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# Artificially induced phased siRNAs promote virus resistance in transgenic plants

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ABSTRACT

We previously developed transgenic tobacco plants that were resistant to two geminiviruses. We generated resistance using RNAi constructs that produced trans-acting siRNA (tasiRNA) like secondary siRNAs known as phased siRNA (phasiRNA) that targeted several regions of Tomato Leaf Curl New Delhi Virus (ToLCNDV) and Tomato Leaf Curl Gujarat Virus (ToLCGV) transcripts encoding the RNA silencing suppressor proteins AC2 and AC4. Here, we performed degradome analysis to determine the precise cleavage sites of RNA-RNA interaction between phasiRNA and viral transcripts. We then applied our RNAi technology in tomato, which is the natural host for ToLCNDV and ToLCGV. The relative ease of developing and using phasiRNA constructs represents a significant technical advance in imparting virus resistance in crops and/or important model systems.

## 1. Introduction

RNA silencing is an endogenous, conserved eukaryotic mechanism whereby small RNAs (sRNAs) regulate gene expression. In plants, RNA silencing is a mechanism to degrade cellular and viral RNAs in a sequence specific manner at the post-transcriptional level (Hamilton et al., 1999; Agrawal et al., 2003). At the transcriptional level, sequence specific nascent RNAs attract methylation and heterochromatin components to the DNA resulting in inhibition of transcription (Calarco et al., 2012; Henderson et al., 2006). Both transcriptional and post-transcriptional gene silencing are considered as components of antiviral defence in plants. Double stranded RNA (dsRNA) triggers RNA silencing where dsRNA is processed into 21 to 24 nucleotide (nt) molecules called small interfering RNA (siRNA) and microRNA (miRNA). SiRNAs are derived from both endogenous genomic regions and exogenous infecting viral genomes (Pumplin and Voinnet, 2013). Key molecules involved in siRNA/miRNA include the type III RNA endonuclease Dicer like protein (DCL), RNA-dependent RNA polymerase (RdRP) and Argonaute (AGO, https://www.sciencedirect.com/science/article/pii/S0042682215003724 Bartel, 2004; Tomari and Zamore, 2005).

RdRP is further involved in siRNA amplification that ensures persistence plus systemic spread of RNA silencing even in the absence of the initial dsRNA trigger (Agarwal et al., 2003). Trans-acting siRNAs (tasiRNAs) are plant-specific siRNAs that are transcribed from specific loci known as *TAS* that undergo maturation after the precursor transcript is first targeted by a miRNA. Some of these miRNA-mediated cleavage products are converted into dsRNA by RNA-dependent RNA polymerase 6 (RDR6). This subset of dsRNA is then cleaved into 21 nt segments/phases known as phased siRNA (phasiRNA) by DCL4 (Howell et al., 2007). In principle, tasiRNA/phasiRNA machinery can be exploited to produce specific antiviral sRNAs (Carbonell et al., 2014; Singh et al., 2015).

Most plant viruses have evolved to produce proteins capable of suppressing the host RNA silencing (Csorba et al., 2015; Sanan-Mishra et al., 2017). Viral RNA silencing suppressors have been used as targets for the generation of transgenic plants with viral resistance (Niu et al., 2006; Van Vu et al., 2013). Geminiviruses are pathogenic viruses with small, single stranded DNA genomes. Most geminiviruses belong to the genus Begomovirus and encode AC2, AC4 and AV2 proteins that suppress host RNA silencing in order to support their infection (Van Vu et al., 2013). We previously reported the design of gene silencing binary vectors utilising features of tasiRNA/phasiRNA biogenesis to produce

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**Table 1**
General Information of small RNA libraries.

| Construct | Total reads | Accepted reads (after removing reads with N) | Percentage Accepted | Adapter removed | Percentage Accepted reads (after adapter removal) | Removal (HD adapter contains 6 random N at the end) | Non-redundant (unique reads) | Percentage reads mapping to *N. tabacum* genome (redundant) | Percentage reads mapping to *N. tabacum* genome (non-redundant) | Percentage reads mapping to ToLCNDV (redundant) | Percentage reads mapping to ToLCNDV (non-redundant) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| AC2_sRNA1 | 12278571 | 12273289 | 99.96 | 11645048 | 94.88 | 11184115 | 3303710 | 91.18 | 86.25 | 0.01 | 0.01 |
| AC2_sRNA2 | 12420478 | 12415190 | 99.96 | 11895090 | 95.81 | 11786556 | 4250353 | 91.32 | 87.33 | 0.01 | 0.01 |

phasiRNA that target viral *AC2* transcript (construct name TRiV-AC2) and viral *AC4* transcript (construct name TRiV-AC4). Both constructs conferred high resistance against the geminiviruses Tomato Leaf Curl New Delhi Virus (ToLCNDV) and Tomato Leaf Curl Gujarat Virus (ToLCGV) in our tobacco model system (Singh et al., 2015).

