellites have been found to be associated with bipartite begomoviruses as well (10–14).

Betasatellites are approximately half the size (~1,350 bp) of their helper begomoviruses and encode a single protein, βC1, which is a pathogenicity determinant (6, 8, 9, 15–18). The βC1 protein suppresses silencing, upregulates viral DNA levels in plants, assists with virus movement, and modulates virus host range (16–23). Betasatellites share only the nonanucleotide [TAA TATTAC] sequence with the helper begomovirus, which is believed to serve as the origin of replication (6, 8, 9). Some monopartite begomoviruses are either poorly infectious and/or induce atypical symptoms in the absence of the betasatellite (15, 16, 22). Alphasatellites are also approximately half the size (~1,350 bp) of their helper begomoviruses and encode a single product, which is similar to the replication-associated protein (Rep; a rolling-circle replication initiator protein) of another family of single-stranded DNA viruses, the nanoviruses (7–9). Unlike betasatellites, the presence of alphasatellites, as in the case of Ageratum yellow vein Singapore alphasatellite (AYVSGA), Tobacco curly shoot alphasatellite (TbCSA), *Gossypium darwinii* symptomless alphasatellite (GDarSLA), and *Gossypium mustelinum* symptomless alphasatellite (GMusSLA) in plants infected with begomovirus-betasatellite

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Address correspondence to Rakesh Tuli, rakeshtuli@hotmail.com.

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complexes, reduces the accumulation of betasatellite (24–26) and attenuates viral symptoms (24, 25).

The geminivirus-encoded Rep protein binds to iterated DNA motifs (termed iterons), which is a key step in initiating replication of viral DNA. The interaction is specific and usually limits Rep transreplication to its own genomic components (22, 27, 28). However, the betasatellites are transreplicated by a diverse range of begomoviruses (8, 9, 14, 17, 22, 23, 29–32), thus suggesting a more relaxed interaction between betasatellites and their helper begomoviruses. For example, Ageratum yellow vein betasatellite (AYVB) and Cotton leaf curl Multan betasatellite (CLCuMB) are transreplicated by noncognate helper begomoviruses (14, 17, 22, 30, 31). A defective betasatellite, *Tomato leaf curl virus* (ToLCV) sat-DNA, is transreplicated by a noncognate begomovirus, *African cassava mosaic virus* (ACMV), and even by a curtovirus, *Beet curly top virus* (32).

The majority of mastreviruses infect monocot plants and are transmitted by leafhoppers (33–35). The genome of mastreviruses comprises four open reading frames (ORFs). Two ORFs, located on the virion-sense strand, encode the movement protein (MP) and the coat protein (CP). The other two ORFs, RepA and RepB, are transcribed from the complementary-sense strand. The protein RepA is translated from a nonspliced transcript, whereas Rep is translated from a spliced transcript that fuses RepA and RepB (33, 36). The ORFs on the virion and complementary strands are separated by a small intergenic region (SIR) and a large intergenic region (LIR). Rep is required for viral DNA replication, whereas RepA interacts with plant retinoblastoma-related proteins to regulate the host cell cycle (33, 36, 37). The viral genome is encapsidated in CP, and the movement is mediated by both MP and CP (33, 36). No mastrevirus has yet been reported to be associated with satellites.

The study presented here reports the first identification of the association of satellites with a mastrevirus, *Wheat dwarf India virus* (WDIV), which causes dwarfing in wheat. The role of the detected satellites (two alphasatellites and a betasatellite) in the accumulation of WDIV and the severity of disease symptoms was studied. The ability of the satellites identified in wheat to interact with begomoviruses, with which they were first identified, was examined.

MATERIALS AND METHODS

Virus source. Leaf samples from 450 symptomatic wheat plants were collected between 2010 and 2012 from research farms located in Mohali (30°42′N, 76°42′E) and Wellington (11°22′N, 76°47′E) in India. The symptomatic plants were selected on the basis of dwarfing (Fig. 1A), yellowing (Fig. 1B), or yellowing and dwarfing (Fig. 1C). Leaves from 10 asymptomatic plants were taken as negative controls. Total DNA was isolated using the DNeasy plant minikit columns (Qiagen GmbH, Germany).

Detection, amplification, and cloning of WDIV and satellites. WDIV and satellite DNAs were amplified by PCR and rolling-circle amplification (RCA). Approximately 100 ng of DNA from each plant leaf was used for the PCR and RCA reactions. Phusion High-Fidelity DNA polymerase (Thermo Scientific, USA) was used in the PCR assays. RCA was performed using the TempliPhi amplification kit (GE Healthcare, USA). The RCA products were digested with the BamHI, EcoRI, HindIII, KpnI, NdeI, PstI, SacI, SalI, and XbaI restriction endonucleases (New England BioLabs, Ipswich, United Kingdom) to obtain DNA fragments of the desired sizes for WDIV, begomoviruses, and the satellites. The primer pair MF1_FOR/REV (Table 1) was used in the PCR assays to amplify the WDIV genome from wheat samples. Alphasatellites and betasatellite were amplified in PCR assays using the primer pairs nanofor/nanorev and β01/04 (Table 1), respectively, as described previously (38). The RCA and PCR assays were performed for all of the 460 wheat (450 symptomatic and 10 asymptomatic) samples. The RCA restriction products were cloned into SK+ (Fermentas, European Union) and the PCR products into pDRIVE cloning vectors (Qiagen GmbH, Germany). Five RCA and three PCR clones for the virus, alphasatellite, and betasatellite were sequenced from each of the 80 plant samples (selected out of the 425 virus-positive samples) using an automated sequencer (3730xl DNA analyzer; Applied Biosystems, USA).

Sequence analysis. Similarity searches were performed using BLASTN. The most similar sequences were retrieved from the database for further analysis. Sequence similarities were calculated using multiple sequence alignment (http://www.ebi.ac.uk/Tools/msa/clustalo/) and pairwise global alignment (http://www.ebi.ac.uk/Tools/psa/emboss_needle/nucleotide.html).

Constructs for infectivity of WDIV, begomoviruses, and satellites. The constructs for the infectivity of WDIV (JQ361910) and begomoviruses (*Ageratum enation virus* [AEV; JF728864], *Cotton leaf curl Burewala virus* [CLCuBuV; JN678802], and *Cyamopsis tetragonoloba leaf curl virus* [CyTLCuV; GU385879]) were prepared by cloning head-to-tail tandem repeats of the full-length viral genome in pCAMBIA1301, as described previously (39, 40). The clone for the infectivity of Ageratum yellow leaf curl betasatellite (AYLCB; KC305084) was prepared using primers β01D/04D and β05D/06D (Table 1) in two PCRs. The two PCR products were digested with BamHI and then ligated to generate head-to-tail tandem repeats of the betasatellite DNA. The ligated product was cloned into a pDRIVE cloning vector. One positive clone was digested with XbaI restriction endonuclease. The product released after digestion was ligated at the XbaI site in the binary vector pCAMBIA1301 (CAMBIA, Canberra, Australia). A clone for the infectivity of Cotton leaf curl Multan betasatellite (CLCuMB; HQ257372) was prepared as described for AYLCB. For preparing the constructs for the infectivity of Cotton leaf curl Multan alphasatellite (CLCuMA; KC305093) and Guar leaf curl alphasatellite (GLCuA; KC305095), the pDRIVE vectors having alphasatellites were double digested with HindIII and PstI to yield a 918-bp fragment of the alphasatellite encompassing the predicted origin of replication. The digestion product was introduced into pCAMBIA1301 at the HindIII/PstI site to generate a pCAMBIA-alpha 918-bp intermediate clone. The alphasatellite cloned into the pDRIVE vector was digested with PstI and cloned into the PstI site of the pCAMBIA-alpha 918-bp intermediate clone to generate a 1.7-mer head-to-tail tandem repeat of the alphasatellite DNA. All of the PCR-amplified products were sequenced to confirm the absence of mutations in the clones. Derivatives of the binary vector pCAMBIA1301, having constructs for infectivity of WDIV, begomoviruses, alphasatellites, and betasatellites, were mobilized separately into the *Agrobacterium tumefaciens* strain GV3101 via the freeze-thaw method (41).

