

**Artificial miRNA mediated resistance in tobacco against *Jatropha leaf curl Gujarat virus*
by targeting RNA silencing suppressors**

**Prashant More^{a,b}, Parinita Agarwal^{a*}, Abhishek Anand^c, Neeti Sanan-Mishra^c and
Pradeep K Agarwal^{ab*}**

^aPlant Omics Division,

CSIR-Central Salt and Marine Chemicals Research Institute (CSIR-CSMCRI),

Council of Scientific & Industrial Research (CSIR),

Gijubhai Badheka Marg,

Bhavnagar- 364 002, (Gujarat), INDIA

^bAcademy of Scientific and Innovative Research (AcSIR), Ghaziabad- 201002, India.

^cPlant RNAi Biology Group,

International Centre for Genetic Engineering and Biotechnology

Aruna Asaf Ali Marg

New Delhi-110 067, India

***Corresponding author**

E-mail: pagarwal@csmcri.res.in

E-mail: parinitaa@csmcri.res.in

Telephone +91-278-2564761,

Fax +91-278-2567562

Supplementary Tables

Table S1. Details of the MSAP primers used for methylation polymorphism analysis

MSAP Primer/ Adaptor	Sequence of the primer (5'-3')
Eco-Adaptor1	GACGATGAGTCTAGAA
Eco- Adaptor2	CGTTCTAGACTCATC
HM- Adaptor1	CTCGTAGACTGCGTACC
HM- Adaptor2	AATTGGTACGCAGTC
Preamp-F	GACTGCGTACCAATTCA
Preamp-R	GATGAGTCTAGAACGGT
MSAP-1	P1: GACTGCGTACCAATTCACC P2: GATGAGTCTAGAACGGTCT
MSAP-2	P1: GACTGCGTACCAATTCAGA P2: GATGAGTCTAGAACGGTCA
MSAP-3	P1: GACTGCGTACCAATTCAGC P2: GATGAGTCTAGAACGGTGA
MSAP-4	P1: GACTGCGTACCAATTCACC P2: GATGAGTCTAGAACGGTGA
MSAP-5	P1: GACTGCGTACCAATTCAGA P2: GATGAGTCTAGAACGGTCT
MSAP-6	P1: GACTGCGTACCAATTCACT P2: GATGAGTCTAGAACGGTAT
MSAP-7	P1: GACTGCGTACCAATTCACG P2: GATGAGTCTAGAACGGTAA
MSAP-8	P1: GACTGCGTACCAATTCAGT P2: GATGAGTCTAGAACGGTAG
MSAP-9	P1: GACTGCGTACCAATTCACT P2: GATGAGTCTAGAACGGTAG
MSAP-10	P1: GACTGCGTACCAATTCACG P2: GATGAGTCTAGAACGGTAT
MSAP-11	P1: GACTGCGTACCAATTC AAT

	P2: GATGAGTCTAGAACGGTTC
MSAP-12	P1: GACTGCGTACCAATTCAGT P2: GATGAGTCTAGAACGGTAA
MSAP-13	P1: GACTGCGTACCAATTCACC P2: GATGAGTCTAGAACGGTCA
MSAP-14	P1: GACTGCGTACCAATTCAGA P2: GATGAGTCTAGAACGGTGA
MSAP-15	P1: GACTGCGTACCAATTCAAG P2: GATGAGTCTAGAACGGTCT

Table S2. The concentration of all the detected metabolites in healthy (control) and virus infiltrated leaf tissue of WT and amiRNA transgenics

[illegible]

1	Alkene	1-Undecene	1.18	4.41	3.18	2.28	1.23	2.07	2.02	1.04
2		3-Eicosene	1.40	0.53	2.53	0.61	4.45	1.78	2.15	2.37
3		1-Nonene	1.18	0.79	0.94	1.49	1.93	1.91	1.49	1.63
1	Fatty acids	Stearic acid	11.57	14.74	9.60	10.60	0.00	0.00	0.00	0.00
2		Myristic acid	0.25	0.53	0.53	0.44	0.00	0.00	0.00	0.00