In the current study, we performed next generation sequencing and degradome analysis to investigate cleavage at nucleotide resolution between our TRiV-AC2 produced phasiRNAs and their target *AC2* transcripts. Degradome analysis exploits the stability of cleaved 3' fragments with parallel analysis of RNA ends (PARE) (Llave et al., 2012; German et al., 2008; Addo-Quaye et al., 2008; Gregory et al., 2008; Folkes et al., 2012). We then transferred the knowledge gained from our tobacco model and produced transgenic lines of tomato, a natural host for ToLCNDV and ToLCGV, using both the RNAi constructs TRiV-AC2 as well as TRiV-AC4.

## 2. Results

### 2.1. The TRiV-AC2 construct produces 21 nt phasiRNA

When constructing two sRNA libraries for sequencing using high definition (HD) adapters (Sorefan et al., 2012) for TRiV-AC2, we agroinfiltrated *Nicotiana tabacum* leaves using the agrobacterium strain EHA-105. We collected samples at 6 days post inoculation (dpi). We obtained 12 M (million) reads from both replicates after sequencing of the samples, of which 11 M reads were analysed (Table 1). Size class distributions indicated one major peak at 24 nt that is consistent with several other studies (Mitter et al., 2013; Kravchik et al., 2013, Supplementary Fig. 1). Only 0.1% of total reads mapped to the *AC2* gene. Only sRNA reads with perfect matching to *AC2* transcript were used for analysis. SRNAs with perfect matching to *AC2* were predominantly 21 nt (Fig. 1a). These reads were identified from both strands of the genome, which can be consolidated into one set of values by summing sense and antisense reads that were offset by 2 nt (Fig. 1b). The first expected phasiRNA started at the cleavage site of miR390. This phasiRNA was followed by consecutive phasiRNAs in 21 nt divisions. No miR390-guided cleavage was detected in our negative control construct TRiVM2-AC2, which is a modified TRiV-AC2 construct containing a 4 base pair (bp) mutation in the miR390 binding site.

### 2.2. PhasiRNAs from TRiV-AC2 cleave AC2 in consecutive 21 nt segments

We performed degradome analysis using high definition (HD) adapters (Xu et al., 2015) in tobacco separately expressing TRiV-AC2 and TRiVM2-AC2 (negative control) when the plants were infected with ToLCNDV. We obtained > 35M reads from all libraries (Table 2). Size class distributions indicated two major peaks at 20–21 nt (Supplementary Fig. 2). Systematic identification of phasiRNA targets was accomplished using previously described methods by analysing the 20 and 21 nt reads with the CleaveLand pipeline for target identification (Addo-Quaye et al., 2008) followed by normalisation to total sample size as described in Mortazavi et al. (2008). To remove noise, reads with normalised abundance < 5 were deleted. The identified phasiRNA targets using sRNA sequencing plus degradome analysis are presented as target plots (t-plots) that display the abundance of the signatures relative to their position in the transcript (Fig. 2). We observed high read numbers (Fig. 2a and b) showing cleavage within the 1417–1719 nt region of *AC2* transcript. In the controls, we observed low read numbers of cleavage products (Fig. 2c and d), which may derive from random degradation of transcripts without cleavage by phasiRNA. The 1417–1719 nt region is included in TRiV-AC2 (Fig. 2e).