Agroinoculation. *A. tumefaciens* cultures containing the constructs for infectivity were grown separately in 5 ml Luria-Bertani (LB) medium for 24 h at 28°C and 200 rpm in a rotary shaker. A 100-μl primary culture was inoculated in 200 ml fresh LB medium and incubated overnight (optical density at 550 nm of 1), pelleted, and resuspended in 200 ml infiltration buffer (10 mM morpholineethanesulfonic acid [MES], 10 mM $MgCl_2$, and 100 μM acetosyringone). Leaves of 7-day-old seedlings of 500 wheat plants representing 20 wheat cultivars (Table 2) at the 2- to 3-leaf stage were agroinoculated using a needleless syringe. *Nicotiana tabacum* plants at the 4-leaf stage were also inoculated with the constructs. Inoculated tobacco and wheat plants were maintained in separate plant growth chambers (PGR14; Conviron, Canada) at 25°C and 22°C, respectively.

Detection and quantification of virus and satellites. Total DNA was isolated from the leaves of the agroinoculated plants 27 days postinoculation (dpi). The begomoviruses and WDIV were detected using primers CPB1/B2 and CP01/02, whereas the alphasatellite and betasatellite were detected using the nanofor/nanorev and β01/04 primers, respectively (Table 1). For quantification of WDIV in different parts (leaf, stem, and root) of the wheat plant, total DNA was isolated from each part separately.

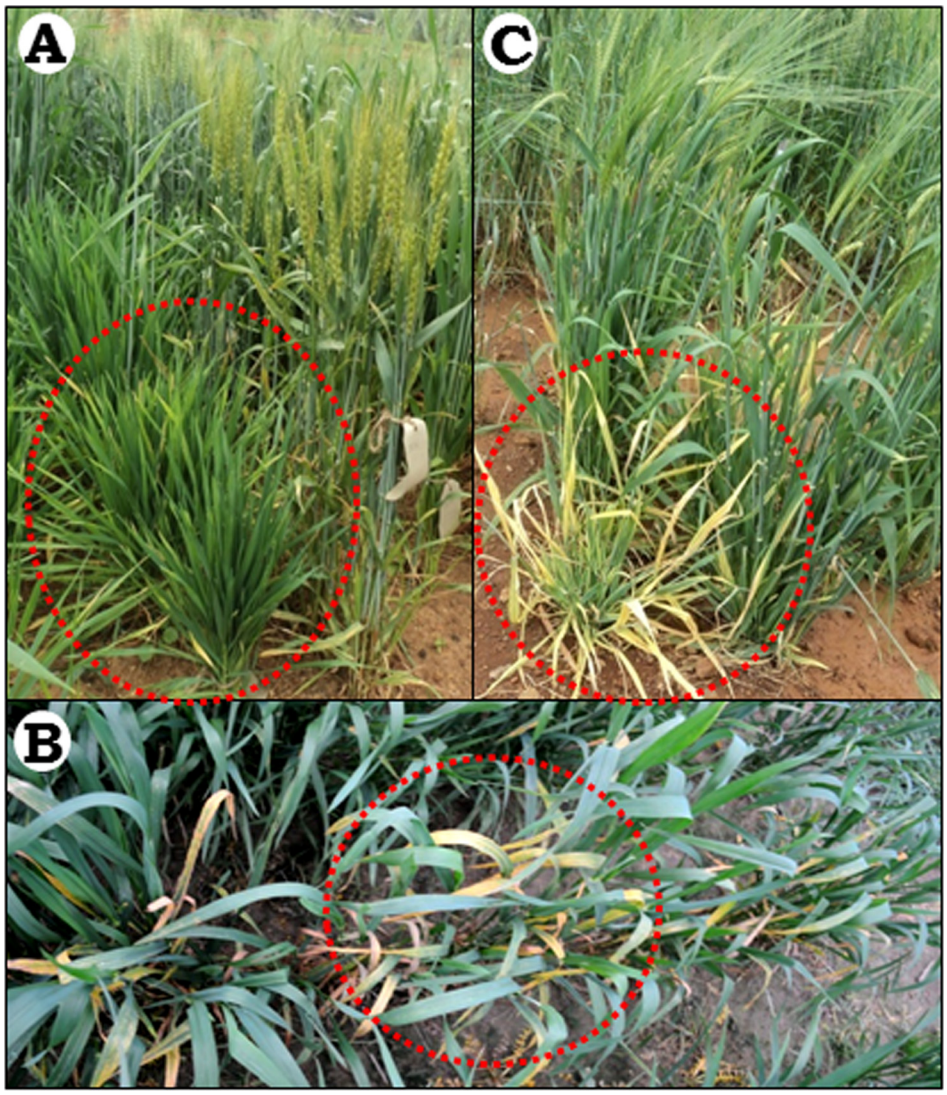

FIG 1 Wheat plants suspected of being infected with the mastrevirus. Wheat plants (red circle) showing dwarfing (A), yellowing (B), and yellowing with dwarfing (C).

TABLE 1 List of the primers used in this study

Name of primer	Nucleotide sequence (5′ to 3′)[a]	Reference or source
MF1_FOR	GCCCTGAACCTACATCTGTCT	34
MF1_REV	TGCCTTATACGTCATAGTCTCG	34
β01	GAAACCACTACGCTACGCAGC	32
β04	ACCCTCCCAGGGGTACACACCG	32
Nanofor	AAGTGGGTCCTGGTTCTA	32
Nanorev	CTGTACAGGTCTCTGGC	32
β01D	ATCTAGAGAAACCACTACGCTACGCAGC	This study
β04D	AGGATCCACCCTCCCAGGGGTACACACCG	This study
β05D	AGGATCCGAAACCACTACGCTACGCAGC	This study
β06D	ATCTAGAACCCTCCCAGGGGTACACACCG	This study
EF1αfor	GCTGTCAAGTTTGCTGAGATCC	This study
EF1αrev	GTACTGAGCGAAGGTCTCCAC	This study
CP01	ATGTCTCAGGTGAAGAAGAGGAC	This study
CP02	TTACTGGTTACCGATACTCTTGA	This study
CPB1	ATTATGTCGAAGCGACCAGCA	This study
CPB2	TGTTCTCATCCATCCAAATCTT	This study

[a] Underlined nucleotide sequences represent the sites for restriction endonucleases BamHI (GGATCC) and XbaI (TCTAGA).

Total DNA from 3 biological replicates (samples from 3 plants) of each satellite-virus combination was pooled for Southern hybridization. The pooled total DNA (3 μg each from leaf, stem, or root) was electrophoresed on a 1% agarose gel and transferred to a Hybond N+ nylon membrane (GE Healthcare, USA) using standard methods (42). For detecting WDIV DNA, the coat protein gene of WDIV was labeled using a DIG High Prime DNA labeling and detection kit (Roche Applied Science, USA). The coat protein genes of AEV, CLCuBuV, and CyTLCuV were labeled and used as probes to detect the respective virus in the inoculated plants. For the detection of CLCuMA, GLCuA, AYLCB, and CLCuMB, the Rep and βC1 genes of the corresponding alphasatellites or betasatellites were used as probes. Hybridization of the probes to DNA on the membrane was detected using the chromogenic substrate nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate (NBT-BCIP) (Roche Diagnostics, Switzerland).