Table S3. List of primers used in the present study

Primer Name	Primer sequence (5'-3')	Purpose
AV 494 AC 1048	GCCYATRTAYAGRAAGCCMAG GGRTTDGARGCATGHGTACATG	Detection of DNA-A
beta01 beta02	GGTACCACTACGCTACGCAGCAGCC GGTACCTACCCTCCCAGGGGTACAC	Detection of DNA-β
PCRC1 PBL1v2040	CTAGCTGCAGCATATTTACRARWATGCCA GCCTCTGCAGCARTGRTCKATCTTCATACA	Detection of DNA-B
V1/V2-I miR-s V1/V2-II miR-a V1/V2-III miR-s V1/V2-IV miR-a	AGTCGAAGTTCAGACGGCGACGTCAGGAGATTCAGTTTGA TGACGTCGCCGTCTGAACTTCGACTGCTGCTGCTACAGCC CTACGTCGCCGACTGAACTTCGATTCTGCTGCTAGGCTG AATCGAAGTTCAGTCGGGGACGTAGAGAGGCCAAAAGTGAA	Amplification of V1/V2 amiRNA
C2/C3-I miR-s C2/C3-II miR-a C2/C3-III miR-s C2/C3-IV miR-a	AGTCTCCCAGGTATAGACGCCATCAGGAGATTCAGTTTGA TGATGGCGTCTATACCTGGGAGACTGCTGCTGCTACAGCC CTATGGCCTCTTTACCTGGGAGATTCTGCTGCTAGGCTG AATCTCCCAGGTAAAGAGGCCATAGAGAGGCCAAAAGTGAA	Amplification of C2/C3 amiRNA
C1/C4-I miR-s C1/C4-II miR-a C1/C4-III miR-s C1/C4-IV miR-a	AGGAGCTGGATTTAGCTCCCTGACAGGAGATTCAGTTTGA TGTCAGGGAGCTAAATCCAGCTCCTGCTGCTGCTACAGCC CTTCAGGCAGCAAAATCCAGCTCTTCCTGCTGCTAGGCTG AAGAGCTGGATTTTGCTGCCTGAAGAGAGGCCAAAAGTGAA	Amplification of C1/C4 amiRNA
G-4368 G-4369	CTGCAAGGCGATTAAGTTGGGTAAC GCGGATAACAATTTACACAGGAAACAG	Amplification of pre-amiRNA
G11491-Xho1 G11494-Kpn1	TCCTCGAGCAGCAGCAGCCACAGCAAA TCGGTACCGCTGCTGATGCTGATGCCAT	Amplification of amiRNA gene
hptII F hptII R	TTCTTTGCCCTCGGACGAGTG ACAGCGTCTCCGACCTGATG	Amplification of hygromycin gene
GusA F GusA R	GATCGCGAAAAGTGTGGAAT TGAGCGTCGCAGAACATTAC	Amplification of gus gene
NtActF NtActR	GATTTGCTGGTGATGATGCTCC GTCTCAAACATGATCTGTG TCATC	Amplification of tobacco actin gene
GFP-F GFP-R	GACCATGGCAAGTAAAGGAG TCCGAGCTCTTAGAGTTCGTCG	Amplification of gfp gene
V1V2StemloopRT C2C3StemloopRT C1C4StemloopRT	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACACGTCG GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACATGGCG GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACCTCAGGG	For cDNA synthesis of amiRNA
V1V2qRT-F C2C3qRT-F C1C4qRT-F Universal-qRT-R	GTTGTCTGAAGTTCAGACGG GTGGTCTCCCAGGTATAGA GTGGGAGCTGGATTTAGCT GTGCAGGGTCCGAGGT	For qRT PCR for relative estimation of amiRNA

Supplementary Figures

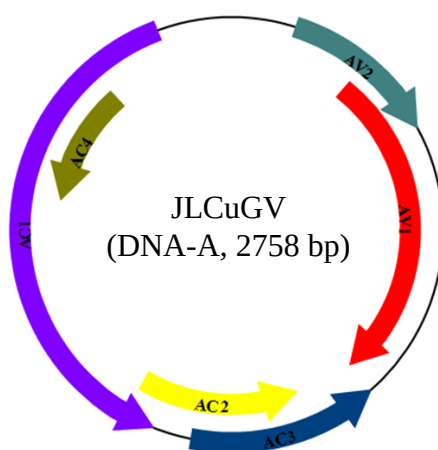


Figure S1. The genome organisation of JLCUGV DNA-A. The direction of arrow represents the orientation of ORF. This genome organisation map was constructed using Serial Cloner 2.6.1 software (freeware available at http://serialbasics.free.fr/Serial_Cloner.html).