### 2.3. The natural host of ToLCNDV, tomato, also produces artificially induced phasiRNA

Because tomato is the natural host for ToLCNDV, we wanted to

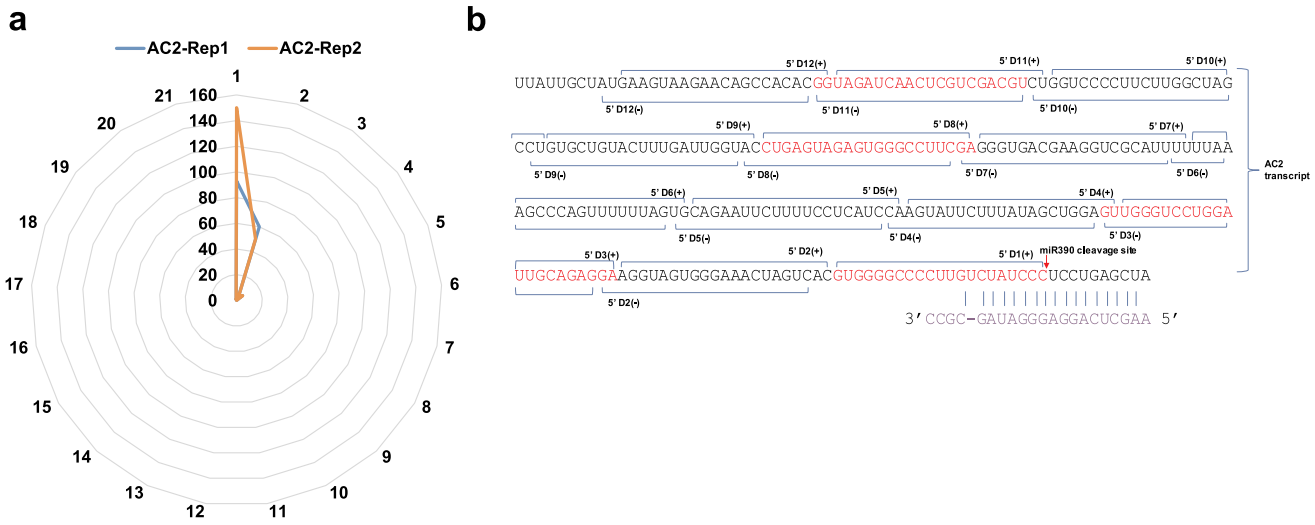


**Fig. 1.** Processing of tasiRNAs directed by miR390 on *AC2* transcript. Panel a depicts radar plot showing the abundance of 21 nt reads (shown in the radial axis) corresponding to the *AC2* transcript sequenced in TRiV-AC2. Position 1 is designated as immediately after the miR390-guided cleavage site. Panel b depicts TRiV-AC2, showing phasiRNA production. TasiRNA are shown in red. MiRNA390 cleavage site in *TAS3* transcript is indicated by the red arrow. The 21 nt phase relative to the miR390 cleavage site is indicated by brackets. AC-Rep1 and AC2-Rep2 are the two replicates used.

tested our RNAi constructs for biotechnological relevance. As in tobacco, we generated transgenic tomato (*Solanum lycopersicum,* cultivar "Pusa Ruby") using the construct TRiV-AC2 described earlier, and TRiV-AC4 derived from viral AC4 transcript (Singh et al., 2015) constructs. Twenty-five lines of $T_0$ plants were analysed for each construct by screening for the presence of the transgene using PCR. Fifteen plants amplifying the expected band were checked for the expression of phasiRNA by northern blot, taken from $T_0$ leaf samples. Nine lines with TRiV-AC2 (AC2-2, AC2-3, AC2-5, AC2-9, AC2-11, AC2-13, AC2-17, AC2-19 and AC2-25) and eight lines with TRiV-AC4 (AC4-4, AC4-6, AC4-9, AC4-10, AC4-14, AC4-15, AC4-17 and AC4-20) accumulated phasiRNAs corresponding to the transgenes with varying levels of accumulation (Fig. 3a and b). Accumulation of siRNAs was highest in line # AC2-13 and line # AC4-20. These plants did not show any abnormality compared to non-transgenic controls (Fig. 3c). We tested tomato plants expressing phasiRNA to determine the copy number of transgenes. Southern analysis indicated the presence of the transgene in a single copy in plant lines AC2-3, AC2-11, AC4-9, AC4-10, AC4-17 and AC4-20 (Fig. 4a and b). All other lines carried two or more copies of the transgene. Lines AC2-3, AC2-11, AC4-9 and AC4-20 carrying a single copy of the transgene were advanced to $T_1$. We tested the expression of phasiRNA by northern blot in $T_1$. Here, 57% of $T_1$ plants showed expression of phasiRNA.

### *2.4. Transgenic tomato plants are resistant to both ToLCNDV and ToLCGV*

As performed in tobacco, we tested the performance of $T_1$ tomato lines expressing TRiV-AC2 and TRiV-AC4 derived phasiRNA against ToLCNDV and the closely related strain ToLCGV. In tomato plants containing TRiV-AC2 and TRiV-AC4, an average of 68% of plants were resistant to both viruses (Table 3). All inoculated plants harbouring negative control constructs showed disease symptoms including upward curling of the leaflet margins, reduction of leaflet area, swelling of veins and stunting at 21 dpi (Fig. 5).

