



Transcriptome-wide identification of host genes targeted by tomato spotted wilt virus-derived small interfering RNAs



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ABSTRACT

RNA silencing mechanism functions as a major defense against invading viruses. The caveat in the RNA silencing mechanism is that the effector small interfering RNAs (siRNAs) act on any RNA transcripts with sequence complementarity irrespective of target's origin. A subset of highly expressed viral small interfering RNAs (vsiRNAs) derived from the tomato spotted wilt virus (TSWV; *Tospovirus: Bunyaviridae*) genome was analyzed for their propensity to downregulate the tomato transcriptome. A total of 11898 putative target sites on tomato transcripts were found to exhibit a propensity for down regulation by TSWV-derived vsiRNAs. In total, 2450 unique vsiRNAs were found to have potential cross-reacting capability with the tomato transcriptome. VsirRNAs were found to potentially target a gamut of host genes involved in basal cellular activities including enzymes, transcription factors, membrane transporters, and cytoskeletal proteins. KEGG pathway annotation of targets revealed that the vsiRNAs were mapped to secondary metabolite biosynthesis, amino acids, starch and sucrose metabolism, and carbon and purine metabolism. Transcripts for protein processing, hormone signalling, and plant-pathogen interactions were the most likely targets from the genetic, environmental information processing, and organismal systems, respectively. qRT-PCR validation of target gene expression showed that none of the selected transcripts from tomato cv. Marglobe showed up regulation, and all were down regulated even upto 20 folds (high affinity glucose transporter). However, the expression levels of transcripts from cv. Red Defender revealed differential regulation as three among the target transcripts showed up regulation (*Cc-nbs-lrr*, resistance protein, AP2-like ethylene-responsive transcription factor, and heat stress transcription factor A3). Accumulation of tomato target mRNAs of corresponding length was proved in both tomato cultivars using 5' RACE analysis. The TSWV-tomato interaction at the sRNA interface points to the ability of tomato cultivars to overcome vsiRNA-mediated targeting of NBS-LRR class R genes. These results suggest the prevalence of vsiRNA-induced RNA silencing of host transcriptome, and the interactome scenario is the first report on the interaction between tospovirus genome-derived siRNAs and tomato transcripts, and provide a deeper understanding of the role of vsiRNAs in pathogenicity and in perturbing host machinery.

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1. Introduction

RNA silencing in eukaryotes is an established phenomenon that plays a fundamental role in gene regulation, genome stability, and defence against the invading pathogens including viruses and transposons (Napoli et al., 1992; Romano and Macino, 1992; Ruiz-Ferrer and Voinnet, 2009; MacLean et al., 2010). RNA silencing

machinery controls the growth and development of an organism (Rubio-Somoza et al., 2009). Plant virus infection triggers the process of RNA silencing with the onset of dsRNAs leading to dicing by host DCL (Dicer-Like) enzymes culminating in the production of 21–24nt length primary small interfering RNAs (siRNAs). The silencing process also involves amplification step wherein host RNA dependent RNA polymerases (RdRps) convert ssRNA into dsRNA substrates to feed DCL processing resulting in the production of secondary siRNAs (Schiebel et al., 1998; Curaba and Chen, 2008; Voinnet, 2008). The small RNAs are recruited onto host Argonaute (AGO) proteins to guide the RNA-Induced Silencing Complex (RISC) to target the complementary transcript or DNA (Mallory and Vaucheret, 2010). The secondary siRNAs are involved in the sys-

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temic spread of silencing signals throughout the plant (Dunoyer and Voinnet, 2005; Dunoyer et al., 2010). Virus infection triggers the process of host RNA silencing because most plant viruses are positive strand RNA viruses, and as such dsRNA intermediates are formed during the process of viral genome replication (Ratcliff et al., 1997; Ghoshal and Sanfaçon, 2015). This process of RNA silencing mainly functions as an antiviral mechanism. However, the caveat in the RNA silencing mechanism is that the effector siRNAs function on the principle of sequence complementarity irrespective of the origin of RNA transcripts. Therefore, sequence complementarity between siRNA and any random transcripts would cause silencing of cognate mRNA. Hence it is plausible for the vsiRNAs to have inadvertent silencing effect on the host transcriptome.

Evidence has revealed that the virus-derived siRNAs (vsiRNA) could function as potential regulators of host transcriptome machinery (Dunoyer and Voinnet, 2005; Donaire et al., 2009; Catalano et al., 2012; Wang et al., 2016; Xia et al., 2016). Furthermore, the successful application of viral vectors to silence the host-derived genes through virus-induced gene silencing (VIGS) attests to the possibility of virus-derived siRNAs' role in cross silencing host transcriptome (Becker and Lange, 2010). In this context, it will be interesting to learn the transcriptome changes at plant-virus interface to gain insights into viral pathogenesis, to identify host susceptibility factors and ultimately to devise novel disease resistant strategies.