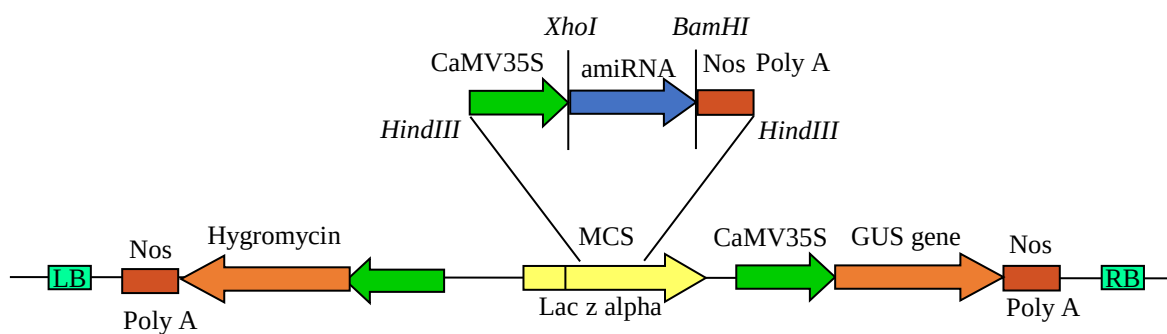
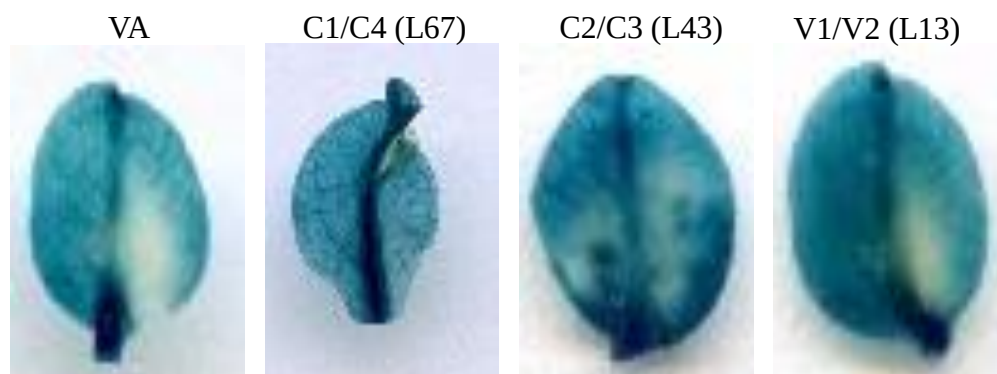
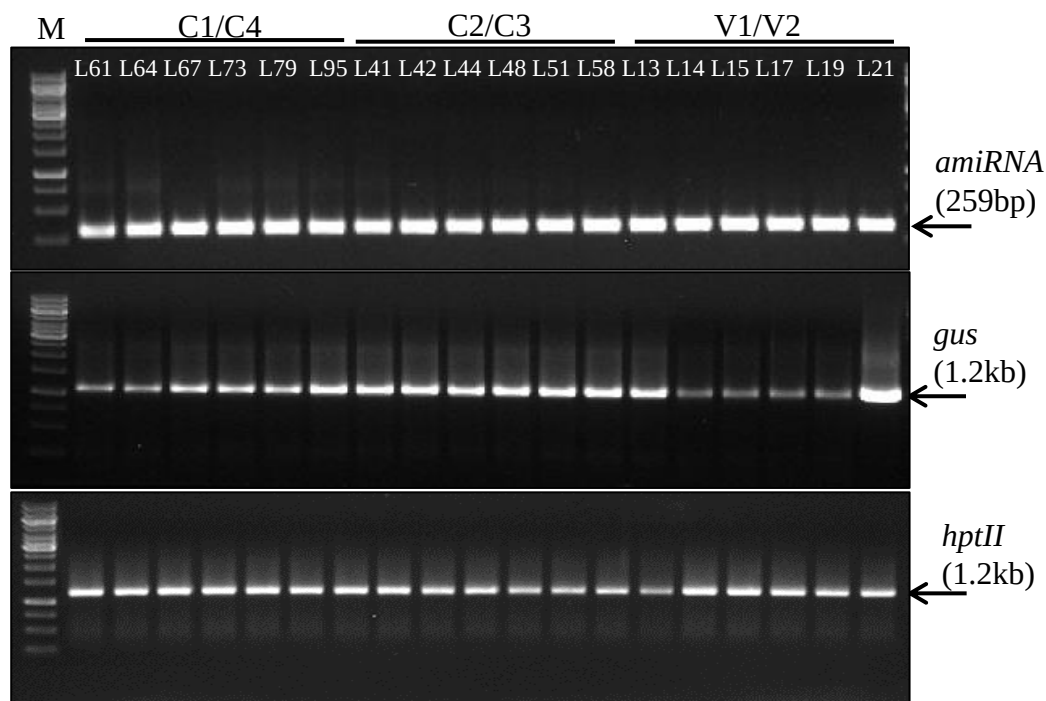


Figure S2. The schematic representation of amiRNA gene construct in pCambia 1301.