## 3. Discussion

We previously reported resistance against ToLCNDV and ToLCGV in transgenic tobacco expressing phasiRNA derived from TRiV-AC2 and TRiV-AC4 constructs (Singh et al., 2015). In the current study, we used next generation sequencing technologies to report that the RNAi constructs produce phasiRNAs that specifically cleave the 1417–1719 nt region of the ToLCNDV transcript *AC2* in 21 nt segments.

Next generation sequencing revealed a phased production of secondary siRNAs, or phased siRNA, derived from an *AC2* sequence starting at the 10$^{th}$ nt from the miR309 binding site. Most of the phasiRNAs derived from the construct followed a 21 nt "register" that has previously been reported for other tasiRNA families (Allen et al., 2005; Montgomery et al., 2008; Carbonell et al., 2014). The definition of "phase" in tasiRNA/phasiRNA biogenesis is strictly reliant on AGO1 (Arribas-Hernández et al., 2016).

SRNA sequencing plus degradome analysis has been used in several plant species for the identification of miRNA targets (Shao et al., 2013). Target plots are used to show that each peak corresponds to one miRNA cleavage site. A single peak is obtained for each miRNA target (German et al., 2008). Here, we used degradome analysis to determine cleaved fragments of the ToLCNDV transcript *AC2*. Since 21 nt secondary siRNAs are produced in phases, several cleavage peaks were observed. Mapping of cleaved RNA fragments to the *AC2* region of the ToLCNDV genome shows that the secondary siRNAs or phasiRNAs targeted the viral transcript in a sequence-specific manner. To the best of our knowledge, this is the first report where the targeting of a plant virus via manipulation of RNA silencing has been verified by degradome analysis.

Finally, we applied our RNAi technology in tomato, which is the natural host of ToLCNDV as well as to a closely related strain ToLCGV. The transgenic plants showed no overall phenotypic difference from the untransformed plants, indicating no possible adverse effects of the PhasiRNAs on the normal growth and development of the plants. The two viruses share about 96% nucleotide identities within the sequences used in the transgenes and thus, the phasiRNAs generated by the constructs presumably could target transcripts generated by the two viruses with equal efficiency. Hence, it is not surprising that the four transgenic plants tested displayed similar resistance against both the viruses.

ToLCNDV has recently been detected to be infecting cucurbitaceous and solanaceous crops in countries of the Medditerranean basin (Moriones et al., 2017; Juarez et al., 2019). This indicates a possible spread of the virus from its previously reported host range of solanaceous crops, being limited mainly to South Asia. Hence, the technology of anti-viral artificial phasiRNA, demonstrated in this report, holds considerable promise to design novel strategies against this emerging virus.

**Table 2**
General information of degradome libraries.

| Samples | Total reads | Accepted (after removing reads with Ns) | Percentage Accepted | Adapter removed | Percentage Accepted | N_removal (HD adapter contains 6 random N at the end) | Non redundant (unique reads) | *N. tabacum* mapping reads (redundant) | *N. tabacum* mapping reads (non-redundant) | ToLCNDV mapping reads (redundant) | ToLCNDV mapping reads (non-redundant) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| AC2_rep1 | 45246503 | 45231592 | 99.97 | 44157594 | 97.63 | 43684222 | 11356597 | 43444819 | 8375905 | 43066 | 1134 |
| AC2_rep2 | 37811376 | 37799043 | 99.97 | 36916821 | 97.67 | 36634076 | 20593780 | 36448502 | 14352692 | 40593 | 1368 |
| Cntrl_rep1 | 41249301 | 41235746 | 99.97 | 40379049 | 97.92 | 39721824 | 13455891 | 39433442 | 8394953 | 121 | 75 |
| Cntrl_rep2 | 90864847 | 90835133 | 99.97 | 89204382 | 98.20 | 88226099 | 14794937 | 87752844 | 9350675 | 200 | 80 |

## 4. Conclusion

We demonstrate sequence specific cleavage of a viral transcript by artificially-induced phasiRNA. This methodology can be applied to other viruses/plants/models and phasiRNAs can potentially emerge as a powerful tool for crop improvement and beyond. The relative ease of developing and using phasiRNA constructs represents a significant technical advancement in imparting desired traits of virus resistance for crop plants in future.