The primers targeting the coat protein gene of WDIV (CP01/02; Table 1) were used to perform real-time PCR and semiquantitative PCR. Elongation factor 1 alpha (EF1αfor/rev; Table 1) was used as an internal control in both the semiquantitative and real-time PCR assays. Total DNA isolated from wheat was adjusted to 50 ng μl^{-1} for use in real-time and

TABLE 2 Disease severity in wheat cultivars agroinoculated with WDIV and the satellites

No.	Wheat cultivar	Disease severity of agroinoculated wheat plants (mean ht of 3 plants in cm ± SD)				
		Control	WDIV	WDIV + CLCuMA	WDIV + AYLCB	WDIV + AYLCB + CLCuMA
1	C 306	71.6 ± 2.56	60.6 ± 2.08	52.3 ± 1.52	39.6 ± 1.52	28.6 ± 2.08
2	DBW 17	69.6 ± 3.05	56.3 ± 1.52	48.3 ± 2.51	41.6 ± 1.52	32 ± 1.73
3	GW 366	67.6 ± 1.52	56.3 ± 1.15	48.6 ± 1.52	46.6 ± 3.51	34.3 ± 2.51
4	HD 2329	66.6 ± 1.52	55.6 ± 2.08	50 ± 2.64	41.3 ± 2.51	32.6 ± 1.52
5	HD 2733	69.6 ± 2.08	56.6 ± 1.52	50.3 ± 1.52	42.6 ± 1.52	33 ± 1.73
6	HI 977	68.6 ± 1.52	57.3 ± 1.52	50 ± 1.73	41.3 ± 1.15	36.3 ± 2.51
7	IITR 26	75.3 ± 1.52	67.6 ± 2.08	59.6 ± 2.08	49.6 ± 2.08	42.3 ± 1.15
8	Kharchia 65	70 ± 2	49.3 ± 2.30	41.6 ± 1.52	36 ± 1.73	27 ± 1.73
9	LOK 1	69 ± 2	56.3 ± 1.52	48.6 ± 2.51	42 ± 1.73	31.6 ± 2.08
10	NI 5439	69 ± 3	56 ± 1.73	49.6 ± 1.52	47 ± 2.08	31.3 ± 1.52
11	NIAW 917	70 ± 2	55.6 ± 1.52	48.3 ± 2.08	45.6 ± 4.50	32.6 ± 2.51
12	NP 4	68.6 ± 2.08	54.6 ± 1.15	43.3 ± 2.51	39.6 ± 1.52	29.6 ± 1.52
13	PBW 343	70.3 ± 1.52	57.3 ± 2.51	50.3 ± 1.52	41.6 ± 2.08	29.6 ± 1.52
14	RAJ 3765	71 ± 2	67.3 ± 1.52	58.3 ± 2.08	50.6 ± 1.15	42 ± 1.73
15	Sonalika	71.6 ± 1.52	70.3 ± 2.51	61.6 ± 2.51	50.6 ± 1.52	44.6 ± 1.52
16	UP 2772	68.3 ± 1.52	55.3 ± 1.52	48.6 ± 1.52	41 ± 1.73	31 ± 1.73
17	WH 291	69.6 ± 1.52	57.6 ± 2.51	51.6 ± 2.08	41.6 ± 1.52	31.3 ± 1.52
18	WH 542	70.3 ± 1.52	57.3 ± 1.52	51.3 ± 2.51	41.3 ± 2.51	30.6 ± 2.08
19	WH 533	70.3 ± 2.51	57.3 ± 3.05	52.3 ± 1.52	42.6 ± 1.15	31 ± 2.64
20	WL 711	71 ± 2	47.6 ± 2.08	40.3 ± 1.52	30.3 ± 1.52	23.6 ± 1.52

semiquantitative PCR in a 25-μl reaction volume containing 30 picomoles of each primer. Semiquantitative PCR and real-time PCR were performed in an Eppendorf Mastercycler Pro (Eppendorf AG, Hamburg, Germany) and in a 7500 Fast real-time PCR system (Applied Biosystems, California, USA), respectively. The Platinum SYBR green chemistry (Fast SYBR green master mix; Life Technologies, Applied Biosystems, USA), with a final fluorescein concentration of 10 nM, was used as per the manufacturer's recommendation. Three biological replicates were amplified separately in real-time PCR assays for each satellite-virus combination of agroinoculated wheat plants.

Detection of virus-specific small RNAs. Low-molecular-weight RNAs were isolated from 2 g of leaf tissue from the infected agroinoculated wheat plants using a mirVana microRNA (miRNA) isolation kit (Ambion, Life Technologies, USA). The small RNAs were eluted in 50% formamide and heated to 65°C for 5 min before loading on a 15% polyacrylamide (29:1) gel containing 7 M urea in 0.5× Tris-borate-EDTA (TBE). Small RNAs from three biological replicates for each combination were pooled and electrophoresed. The segment of the polyacrylamide gel containing RNAs between 10 and 25 nucleotides (nt) was blotted to a Hybond N membrane (GE Healthcare, USA) to transfer the small RNAs. The probe was made by labeling the full-length WDIV genome with [α-^{32}P]dATP. Hybridization was carried out at 42°C overnight in 5× SSC (0.75 M NaCl, 0.075 M trisodium citrate [pH 7.0]), 1× Denhardt solution (0.1% each Ficoll, polyvinylpyrrolidone, and bovine serum albumin), and 0.5% sodium dodecyl sulfate (SDS) with competitor DNA (herring sperm DNA; Sigma). The membranes were then washed in 1× SSC-1% SDS, followed by 0.1× SSC-1% SDS and then 0.01× SSC-1% SDS at 50°C. The washed membranes were exposed to a Kodak phosphorimager screen in a Kodak exposure cassette (Sigma-Aldrich Chemicals Private Limited, India). Hybridization signals were detected using a phosphorimager (Bio-Rad Laboratories, California, USA).

Nucleotide sequence accession numbers. The sequences determined in this study have been deposited in GenBank under accession numbers KC305084, KC305086, KC305087, KC305089, KC305090, KC305091, KC305093, KC305095, JF781306, and HQ257372.

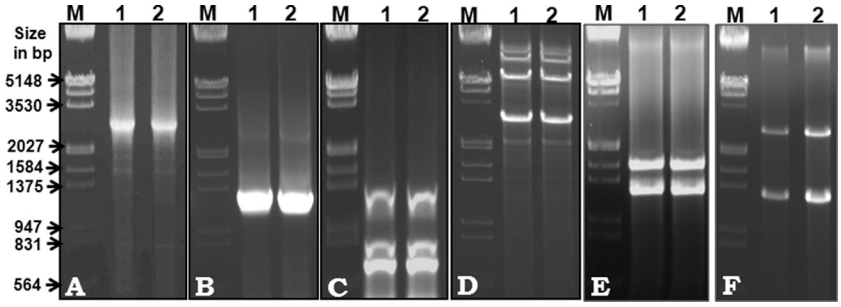

FIG 2 Detection of the virus and the satellite DNAs in symptomatic plant samples. (A) PCR amplicon of ~2.8 kb representing the genome size of WDIV. PCR products of the alphasatellite showing one amplicon of ~1.3 kb (B) and the betasatellite showing multiple amplicons of ~650 bp, ~850 bp, and ~1.3 kb (C). (D) KpnI-digested RCA products showing the fragments of ~2.8 kb and ~5.6 kb and higher multimeric forms in the diseased wheat samples. PstI-digested RCA products showing fragments of ~1.3 and ~1.6 kb (C), and NdeI-digested products showing ~1.3- and ~2.5-kb fragments (D). Lanes 1 and 2 in panels A to F represent the wheat samples from Mohali and Wellington, respectively. M, EcoRI/HindIII-digested λDNA.

TABLE 3 Number of wheat plants found positive for mastrevirus and the associated satellites

Mastrevirus and satellite(s)[a]	No. of positive samples out of 450 samples tested
WDIV	425
WDIV and CLCuMA	356
WDIV and GLCuA	44
WDIV and AYLCB	354
WDIV and AYLCB and AYLCBΔ	133
WDIV and AYLCB and CLCuMA	298
WDIV and AYLCB and GLCuA	56

[a] WDIV, *Wheat dwarf India virus*; AYLCB, Ageratum yellow leaf curl betasatellite; CLCuMA, Cotton leaf curl Multan alphasatellite; GLCuA, Guar leaf curl alphasatellite; AYLCBΔ, deletion mutants of AYLCB.