(a)



(b)



(c)

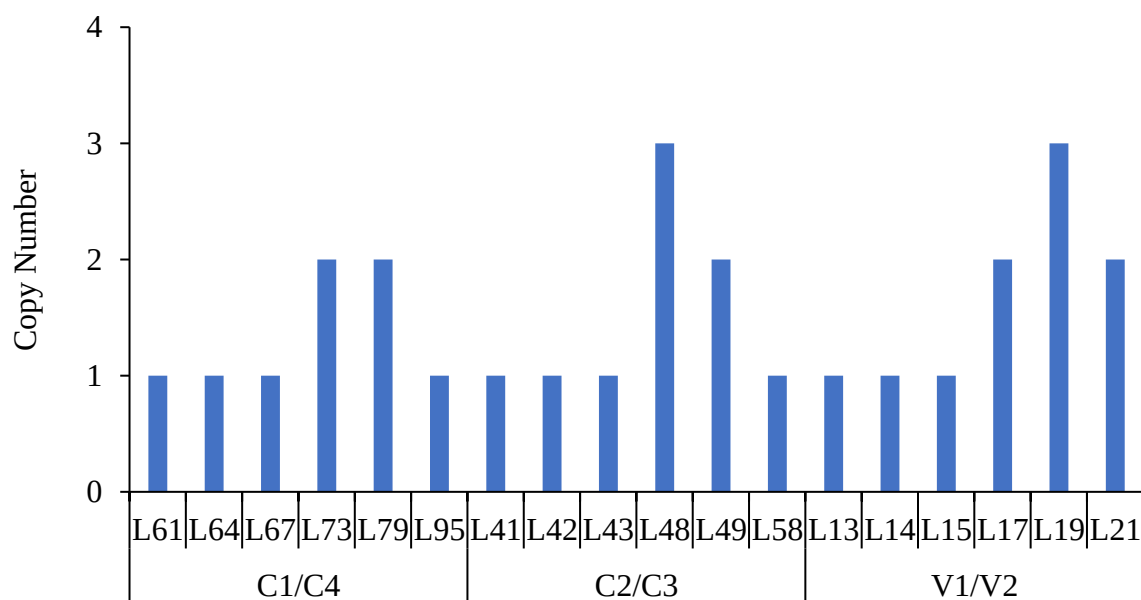


Figure S3. The confirmation of tobacco transgenics with amiRNA integration by GUS assay (a), amplification of integrated amiRNA gene, *gus* and *hptII* gene (b) and the copy number of transgenic lines of three transgenic events (c).

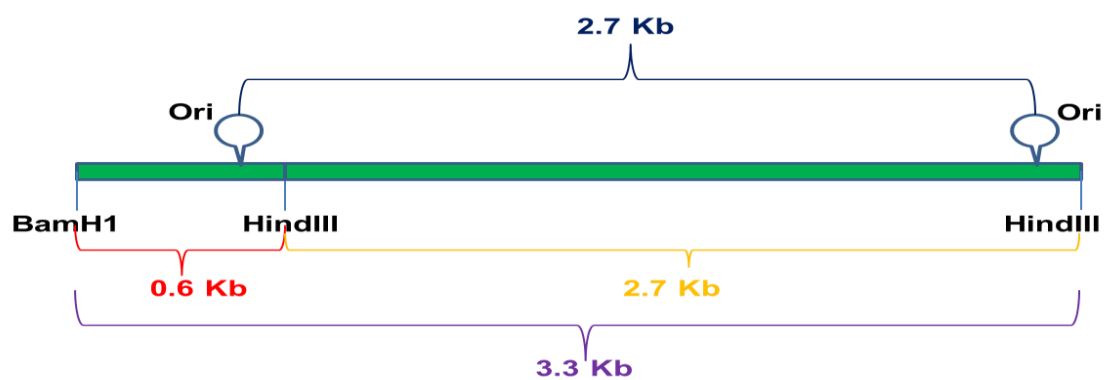
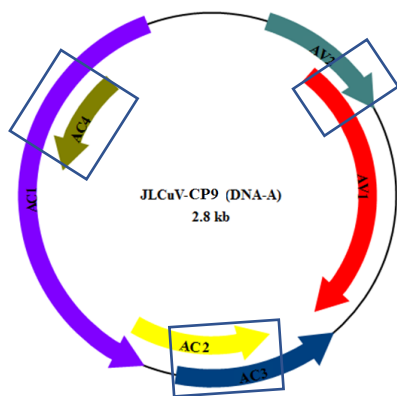