## 5. Materials and methods

### 5.1. Agroinfiltration

Agrobacterium cells containing a TRiV-AC2 plus TRiVM2-AC2 (control) plasmid were grown to an optical density at 600 nm ($OD_{600}$) of 1.0. We centrifuged cells and resuspended in an equal volume of 2-*N*-morpholino ethanesulfonic acid (MES) buffer containing 200 µM of acetosyringone. We incubated the mixture at 28 °C for 1 h before infiltration. Around 4 mL of the mixture was infiltrated on the abaxial surface of each leaf. *Agrobacterium*-mediated transient expression in leaves was achieved through pressure infiltration at the six-leaf stage, as described (Karjee et al., 2008).

### 5.2. Degradome library preparation

*N. tabacum* TRiV-AC2 transgenic plants were inoculated with ToLCNDV by agroinfiltration. The second top leaf tissue (~50 mg) was collected at 21 dpi to prepare degradome libraries in replicates (AC2_rep1 and AC2_rep2). For controls, leaves were collected from non-transgenic plants infected with ToLCNDV. Libraries were generated in replicates (control 1 and control 2). Degradome libraries were generated according to the protocol described (Zhai et al., 2014) with modifications to the 5′PARE RNA adapter (Table 4).

### 5.3. Bioinformatics

We used *N. tabacum BX* genome available on Sol Genomics website (https://solgenomics.net/organism/Nicotiana_tabacum/genome). Fastq files were converted to fasta files and sequence reads with no Ns were kept for further analysis. The first 8 nt of the 3′ adapters were identified and removed followed by four nucleotides on the 5′ and 3′ ends of the reads (that corresponded to the NNNN tags on the HD adapters). Reads were mapped to the tobacco genome with 0 mismatches, in non-redundant format, using PatMaN (Prufer et al., 2008). Abundance of sequenced reads was normalised using the reads per million approach. Degradome analysis was performed using the CleaveLand pipeline.

### 5.4. Northern blot

Total RNA was isolated using Trizol (Sigma, USA) from PCR positive transgenic plants and 300 ng of RNA was resolved on a 12% denaturing urea gel. RNA was transferred to a nylon membrane (Hybond N, Amersham, UK) by electroblotting at 0.8 A for 40 m (Bio-Rad, USA) and cross-linked by UV. Hybridisation was carried out at 37 °C as previously described (Pall et al., 2008). AC1/AC2 and AC1/AC4 sequences were labelled using 3000 Ci/mmol of [α-$^{32}$P] dCTP (Perkin Elmer Life Sciences, USA) and were used as probes for TRiV-AC2. We purified probes in a G25 column (GE Healthcare Life Sciences, UK) according to the manufacturer's protocol. For markers, 22 nt oligonucleotides complementary to TRiV-AC2 were synthesized. Membranes were autoradiographed using the TYPHOON phosphor imager (GE Healthcare Life Sciences). Band intensities for siRNAs were quantified using ImageJ software (http://rsbweb.nih.gov/ij/index.html).