RESULTS

Detection of WDIV and satellites in wheat. Of the 450 symptomatic wheat plant samples tested, 425, representing a total of 30 wheat cultivars (see Table S1 in the supplemental material), yielded an amplicon of ~2.8 kb using WDIV-specific primers (Fig. 2A). Of the 425 WDIV-positive wheat plant samples, 400 and 354 (Table 3) yielded the expected fragments of ~1.3 kb (Fig. 2B and C) using alphasatellite- and betasatellite-specific primers, respectively. Additional fragments of smaller sizes were detected by the betasatellite-specific primers (Fig. 2C). RCA also gave positive results for the same 425 samples. Restriction fragments of ~2.8 kb and ~5.6 kb and longer multimers were obtained upon partial digestion of the RCA products with KpnI (Fig. 2D). Complete digestion of the RCA products with PstI gave restriction fragments of ~1.3 and ~1.6 kb (Fig. 2E), whereas the fragments of ~0.25 (not visible in Fig. 2F), ~1.3, and ~2.5 kb were observed upon digestion with NdeI (Fig. 2F). Restriction endonucleases BamHI, EcoRI, and HindIII yielded fragments matching the size of WDIV (~2.8 kb) or the satellite genome (~1.3 kb; data not shown). Digestion of the RCA products from the wheat samples with SacI, SalI, and XbaI did not yield any fragments. Restriction sites used for the digestion of RCA products for the cloning of WDIV and the satellite DNAs are shown on the physical maps (Fig. 3). Sequencing of the ~0.25-, ~1.6-, ~2.5-, and ~2.8-kb RCA restriction molecules confirmed these as the fragments derived from the genome of WDIV. The fragment of ~5.6 kb comprised two full-length genomes of WDIV. Analysis of the sequences of the ~1.3-kb RCA restriction fragments revealed these to be either the alphasatellites (accession no. KC305093 and KC305095), the betasatellite (accession no. KC305084), or partial fragments of the WDIV genome (data not shown). Sequencing of the PCR-amplified products of WDIV, the alphasatellite, and the betasatellite established the presence of WDIV and the satellites in the infected plants. The additional PCR amplicons of 657, 891, 929, 1,046, and 1,084 bp (accession no. KC305091, KC305090, KC305089,

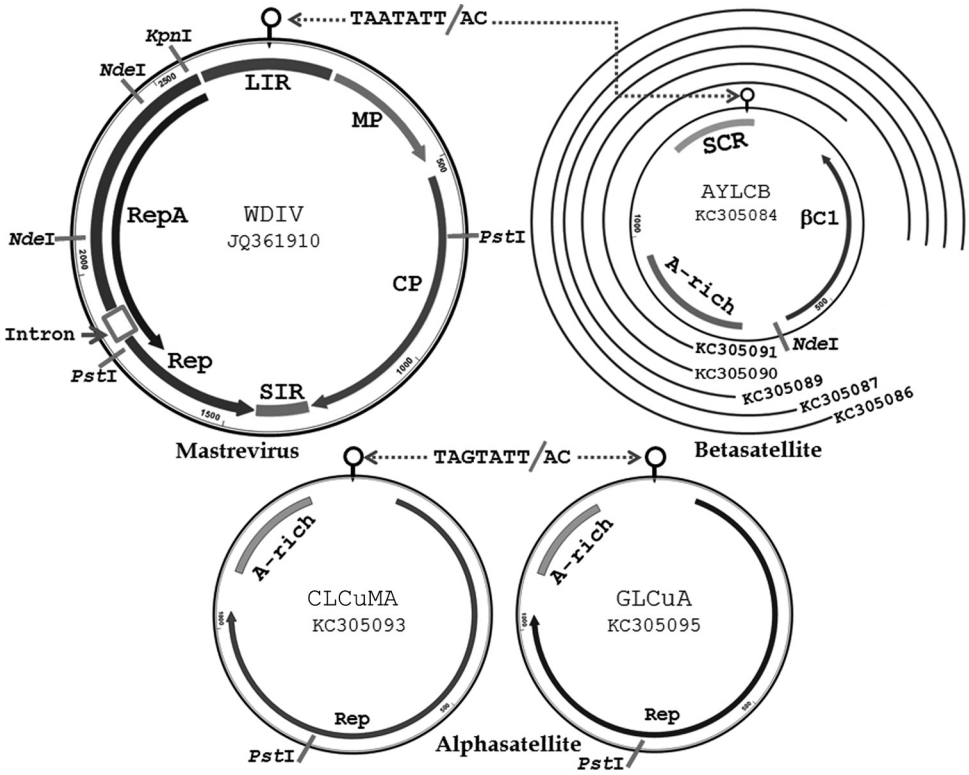

FIG 3 Genomic organization of WDIV, AYLCB, CLCuMA, and GLCuA. The sequences retained in AYLCB deletion mutants are indicated by arcs along with the accession numbers of the defective molecules (657 bp, KC305091; 891 bp, KC305090; 929 bp, KC305089; 1,046 bp, KC305087; and 1,084 bp, KC305086). The positions of the open reading frames (ORFs), coat protein (CP), movement protein (MP), replication-associated protein (Rep), replication-associated protein A (RepA), and βC1 of betasatellite are shown. Other features, including the intron in the Rep gene, the large intergenic region (LIR), the small intergenic region (SIR), the A-rich region, and the satellite conserved region (SCR), are also shown. The nonanucleotide sequences of WDIV and AYLCB are identical [TAATATT|AC], whereas TAGTATT|AC is present in the alphasatellites. Recognition sites for the restriction endonucleases KpnI, NdeI, and PstI, used for cloning from the RCA products, are shown on the genome maps.