Figure S4. Schematic representation of infectious clone of DNA-A of JLCuGV. The hairpin structure represents the origin of replication (*ori*) of geminivirus.

(a)



(b)

Designed amiRNA of AV1/AV2 targeting multiple geminiviruses simultaneously

	Description	max score	total score	query cover	E value	Ident	Accession
Tomato	Tomato yellow leaf curl Thailand virus isolate YN2529 AV2 protein (AV2) gene, complete cds, and AV1 protein (AV1) gene, partial cds	42.1	42.1	100%	0.053	100%	KJ552115.1
	Tomato yellow leaf curl Thailand virus isolate YN2836 AV2 protein (AV2) gene, complete cds, and AV1 protein (AV1) gene, partial cds	42.1	42.1	100%	0.053	100%	KJ552115.1
	Tomato yellow leaf curl Thailand virus isolate YN2846 AV2 protein (AV2) gene, complete cds, and AV1 protein (AV1) gene, partial cds	42.1	42.1	100%	0.053	100%	KJ552115.1
	Tomato yellow leaf curl Thailand virus isolate YN2899 AV2 protein (AV2) gene, complete cds, and AV1 protein (AV1) gene, partial cds	42.1	42.1	100%	0.053	100%	KJ552115.1
	Tomato yellow leaf curl Thailand virus isolate YN2899 AV2 protein (AV2) gene, complete cds, and AV1 protein (AV1) gene, partial cds	42.1	42.1	100%	0.053	100%	KJ552115.1
	Tomato yellow leaf curl Thailand virus isolate YN2899 AV2 protein (AV2) gene, complete cds, and AV1 protein (AV1) gene, partial cds	42.1	42.1	100%	0.053	100%	KJ552115.1
Ageratum	Tomato yellow leaf curl China virus isolate YN2841 AV2 protein (AV2) gene, complete cds, and AV1 protein (AV1) gene, partial cds	42.1	42.1	100%	0.053	100%	KJ563132.1
	Ageratum yellow vein China virus isolate YN2895 AV2 protein (AV2) gene, complete cds, and AV1 protein (AV1) gene, partial cds	42.1	42.1	100%	0.053	100%	KJ563132.1
	Ageratum yellow vein virus (Malaysia) Tomato Leaf curl 2011 segment DNA-A, complete sequence	42.1	42.1	100%	0.053	100%	KM023147.2
	Ageratum yellow vein virus China segment DNA-A, complete sequence, isolate ACV/DNA_62-2	42.1	42.1	100%	0.053	100%	LC051140.3
	Ageratum yellow vein virus China segment DNA-A, complete sequence, isolate ACV/DNA_63-2	42.1	42.1	100%	0.053	100%	LC051138.1
	Ageratum yellow vein virus China segment DNA-A, complete sequence, isolate ACV/DNA_64-2	42.1	42.1	100%	0.053	100%	LC051137.1
Jatropha	Tomato yellow leaf curl Thailand virus isolate SPH3.1 segment DNA-A, complete sequence	42.1	42.1	100%	0.053	100%	KJ322144.3
	Tomato yellow leaf curl Thailand virus CP gene for coat protein, isolate P26	42.1	42.1	100%	0.053	100%	LC052933.1
	Tomato yellow leaf curl Thailand virus CP gene for coat protein, isolate SP3	42.1	42.1	100%	0.053	100%	LC052933.1
	Tomato yellow leaf curl Thailand virus CP gene for coat protein, isolate SP3	42.1	42.1	100%	0.053	100%	LC052933.1
	Jatropha leaf yellow mosaic Katamaghat virus isolate Katamaghat 2 segment DNA-A, complete sequence	42.1	42.1	100%	0.053	100%	KJ135229.3
	Jatropha leaf yellow mosaic Katamaghat virus segment DNA-A, complete sequence	42.1	42.1	100%	0.053	100%	KJ135229.3

(c)