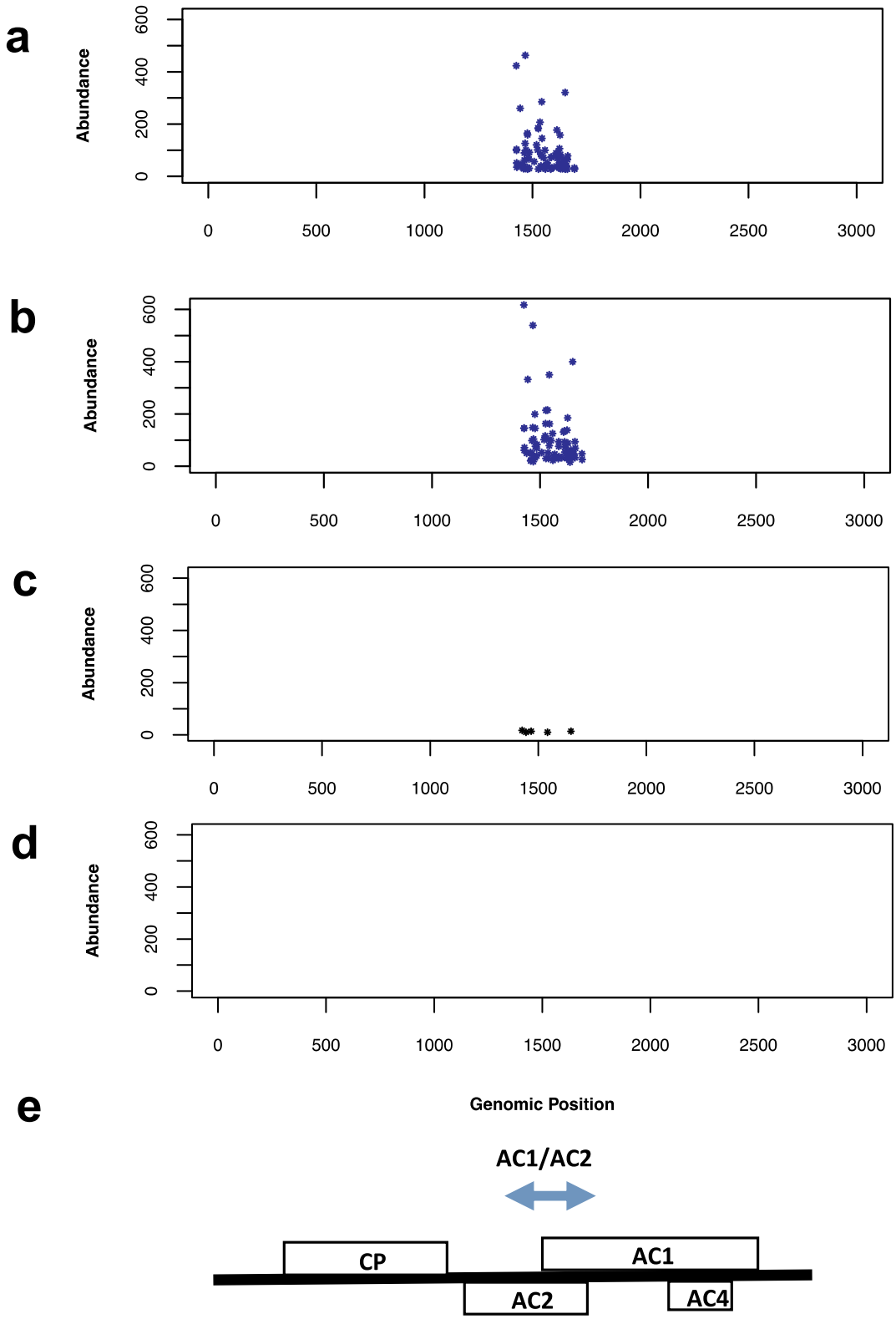


**Fig. 2.** Target plots (t-plots) of phasiRNA produced from the TRiV-AC2 construct. T-plot profiling from tobacco plants expressing TRiV-AC2 (panels a & b) and from tobacco plants expressing TRiVM2-AC2 (control, panels c & d), following infection with ToLCNDV. Panel e shows a schematic representation of position of AC1, AC2, AC4 and CP genes on DNA-A of ToLCNDV. Arrows represent the fragments used for cloning in TRiV-AC2 vector. The y-axis measures the normalised reads, representing reads per million, for the degradome signals. The x-axis represents the position of the cleavage signals on the target transcripts. Black and blue in TRiV-AC2 and TRiVM2-AC2, respectively, mark the cleavage signals.

