Artificially induced phased siRNAs promote virus resistance in transgenic plants

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SUMMARY

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 apply 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.

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) (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 siRNAs from their own genomes to suppress host RNA silencing (Csorba et al., 2015, Sanan-Mishra et al, 2017). Viral RNA silencing suppressors are a significant target 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 phasiRNA that target viral *AC2* transcript (construct name TRiV-AC2) and viral *AC4* transcript (construct name TRiV-AC4). Both constructs conferred high resistance against 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 and TRiV-AC4 produced phasiRNAs and their target *AC2* and *AC4* 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 transfer from our tobacco model and apply the RNAi constructs in tomato, which is the natural host for ToLCNDV and ToLCGV.

RESULTS

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 d post inoculation (dpi). We obtained 12M (million) reads from both replicates of which 11M reads were analysed (Supplementary 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 Figure 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 (Figure 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 (Figure 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.

PhasiRNAs from TRiV-AC2 cleave *AC2* in consecutive 21 nt segments

We performed degradome analysis using HD adapters 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 (Supplementary

Table 2). Size class distributions indicated two major peaks at 20-21 nt (Supplementary Figure 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 (Figure 2). In the controls, we observed low read numbers of cleavage products (Figure 2a, 2b), which may derive from random degradation of transcripts without cleavage by phasiRNA. We observed high read numbers (Figure 2c, 2d) showing cleavage within the 1,417-1,719 nt region of *AC2* transcript, which is the region cloned in TRiV-AC2 (Figure 2e).

The natural host of ToLCNDV, Tomato, also produce artificially induced phasiRNA

Because tomato (cultivar “Pusa Ruby”) is the natural host for ToLCNDV, we wanted to test our RNAi constructs for biotechnological relevance. As in tobacco, we generated transgenic tomato (*Solanum lycopersicum*) using TRiV-AC2 (this study) and TRiV-AC4 (previous study) 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, AC2-25) and eight lines with TRiV-AC4 (AC4-4, AC4-6, AC4-9, AC4-10, AC4-14, AC4-15, AC4-17, AC4-

20) accumulated phasiRNAs corresponding to the transgenes with varying levels of accumulation (Figure 3a, 3b). Accumulation of siRNAs was highest in line #13 and line #20. These plants showed a visible phenotype from non-transgenic controls (Figure 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 (Figure 4a, 4b). 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 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.

Transgenic tomato plants are resistant to both ToLCNDV and ToLCGV

As performed in tobacco, we tested the performance of tomato 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 (Supplementary 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 (Figure 5).

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 1,417-1,719 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 10th 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 apply our RNAi technology in tomato, which is the natural host of ToLCNDV plus a closely related strain ToLCGV.

Conclusion

We demonstrate sequence specific cleavage of a viral transcript by artificially induced phasiRNA. This methodology can be applied to other viruses/plants/models so 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.

MATERIALS AND METHODS

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.

Transformation

The TRiV-AC2 construct was used to transform *N. tabacum* and *S. lycopersicum* employing the modified *A. tumefaciens* mediated transformation protocol (Lloyd et al., 1986). Plants were selected using 100 mg/L of kanamycin. Genomic DNA of the regenerated seedlings was isolated using the DNeasy plant mini kit (Qiagen,

USA). We used PCR to screen for integration using 35S-(TB)/AC2REPR (Supplementary Table 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 labeled 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 synthesised. 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).

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 [γ-^{32}P] ATP (PerkinElmer Life Sciences, 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).

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.

Degradome library preparation

N. tabacum TRiV-AC2 transgenic plants were inoculated with ToLCNDV by agroinfiltration. The second top leaf samples were 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 (Supplementary Table 4).

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 mis-matches, 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.

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.

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Figure 1. Processing of tasiRNAs directed by miR390 on *AC2* transcript. **a** Radar plot showing the abundance of 21 nt reads corresponding to the *AC2* transcript sequenced in TRiV-AC2. Position 1 is designated as immediately after the miR390 guided cleavage site. **b** TRiV-AC2 showing phasiRNA production. TasiRNA are shown in red. The miRNA390 cleavage site in *TAS3* transcript is shown by the red arrow. The 21 nt phase relative to the miR390 cleavage site is indicated by brackets.

Figure 2. Target plots (t-plots) of phasiRNA produced from the TRiV-AC2 construct. **a** & **b** T plot profiling from tobacco plants expressing TRiV-AC2. **c** & **d** T plot profiling from tobacco plants expressing TRiVM2-AC2 (control). All plants carrying the construct were infected with ToLCNDV. The y-axis measures the normalised reads, using the reads per million approach, for the degradome signals. The y-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.

Figure 3. a Northern blot shows phasiRNA in T_0 transgenic tomato plants with TRiV-AC2. **b** Northern blot shows phasiRNA in T_0 transgenic tomato plants with TRiV-AC4. L indicates size marker for 21 nt. M indicates untransformed plants as controls. Lane numbers represent plant identifier number. U6 is loading control. **c** T_1 transgenic tomato using TRiV-AC2 line #3 and TRiV-AC4 line #20 as examples when compared to wild type (WT).

Figure 4. Southern blot shows copy number of the transgene in T_0 transgenic tomato plants. **a** TRiV-AC2. **b** TRiV-AC4. Lane number indicates plant line identifier. M represents mock transformed plants. TRiV-AC2 and TRiV-AC4 plasmids were loaded as positive controls in lane L. We digested DNA with *Eco*R1.

Figure 5. Resistance assay of transgenic tomato plants. **a** TRiV-AC2 with negative control. **b** TRiV-AC4 with negative control. All plants were inoculated with ToLCNDV. Photograph was taken at 30 dpi.

Supplementary Table 1. General Information of small RNA libraries

Supplementary Table 2. General information of degradome libraries

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

Supplementary Table 4. Primers used in this study.

Supplementary Figure 1. Overall summary of small RNA libraries. **a** Size class distribution for total (redundant) reads. **b** size class distribution for unique (non-redundant) reads.

Supplementary Figure 2. Overall summary of degradome libraries. **a** Size class distribution for total (redundant) reads. **b** Size class distribution for unique (non-redundant) reads.