Designed amiRNA of AC2/AC3 targeting multiple geminiviruses simultaneously

	Description	max score	total score	query cover	E value	Ident	Accession
Jatropha	Jatropha leaf curl virus isolate Gujarat, complete genome	42.1	42.1	100%	0.053	100%	KM411359.1
	Sri Lankan cassava mosaic virus isolate Malapouram segment DNA-A, complete sequence	42.1	42.1	100%	0.053	100%	KR611577.1
	Jatropha leaf crumple India virus IJ, curcas, Jodhpur isolate SKJ3, complete genome	42.1	42.1	100%	0.053	100%	KM189819.1
	Jatropha leaf crumple India virus IJ, curcas, Jodhpur isolate SKJ2, complete genome	42.1	42.1	100%	0.053	100%	KM189819.1
Pepper	Jatropha leaf crumple virus isolate SKJ1, complete genome	42.1	42.1	100%	0.053	100%	KM023146.1
	Pepper leaf curl Lahore virus isolate Lucknow segment DNA-A, complete sequence	42.1	42.1	100%	0.053	100%	KJ135234.2
	Jatropha mosaic India virus (Jatropha) isolate Gujarat2 coat protein (AV1) gene, partial cds, replication enhancer protein (AC3) and transcriptional activator protein (AC2) gene	42.1	42.1	100%	0.053	100%	JN07768.1
	Jatropha mosaic India virus (Jatropha) isolate Gujarat2 coat protein (AV1) gene, partial cds, replication enhancer protein (AC3) and transcriptional activator protein (AC2) gene	42.1	42.1	100%	0.053	100%	JN07768.1
Jatropha	Jatropha mosaic India virus isolate Aliqah coat protein (AV1) gene, partial cds, replication enhancer protein (AC3) and transcriptional activator protein (AC2) gene	42.1	42.1	100%	0.053	100%	HQ810408.1
	Jatropha mosaic India virus isolate Lucknow coat protein (AV1) gene, partial cds, replication enhancer protein (AC3) and transcriptional activator protein (AC2) gene	42.1	42.1	100%	0.053	100%	HQ810408.1
	Jatropha mosaic India virus isolate Lucknow strain SK-2 segment DNA-A, complete sequence	42.1	42.1	100%	0.053	100%	HM230683.1
	Jatropha mosaic India virus isolate Raiathan coat protein (AV1) gene, partial cds, BEN protein (AC3) and transcriptional activator protein (AC2) genes, complete c	42.1	42.1	100%	0.053	100%	GU080292.1
Cassava	Jatropha mosaic India virus isolate Lucknow coat protein (AV1) gene, partial cds, replication enhancer protein (AC3) and transcriptional activator protein (AC2) gene	42.1	42.1	100%	0.053	100%	GU574210.1
	Jatropha mosaic India virus isolate Lucknow strain 1 segment DNA-A, AV1 gene, partial cds, AC3 and AC2 genes, complete cds, and AC1 gene, partial cds	42.1	42.1	100%	0.053	100%	FJ346232.1
	Sri Lankan cassava mosaic virus recombinant defective DNA-A, clone DBA1 (SLM/C026a)	42.1	42.1	100%	0.053	100%	AM28432.1
	Sri Lankan cassava mosaic virus (Colombol) DNA-A, complete genome, isolate SLM/C01	42.1	42.1	100%	0.053	100%	AJ34737.1

(d)