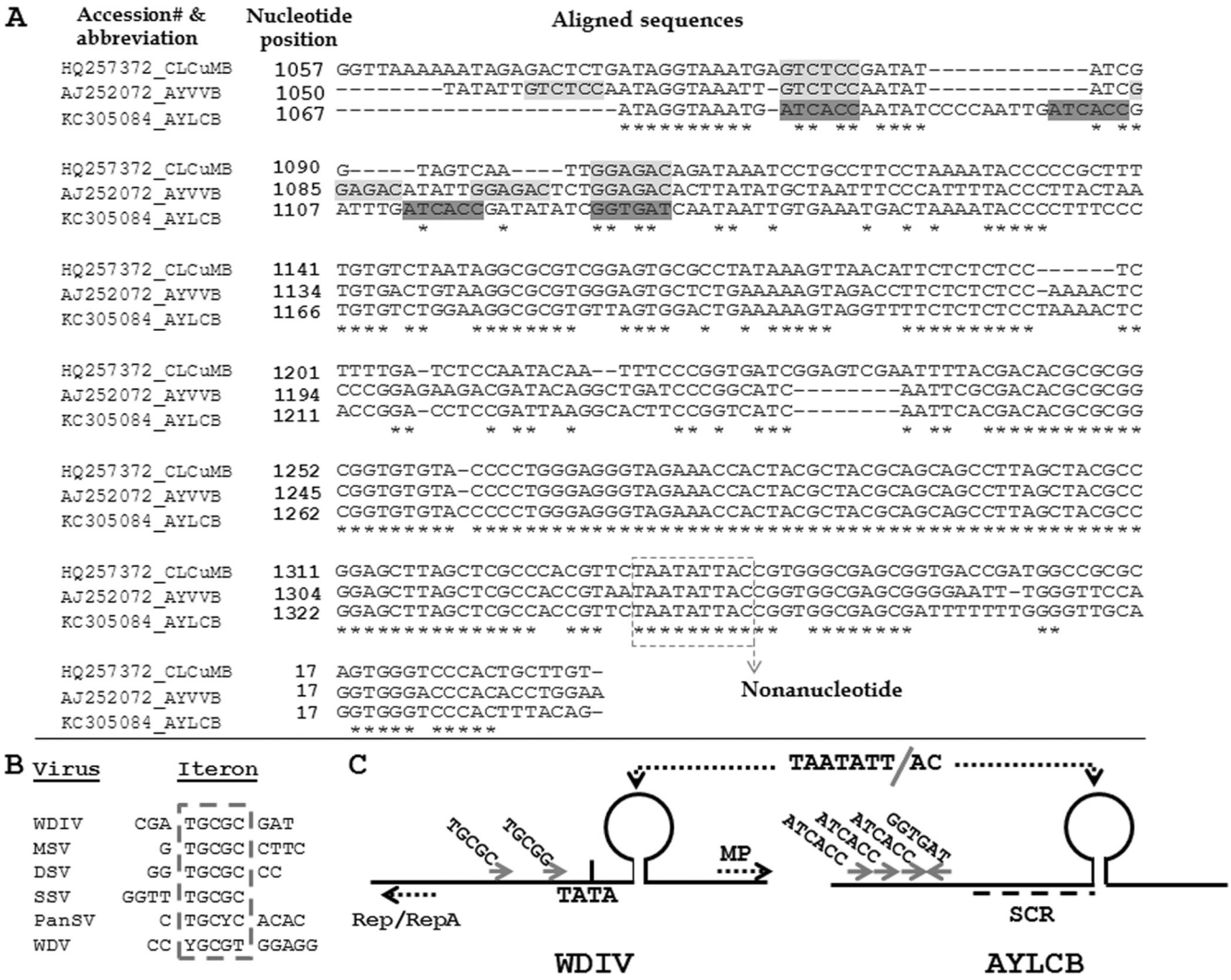

FIG 4 Alignment of the satellite conserved region (SCR) of the betasatellite sequences. SCR and the nucleotide sequence region (1,050 to 1,268 bp) of AYVB suggested to be responsible for transreplication, as well as the nonanucleotide region, are aligned with the corresponding regions of AYLCB and CLCuMB. Iteron-like sequences of AYVB (GTCTCC and GGAGAC) and AYLCB (ATCACC and GGTGAT) located within the region responsible for transreplication of the betasatellites are highlighted. Conserved nucleotides are denoted by asterisks. (A) Nucleotide positions at the start of the alignment of each sequence are shown. (B) The predicted iterons of MSV, DSV, SSV, PanSV, and WDV are aligned with the corresponding region of WDIV. (C) Schematic representations of the reiterated motifs on WDIV, the iteron-like sequences on AYLCB, the TATA box on WDIV, the nonanucleotide sequence present in hairpins on WDIV and AYLCB, the start site of the ORFs on both the virion (MP) and the complementary strand (Rep/RepA) of WDIV, and the SCR on AYLCB are shown.

KC305087, KC305086, respectively), obtained by using the betasatellite-specific primers, represented deletion mutants of the betasatellite. Digestion of the RCA products from the asymptomatic plants used as negative controls yielded no bands diagnostic of either the virus or the satellites. No satellite was detected in the symptomatic WDIV-negative samples. No sequence suggesting the presence of a begomovirus was obtained from any of the wheat samples investigated in this study.

Sequence analysis. The virus genome amplified from the plant samples collected from the two field locations was 2,783 bp long (clones, WDIV-[IN:Wel9:12] and WDIV-[IN:Moh6:12]). Analysis of the sequence revealed a genome organization typical of a mastrevirus (Fig. 3). Similarity searches showed 99.5 to 100% nucleotide sequence identity to WDIV (accession no. JF781306, JQ361910, and JQ361911), thus establishing the presence of WDIV in wheat plant samples.

The betasatellite identified in WDIV-infected wheat plants showed a high level of nucleotide sequence identity (99%) to AYLCB from *Ageratum conyzoides* (43), thus establishing the presence of AYLCB in wheat. Analysis of the full length as well as the deletion mutants of the betasatellite revealed three conserved features: a predicted stem-loop structure with the loop sequence TA ATATTAC (the nonanucleotide sequence), an adenine (A)-rich region, and a highly conserved region, known as the satellite conserved region (SCR), found in all betasatellites. The full-length betasatellite molecule contained a full-length βC1 gene, while the deletion mutants contained partial deletions of βC1; βC1 was absent from the 657-bp molecule (Fig. 3).

The alignment of the SCR nucleotide sequence of AYVB (AJ252072) as well as the sequence between 1,050 and 1,268 bp (predicted to be responsible for transreplication [44]) with the nucleotide sequences of the corresponding regions in AYLCB

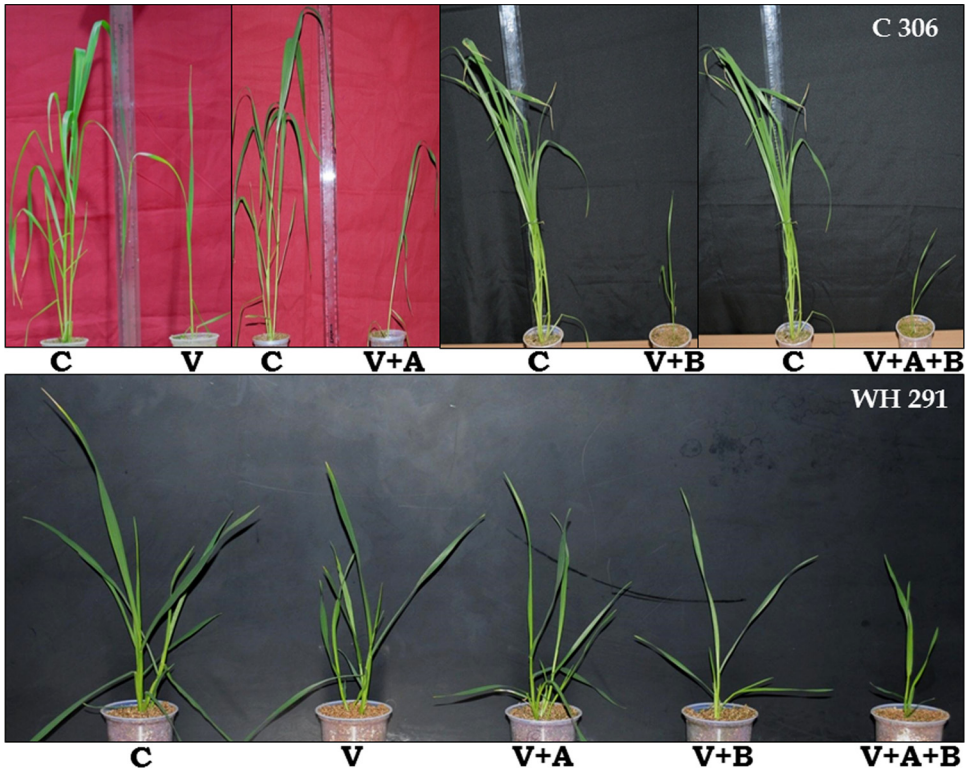

FIG 5 Effects of the satellites on the severity of disease in wheat plants infected with WDIV. Mock-inoculated wheat plants (C) and wheat plants inoculated with WDIV (V), WDIV with CLCuMA (V+A), WDIV with AYLCB (V+B), and WDIV together with CLCuMA and AYLCB (V+A+B) are shown at 42 dpi. Results for the two cultivars (C 306 and WH 291) with different levels of susceptibility are shown. Details regarding the susceptibility of the cultivars are given in Table 2.

identified from wheat (KC305084) and CLCuMB from cotton (HQ257372) revealed many conserved sequence regions (Fig. 4A). AYLCB contained the repeats of ATCACC and its complementary sequence GGTGAT, which were absent in CLCuMB and AYVB (Fig. 4A). The repeats of GTCTCC and GGAGAC, suggested to be important in the transreplication of AYVB (44), were present in CLCuMB (Fig. 4A) but not in AYLCB. Comparison of the predicted iterons (27) of *Maize streak virus* (MSV), *Digitaria streak virus* (DSV), *Sugarcane streak virus* (SSV), *Panicum streak virus* (PanSV), and *Wheat dwarf virus* (WDV) with the corresponding region in WDIV indicated that TGCGC is the probable WDIV iteron (Fig. 4B). The reiterated motifs in WDIV and AYLCB detected in this study (Fig. 4C) display the features (44, 45) associated with iteron-like sequences.

Two types of alphasatellites were detected in different wheat samples. Both of these alphasatellites were ~1.3 kb long and shared 77% identity with one another. In a similarity search, one alphasatellite showed an identity of 99% with Cotton leaf curl Multan alphasatellite (CLCuMA) from okra (46), and the other showed an identity of 93% with Guar leaf curl alphasatellite (GLCuA) from *Cyamopsis tetragonoloba* (38), thus establishing the presence of CLCuMA and GLCuA in wheat. Both of these alphasatellite molecules possessed three highly conserved features: a loop sequence TAGTATTAC (nonanucleotide sequence), an adenine (A)-rich region, and a gene encoding a Rep (Fig. 3).