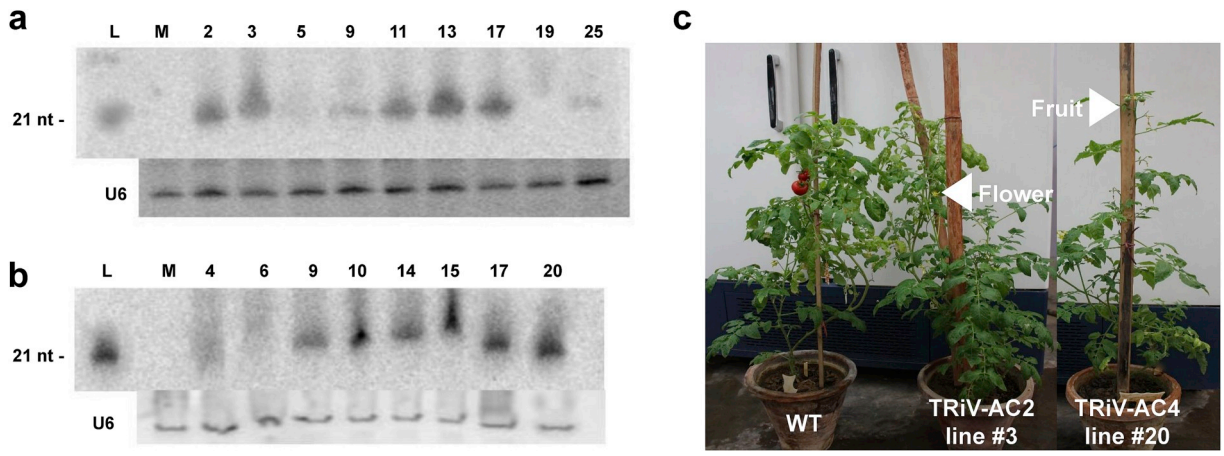


**Fig. 3.** Molecular and phenotypic analysis of transgenic tomato plants. Northern blot showing phasiRNA in $T_0$ transgenic tomato plants containing TRiV-AC2 (Panel a) and in $T_0$ transgenic tomato plants with TRiV-AC4 (panel b). L indicates size marker for 21 nt. M indicates untransformed plants as controls. Lane numbers represent plant identifier number. U6 is loading control. Panel c, $T_1$ transgenic tomato plants TRiV-AC2 line #3 and TRiV-AC4 line #20 compared to wild type (WT). The plants are six weeks old.

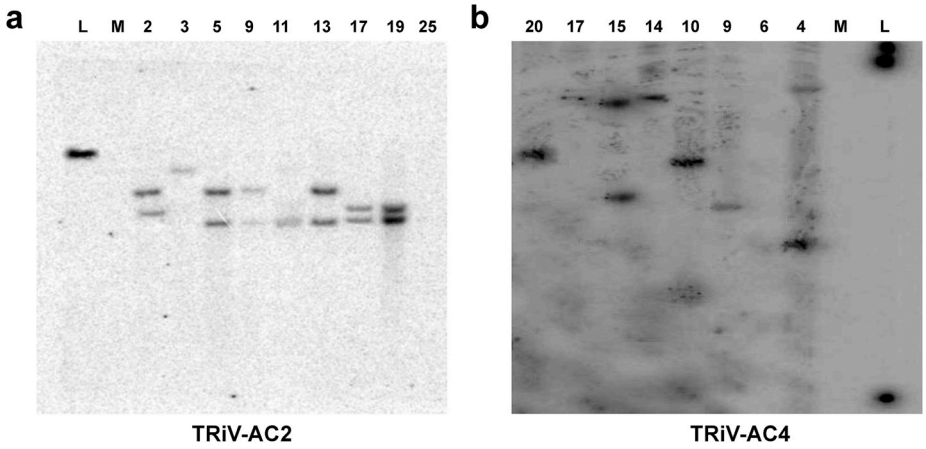


**Fig. 4.** Southern blot depicting the copy number of the transgene in $T_0$ transgenic tomato plants. Panel a, TRiV-AC2 and panel b, TRiV-AC4. Lane numbers indicate plant line identifier. M represents mock transformed plants. TRiV-AC2 and TRiV-AC4 plasmids were loaded as positive controls in lane L. DNA was digested with *Eco*RI, which is a non-cutter for sequences between T-left and T-right border.

**Table 3**
Virus resistance assay performed for $T_1$ transgenic tomato plants challenged separately with ToLCNDV and ToLCGV.

| Constructs | Progeny of $T_0$ transgenic lines | Number of non-symptomatic plants/ number of plants challenged | |
|---|---|---|---|
| | | ToLCNDV | ToLCGV |
| TRiV-AC2 | Line 3 | 15/21 | 8/15 |
| | Line 11 | 14/20 | 7/12 |
| TRiV-AC4 | Line 9 | 16/23 | 9/18 |
| | Line 20 | 12/20 | 07/14 |
| Untransformed control | | 0/10 | 0/10 |
| TRiVM2-AC2 | Line 5 | 0/10 | 0/10 |
| TRiVM2-AC4 | Line 7 | 0/10 | 0/10 |

### 5.5. Southern blot

The nucleotide sequence between left and right border on T-DNA in the TRiV-AC2 vector was checked for the non-cutter. *Eco*R1 was found to be one of the non-cutters and was used to digest genomic DNA. Genomic DNA from $T_0$ transgenic plants was digested with *Eco*R1 and was resolved on a 1.2% agarose gel. TRiV-AC2 plasmid was loaded as a positive control. DNA was blotted onto Hybond N+ (GE Healthcare Life Sciences, UK) by overnight capillary transfer and hybridised with the *AC1/AC2* and *AC1/AC4* sequence amplified by PCR. Probes were end labelled using 6000 Ci/mmol [$\gamma$-$^{32}$P] ATP (PerkinElmer Life Sciences,