Designed amiRNA of AC1/AC4 targeting multiple geminiviruses simultaneously

	Description	max score	total score	query cover	E value	Ident	Accession
Papaya	Ageratum leaf curl virus isolate C1, complete genome	42.1	42.1	100%	0.053	100%	KJ37491.1
	Senna leaf curl virus isolate Mohali, complete genome	42.1	42.1	100%	0.053	100%	KR52742.1
	Indian cassava mosaic virus strain Salem 2011 segment DNA-A, complete sequence	42.1	42.1	100%	0.053	100%	KJ550950.1
	Papaya leaf curl virus isolate Korea, complete genome	42.1	42.1	100%	0.053	100%	KJ266873.1
Pepper	Tomato yellow leaf curl Yunnan virus isolate YN4266, complete genome	42.1	42.1	100%	0.053	100%	KJ975400.1
	Pepper leaf curl Yunnan virus isolate YN4533, complete genome	42.1	42.1	100%	0.053	100%	KJ975395.1
	Sida yellow net virus isolate RJ028 segment DNA-A, complete sequence	42.1	42.1	100%	0.053	100%	KJ99357.1
	Sida yellow net virus isolate RJ018 segment DNA-A, complete sequence	42.1	42.1	100%	0.053	100%	KJ99356.1
Tomato	Sida yellow net virus isolate AM012 segment DNA-A, complete sequence	42.1	42.1	100%	0.053	100%	KJ99355.1
	Tomato yellow leaf curl virus isolate RG, complete genome	42.1	42.1	100%	0.053	100%	KJ248482.1
	Tomato yellow leaf curl Shuangbai virus - YN4536L complete genome	42.1	42.1	100%	0.053	100%	KJ962229.1
	Indian cassava mosaic virus isolate TM4 segment DNA-A, complete sequence	42.1	42.1	100%	0.053	100%	KJ208385.1
Cassava	Papaya leaf curl Guangdong virus DNA, complete genome, isolate RG-2	42.1	42.1	100%	0.053	100%	LC089756.1
	Ageratum yellow vein China virus isolate BS11, complete genome	42.1	42.1	100%	0.053	100%	KJ954390.1
	Ageratum yellow vein China virus isolate QZ1, complete genome	42.1	42.1	100%	0.053	100%	KJ954389.1
	Ageratum yellow vein China virus isolate NN3, complete genome	42.1	42.1	100%	0.053	100%	KJ954388.1

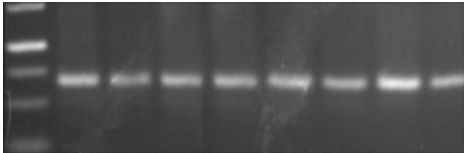
Figure S5. Designing and construction of artificial miRNA against the identified silencing suppressors by taking the overlapping regions used to generate three amiRNAs to target six genes (a), the three amiRNA designed as V1/V2 (b), C2/C3 (c) and C1/C4 (d), which can target the geminiviruses of other economically important crops like tomato, papaya, pepper, cassava. The genome organisation map (a) was constructed using Serial Cloner 2.6.1 software (freeware available at http://serialbasics.free.fr/Serial_Cloner.html).