Association of the satellites enhances viral symptoms. The wheat plants of the 20 cultivars were inoculated with the clones for the infectivity of WDIV, with or without the satellites. All 20 wheat cultivars showed symptoms following agroinoculation. The symptoms (dwarfing) became visible at 14 or 18 dpi, respectively, in the plants inoculated with WDIV in the presence or absence of one or both of the satellites. The agroinoculated plants were screened for the presence of WDIV and the satellites. Mock-inoculated (pCAMBIA1301) plants did not contain WDIV or the satellites. Of the 400 plants inoculated with the constructs for infectivity (WDIV, with or without the satellites), 265 samples yielded a PCR amplicon of ~2.8 kb, indicating the presence of the WDIV genome. The amplicons of ~1.3 kb for the alphasatellite and the betasatellite were detected in the plants inoculated with WDIV and the satellites. Sequencing of the amplified products confirmed the presence of WDIV, the alphasatellite, and the betasatellite. The plants harboring WDIV showed dwarfing compared to the mock-inoculated (control) plants (Fig. 5), whereas the plants having WDIV and any of the two satellites (CLCuMA or AYLCB) showed more severe stunting (Table 2). Coinfection of WDIV with GLCuA had a similar effect with regard to enhancement of the symptoms of WDIV (data not shown). Plants containing WDIV and both the satellites (CLCuMA and AYLCB) showed extreme stunting (Fig. 5, Table 2). Unlike the infected field plants, the plants inoculated in the laboratory showed stunting but no leaf yellowing.

The disease severity was recorded on the basis of the height of the inoculated plants at 42 dpi. Table 2 shows the relative susceptibility of 20 wheat cultivars agroinoculated with WDIV, with or without the two satellites. Two cultivars, WL 711 and Kharchia 65, were the most susceptible among the 20 cultivars tested in this study (Table 2). The WL 711 plants inoculated with WDIV and any of the satellites died within 14 to 16 days of agroinoculation,

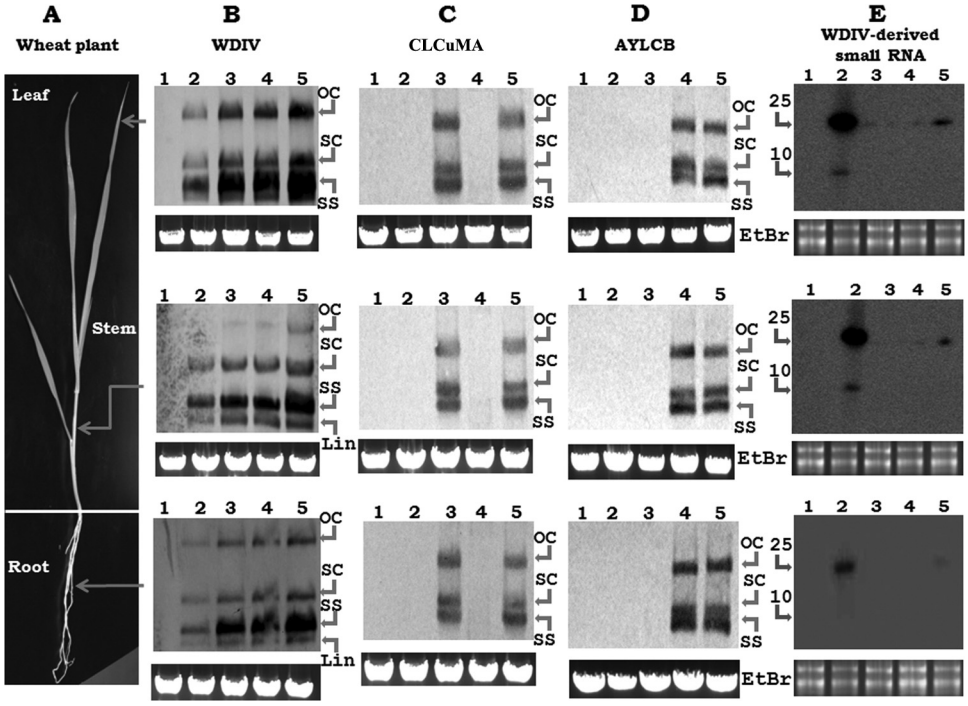

FIG 6 Accumulation of virus and virus-derived siRNA in different tissues of wheat plants infected with WDIV or WDIV with the satellites. (A) Agroinoculated wheat plants were divided into three parts: root, stem, and leaf. Southern hybridization (B, C, D) shows the presence of WDIV, the alphasatellite, and the betasatellite, respectively, in the leaves, stems, and roots of agroinoculated wheat plants. The CP gene of WDIV (JQ361910), the βC1 gene of the betasatellite (KC305084), and the Rep gene of the alphasatellite (KC305093) were used as probes. Different forms of viral/satellite DNA are labeled as follows: OC, open circular; SC, supercoiled; SS, single stranded; and Lin, linear. The ethidium bromide (EtBr)-stained gel at the bottom of panels B, C, and D shows comparable loading of samples. (E) Blots of RNA show virus-specific small RNAs produced in leaves, stems, and roots of agroinoculated wheat plants. Blots were probed with the [α-^{32}P]dATP-labeled WDIV full-length genome (JQ361910). Each lane was loaded with 20 μg low-molecular-weight RNA. The portion of the gel representing RNAs of 25 and 10 nt was blotted onto the membrane. (E) The RNA bands of 25 and 10 nt are visible in WDIV-inoculated samples. WDIV- and satellite-inoculated samples show bands corresponding to 25 nt only. The EtBr-stained gel shows comparable rRNA loading in all lanes. Total DNA and low-molecular-weight RNA were analyzed from mock (1)-, WDIV (2)-, WDIV and CLCuMA (3)-, WDIV and AYLCB (4)-, and WDIV together with CLCuMA and AYLCB (5)-inoculated wheat plant samples.

while the plants inoculated with the virus alone survived with moderate dwarfing (Table 2). In the case of Kharchia 65, the plants showed extreme dwarfing but survived following inoculation with WDIV along with the satellites. Three cultivars, IITR 26, RAJ 3765, and Sonalika, showed only mild dwarfing upon infection with WDIV alone and moderate dwarfing when infected with WDIV along with the satellites (Table 2).

Virus accumulation. WDIV accumulated in all three parts (root, shoot, and leaf) of the inoculated plants (Fig. 6A, Fig. 7A). All three methods, Southern hybridization (Fig. 6B), real-time PCR (Fig. 7B), and semiquantitative PCR (Fig. 7C), revealed that the accumulation of WDIV was highest in all parts of the plant inoculated with WDIV together with the alphasatellite and betasatellite. The next highest levels of WDIV accumulation were induced by inoculation with WDIV and the betasatellite, followed by inoculation with WDIV and the alphasatellite (Fig. 6B and 7B and C). The alphasatellite and betasatellite also accumulated in all parts of the plants (Fig. 6C and D).

Satellites suppress the formation of virus-specific small RNAs. RNA blots of small RNAs probed for virus sequences revealed that the accumulation of virus-specific small interfering RNAs (siRNAs) was highest in WDIV-infected wheat plants (Fig. 6E). The virus-specific siRNA levels were very low in plants inoculated with WDIV and the alphasatellite or the betasatellite or both the alphasatellite and the betasatellite. Low-molecular-weight RNA bands of 25 and 10 nt were present in WDIV-inoculated samples. In plants infected with WDIV along with the satellites, however, a weak 25-nt band was visible, while the 10-nt band was not visible (Fig. 6E).