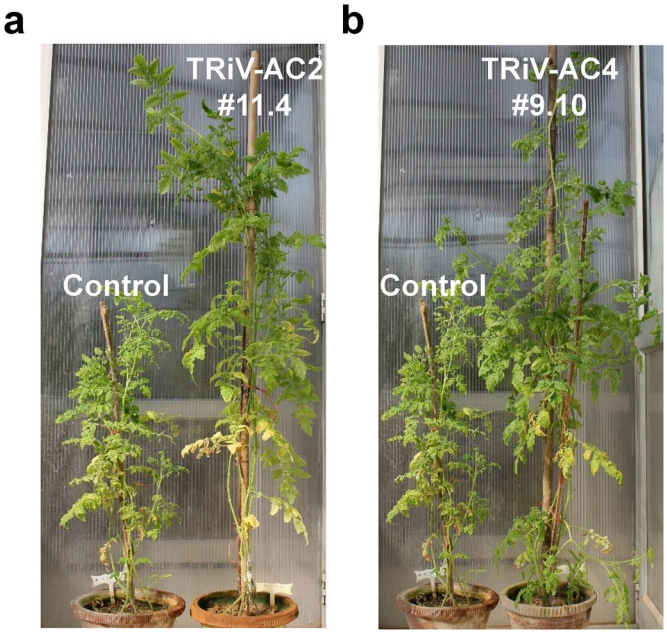


**Fig. 5.** Transgenic and non-transgenic tomato plants at 30 dpi, following challenge with Tomato leaf curl New Delhi virus. Panel a, Transgenic line TRiV-AC2 #11.4 (containing TRiV-AC2 as transgene) to the right and non-transgenic control plant to the left and panel b, transgenic line TRiV-AC4 #9.10 (containing TRiV-AC4 as transgene) to the right and non-transgenic control plant to the left. The plants are eight weeks old.

**Table 4**
Primers used in this study.

| Construct name | Primer name | Sequence (5'- > 3′) |
|---|---|---|
| For screening transgenic plants. | 35S-(TB) | TGACGCACAATCCCACTATC |
| TRiV-AC2-size markers for northern | AC2SM20 | TTCCCACTACCTTCCTCTGC |
| | AC2SM22 | TCTTCCCACTACCTTCCTCTGC |
| TRiV-AC4 size marker for northern | AC4SM20 | AGCTGATCGTCCATCGACTT |
| | AC4SM22 | CCAGCTGATCGTCCATCGACTT |
| ToLCNDV-AC2 | AC2REPF | GAGCTCTTCCCACTACCTTCCTCTGC |
| | AC2REPR | ACTAGTTAAACACGCCATTCTCTGCTT |
| ToLCNDV-AC4 | AC4REPF | GGGGTACCAGCTGATCGTCCATCGACTT |
| | AC4REPR | GGAATTCCTCCTCACTTGCATGTGCTC |
| Primers for RCA | β-actin(TB)F | ACAATGAGCTCCGAGTTGCT |
| | β-actin(TB)R | TTGATCTTCATGCTGCTTGG |
| | β-actin(TM)F | AGGCTGTGCTTTCTCTGTATG |
| | β-actin(TM)R | GCTTTTCCTTCATGTCACGTAC |
| sRNA Library Preparation | 5′ HD adapter | GUUCAGAGUUCUACAGUCCGACGAUCNNNN |
| | 3′ HD adapter | NNNNTGGAATTCTCGGGTGCCAAGG |
| Degradome library | 5′-PARE RNA adapter | CGACAGGTTCAGAGTTCTACAGNNNNNNTCCGAC |

USA) by T4 Polynucleotide Kinase (T4 PNK – Fermentas, Lithuania) and purified by G25 column (GE Healthcare Life Sciences, UK) according to supplier's protocol. Hybridisation was carried out at 37 °C using standard protocol. The membranes were autoradiographed using phosphor imager- TYPHOON (GE Healthcare Life Sciences, UK).

### 5.6. Small RNA library preparation

Tobacco plants were infiltrated with TRiV-AC2 according to the method described previously (Singh et al., 2015). Total RNA was isolated from infiltrated leaves using TRI Reagent Solution (Ambion) at 6 dpi following the manufacturer's protocol. SRNA fractions from total RNA were isolated using the mirVana miRNA isolation kit (Ambion). A total of 2 µg of sRNA from each sample was ligated to 3′ and 5′ HD adapters (Sorefan et al., 2012). Libraries were generated as previously described (Xu et al., 2015). Libraries were sequenced on the Illumina HiSeq2500 set to 50 bp single end sequencing.

### Data availbility

All data regarding this study is available within the article, in the supplementary files or is available from the lead author on request. Raw sequencing data has been deposited in GEO under the accession numbers GSE85816, GSM2284961 and GSM2284962.

## Conflicts of interest

The authors wish to state that no conflicts of interest exist with any of the authors in this manuscript.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virol.2019.08.032.

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