Original Figures

GFP

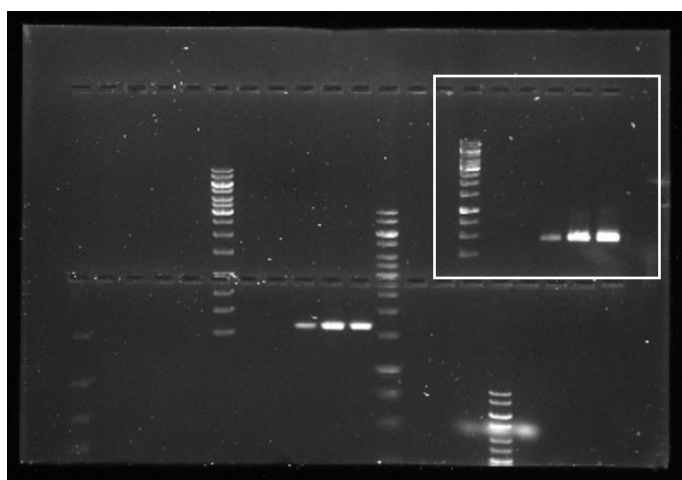


Actin

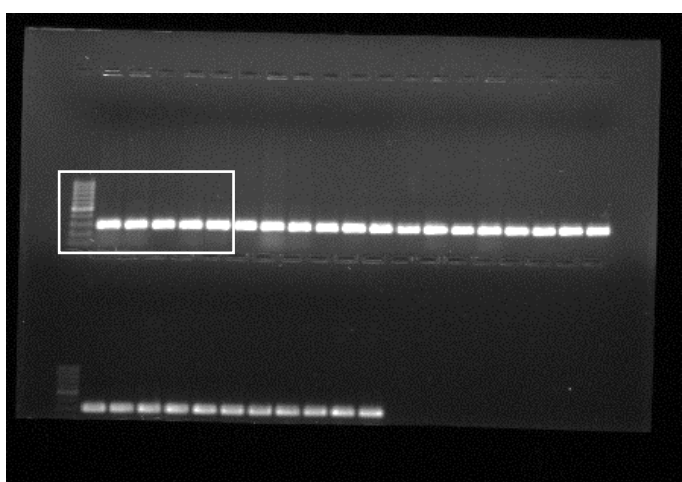


Original Fig. 1c: It is a part of Fig. 1c

PCR

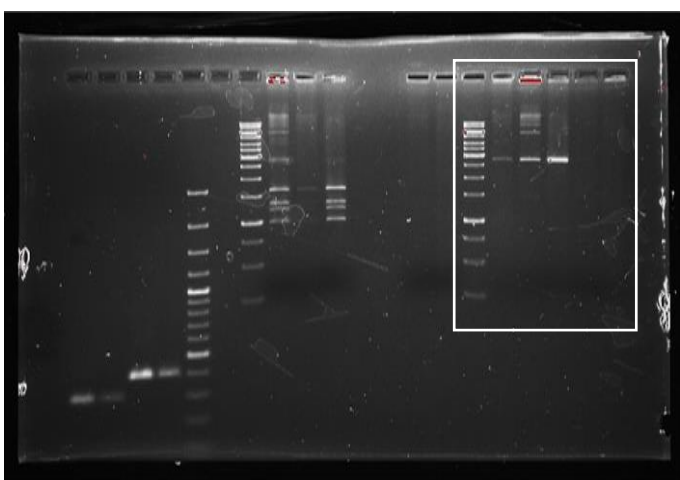


Actin

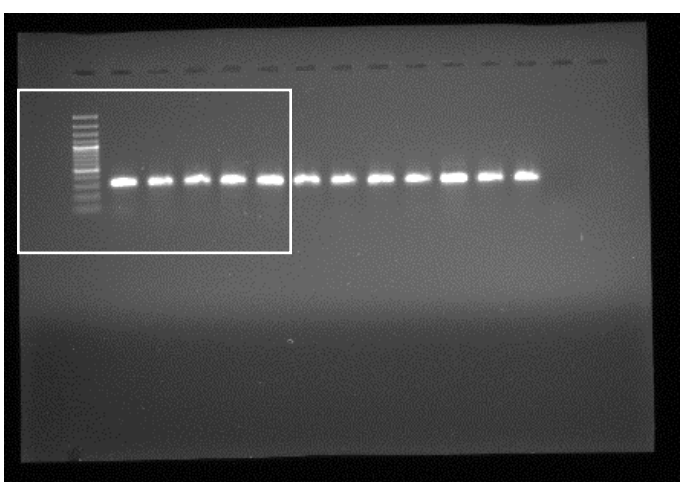


Original Fig. 2f: The boxed area is a part of Fig. 2f

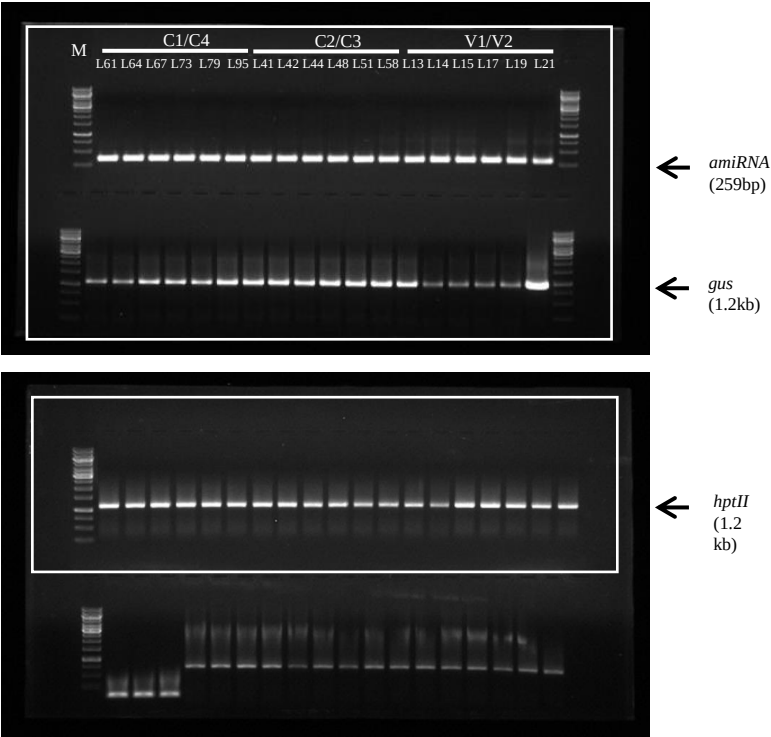
RCA



Actin



Original Fig. 2g: The boxed area is a part of Fig. 2g



Original Fig. S3b: The boxed area is a part of Fig. S3b