Replication promiscuity of the satellites. The wheat plants inoculated with WDIV and AYLCB yielded the expected amplicon using betasatellite-specific β01/04 primers. No amplicon was observed in wheat plants inoculated with WDIV and CLCuMB. Southern hybridization using the βC1 gene as a probe and total DNA from WDIV- and CLCuMB-inoculated plants also revealed the absence of any band (data not shown). These results show that CLCuMB was not maintained by WDIV in wheat. The ability of AYLCB, CLCuMA, and GLCuA isolated from WDIV-infected wheat plants to interact with the begomoviruses with which these satellites are known to be associated was examined. This was performed by inoculating tobacco plants with the clones for the infectivity of AYLCB, CLCuMA, or GLCuA along with their respective begomoviruses (AEV, CLCuBuV, or CyTLCuV). AYLCB, CLCuMA, or GLCuA inoculated into wheat with WDIV (Fig. 8A, B, and C) or into tobacco with begomoviruses (AEV or CLCuBuV or CyTLCuV) (Fig. 8D, E, and F) were efficiently maintained by these viruses in their respective hosts (Fig. 8G). Southern hybridization further established the accumulations of WDIV in wheat

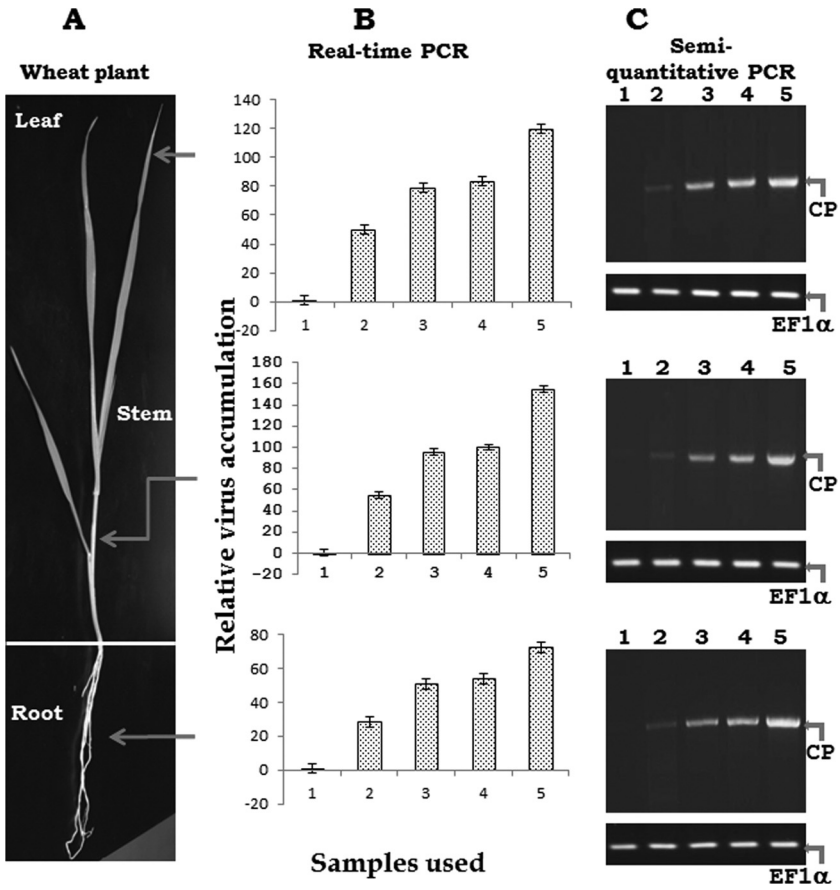

FIG 7 Accumulation of virus in different tissues of wheat plants infected with WDIV or WDIV with the satellites. (A) Agroinoculated wheat plants divided in three parts, root, stem, and leaf, for quantification of WDIV. Real-time PCR (B) and semiquantitative PCR (C), based on three replicates, showing analysis of virus collected from leaf, stem, and root of agroinoculated wheat plants. The CP gene of WDIV was amplified in the real-time PCR (B) and semiquantitative PCR (C). Elongation factor 1 alpha (EF1α) used as the internal control in semiquantitative PCR is shown below the CP amplicon (C).

and the begomoviruses (AEV, CLCuBuV, and CyTLCuV) in tobacco (Fig. 8H).

DISCUSSION

The associations of satellites (betasatellites or alphasatellites or both) with monopartite begomoviruses have been reported for numerous plant diseases (6–9) and, in a few cases, with bipartite begomoviruses (10–14). Betasatellites play an important role in the pathogenesis of begomoviruses, whereas less is known about the contribution of alphasatellites to the pathogenesis of begomovirus-betasatellite disease complexes (7–9, 47, 48). The study presented here reports the association of a betasatellite (AYLCB) and two species of alphasatellites (CLCuMA or GLCuA) with a mastrevirus (WDIV) in field infections of wheat. This study is also the first in which satellites have been reported in a monocotyledonous plant.

With the exception of WDIV, no other geminivirus was detected in any of the symptomatic wheat plants; however, not all symptomatic plant samples collected from the field tested positive for the presence of WDIV. In such cases, the symptoms may have been due to other viruses (RNA virus) or even other stress factors. The absence of begomoviruses and the detection of AYLCB with WDIV in field samples suggest that AYLCB can be transreplicated by WDIV. This prediction was confirmed by inoculating wheat plants with WDIV and AYLCB and then detecting the two in the systemically infected leaves; however, another betasatellite, CLCuMB, reported to interact with a number of noncognate begomoviruses (13, 14, 17, 22), was not maintained by WDIV in wheat. The association of AYLCB with WDIV resulted in enhancement of the disease symptoms and the accumulation of WDIV in wheat, which is consistent with the role of betasatellites (13, 14, 16, 20, 40). AYLCB was maintained as efficiently by AEV as it was by WDIV.

Transreplication of geminivirus genomic components normally requires geminiviruses to have their own Rep (27, 28, 44, 45), while the betasatellites may, in many cases, also be transreplicated by noncognate begomoviruses (8–10, 13, 14, 21–23, 30). In most cases, the betasatellites do not contain the iteron sequences of the viruses with which they are associated. Instead, they have been postulated to contain iteron-like sequences (22). The present study shows that a mastrevirus (WDIV) Rep can interact with a betasatellite (AYLCB) to initiate replication, which suggests that there are iteron-like sequences within AYLCB that functionally mimic the predicted iterons of WDIV (Fig. 4). The interaction clearly occurs, as the betasatellite is maintained in wheat by WDIV, but further work is required to define the iteron-like sequences that may be at the heart of the interaction.

Apart from AYLCB, two species of alphasatellites (CLCuMA

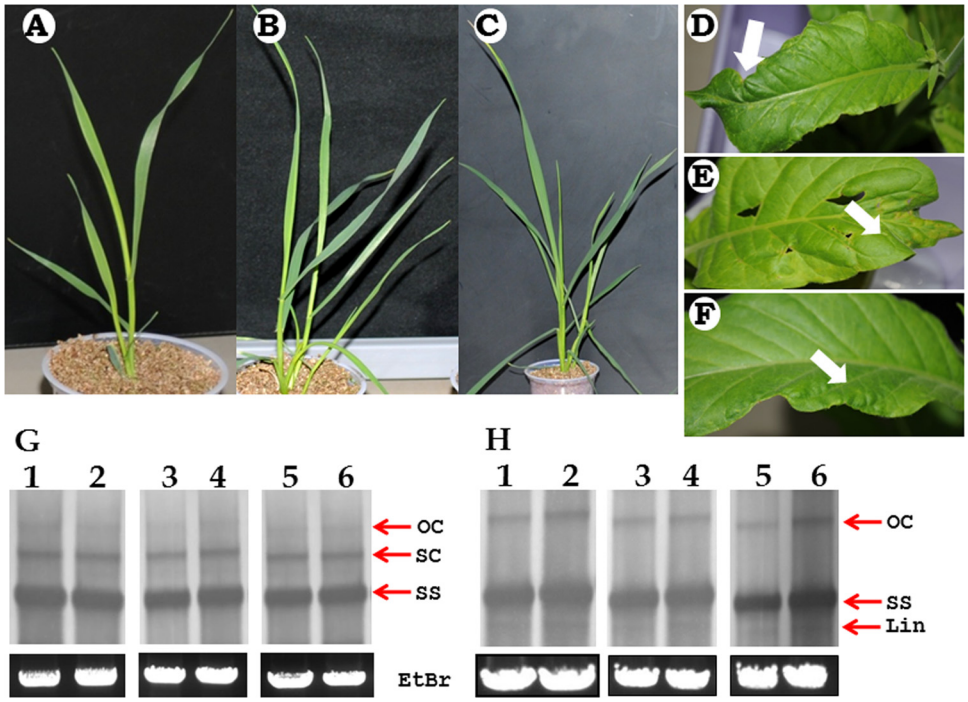

FIG 8 Replication promiscuity of the satellites. Wheat inoculated with WDIV and AYLCB (A), WDIV and CLCuMA (B), and WDIV and GLCuA (C). Tobacco inoculated with AEV and AYLCB (D), CLCuBuV and CLCuMA (E), and CyTLCV and GLCuA (F). Lanes 1, 2, 3, 4, 5, and 6 of panels G and H show Southern blot analyses of samples from inoculated plants shown in panels A, D, B, E, C, and F, respectively. (G) The βC1 and Rep genes were used as probes for detecting betasatellite and alphasatellite, respectively. (H) Coat protein genes of WDIV, AEV, CLCuBuV, and CyTLCuV were used as probes for detecting WDIV, AEV, CLCuBuV, and CyTLCuV, respectively. Different replicative forms of the viral/satellite DNA are labeled as follows: OC, open circular; SC, supercoiled; and SS, single stranded. Arrows show curling symptoms on tobacco leaves.

and GLCuA) were found to be associated with WDIV in natural infections of wheat. Unlike betasatellites, where the βC1 proteins have been reported as the determinants of pathogenicity and as silencing suppressors (15–20), the alphasatellites are believed to have a role in either attenuating viral symptoms (24, 25) or in overcoming host defense (26). In contrast, in the presence of CLCuMA or GLCuA, a higher level of accumulation of WDIV was documented in this study. Although alphasatellites are capable of autonomous replication in permissible plant cells, they depend on helper virus-encoded proteins for movement within and between plants (8). The two alphasatellites were maintained in wheat by WDIV as efficiently as by the begomoviruses (CLCuBuV and CyTLCuV) in tobacco and are thus typical of the earlier described satellites (38, 46).

RNA silencing is an antiviral defense mechanism. A hallmark of RNA silencing is the presence of siRNAs composed of 21 to 25 nucleotides that originate from the viral genome. The siRNAs generated by the cleavage of larger double-stranded RNAs play a key role in host defense against viruses (49, 50). Many plant viruses and associated satellites encode RNA silencing suppressor proteins that play important roles in counterdefense to the host defense machinery (19, 20, 51). In the present study, association with the alphasatellites (either CLCuMA or GLCuA) and the betasatellite (AYLCB) increased WDIV accumulation in infected wheat plants and reduced the production of virus-specific small RNAs. This observation substantiates the silencing suppressor activity of the associated satellites and provides a possible explanation for the selective advantage of the association of alpha- and betasatellites with WDIV in wheat. The involvement of the satellites in symptom severity of a mastrevirus can have implications with regard to the economic impact of the virus on crop yield; however, the prevalence of the WDIV disease complex in natural infection as well as the impact of a mastrevirus associated with satellites require further investigation under field conditions.

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AUTHOR CORRECTION

Correction for Kumar et al., Association of Satellites with a Mastrevirus in Natural Infection: Complexity of *Wheat Dwarf India Virus* Disease

Jitendra Kumar, Jitesh Kumar, Sudhir P. Singh, Rakesh Tuli

National Agri-Food Biotechnology Institute, Mohali, Punjab, India

Volume 88, no. 12, p. 7093–7104, 2014. Page 7100: Fig 6. and its legend should appear as shown below.

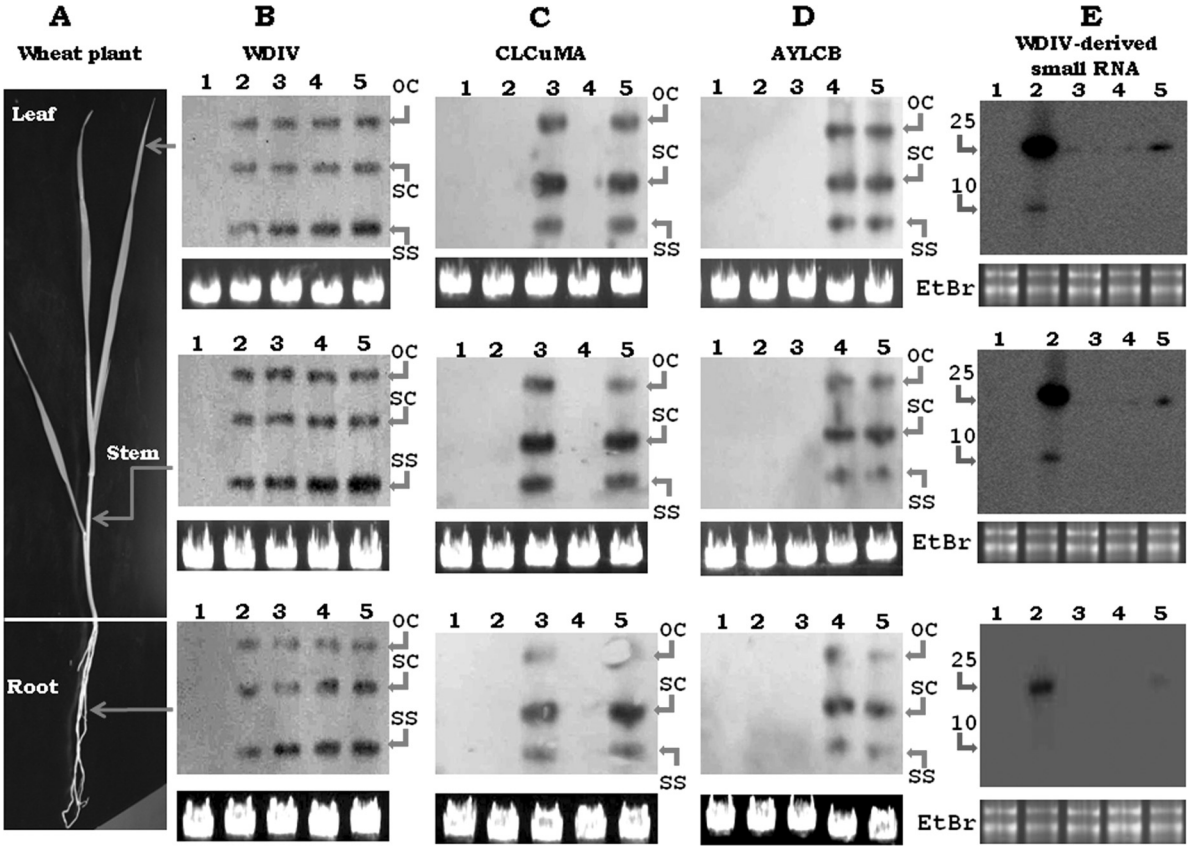

FIG 6 Accumulation of virus and virus-derived siRNA in different tissues of wheat plants infected with WDIV or WDIV with the satellites. (A) Agroinoculated wheat plants were divided into three parts: root, stem, and leaf. Southern hybridization (B, C, D) shows the presence of WDIV, the alphasatellite, and the betasatellite, respectively, in the leaves, stems, and roots of agroinoculated wheat plants. The CP gene of WDIV (JQ361910), the βC1 gene of the betasatellite (KC305084), and the Rep gene of the alphasatellite (KC305093) were used as probes. Different forms of viral/satellite DNA are labeled as follows: OC (open circular), SC (supercoiled), and SS (single stranded). The ethidium bromide (EtBr)-stained gel (low melting point agarose) at the bottom of panels B, C, and D shows comparable loading of samples. Blots of RNA (E) show virus-specific small RNAs produced in leaves, stems, and roots of agroinoculated wheat plants. Blots were probed with [α-^{32}P]dATP-labeled WDIV full-length genome (JQ361910). Each lane was loaded with 20 μg low-molecular-weight RNA. The portion of the gel representing RNAs of 25 and 10 nucleotides (nt) was blotted onto the membrane. The RNA bands of 25 and 10 nt are visible in WDIV-inoculated samples. (E) WDIV- and satellite-inoculated samples show bands corresponding to 25 nt only. The EtBr-stained gel shows comparable rRNA loading in all lanes. Total DNA and low-molecular-weight RNA were analyzed from mock (1)-, WDIV (2)-, WDIV and CLCuMA (3)-, WDIV and AYLCB (4)-, and WDIV together with CLCuMA and AYLCB (5)-inoculated wheat plant samples.

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