Tospoviruses (Family: *Bunyaviridae*; Genus: *Tospovirus*), transmitted by thrips (Thysanoptera, Thripidae), are important pathogens of several field and horticultural crops worldwide. Annual losses due to tospovirus infection are estimated to be more than US\$1 billion (Pappu et al., 2009). Tomato spotted wilt virus (TSWV) is one of the more than 30 known tospoviruses that causes severe economic damage in a wide range of crops including beans, groundnut (peanut), lettuce, pepper, potato, tobacco, and tomato (Pappu et al., 2009; Oliver and Whitfield, 2016). We recently identified and characterized the small RNA profiles of TSWV from infected tomato and *N. benthamiana* plants (Mitter et al., 2013). The vsiRNAs that were expressed in high frequency in tomato were considered for further *in silico* investigation followed by experimental validation using qRT-PCR and RACE techniques. Here, we present the transcriptome-wide identification and validation of target genes that are potential, and inadvertent targets for vsiRNAs.

2. Materials and methods

The data described here has not been obtained directly or indirectly from human or animal subjects. The study has not been conducted on human/animal subjects hence the research was not subject to the Institutional Review Board's review.

2.1. Data acquisition

The vsiRNA profile from TSWV-infected tomato plants (Mitter et al., 2013) was used in studying the interaction with the tomato transcriptome. Tomato-specific vsiRNA degradome data was obtained from the small RNA profile data deposited in the NCBI Bio-project database (submission ID: SRP028288) and accessible at <http://trace.ncbi.nlm.nih.gov/Traces/sra/?study=SRP028288>). The complete expression profile of vsiRNAs was categorized into 'low' (<50 reads), 'high' (>= 50 reads) and 'very high expression' (> = 1000 reads) based on the copy or count numbers (relative abundance). The vsiRNA reads aligned to TSWV genome that are characterized as high copy number and varying from 21–24nt in length were obtained from the parent dataset for the analysis.

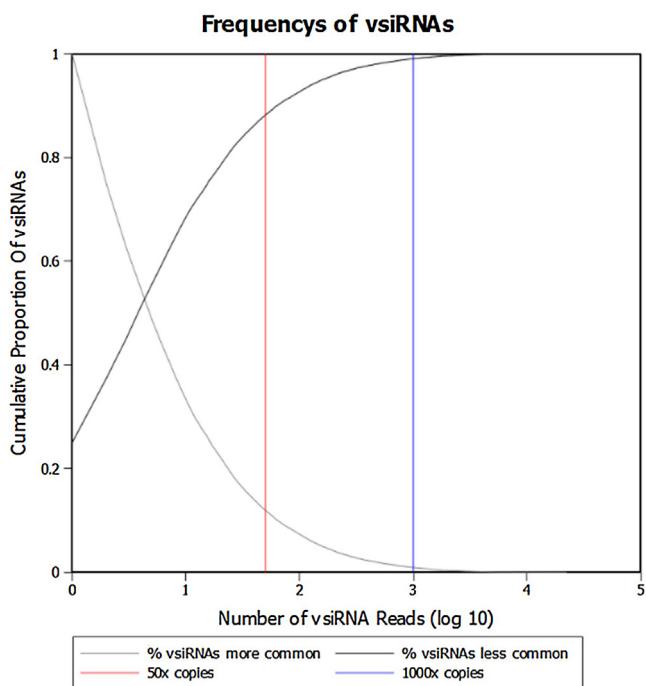


Fig. 1. Frequency distribution of vsiRNAs derived from tomato spotted wilt virus-infected tomato. The plot depicts the cumulative proportion of vsiRNAs obtained from a degradome library against the read count in log scale. The vsiRNA profile has been categorized as "low" (<50 copies) high (>50 copies) and "very high" (>1000 copies) based on the copy numbers. For tomato transcriptome, target prediction high copy vsiRNAs (>50copies) alone were used as a query in psRobot program.

2.2. Target prediction

In order to define vsiRNA threshold for using in target prediction studies, a plot was created to determine the distribution of abundance of all the detected vsiRNAs (distinct 21–24nt reads) (Fig. 1). The distribution plot revealed that most vsiRNAs are very rare (25% having only one copy). In order to perform the target prediction analysis, vsiRNAs with 50 or more copies (red line in Fig. 1) were considered for analysis (i.e. the 'high' and 'very high' abundant categories). These vsiRNAs amount to a little over 10% of all the detected vsiRNAs in tomato. Further, a category of very highly abundant vsiRNAs ($N = 222$) corresponding to 1000 or more counts from small RNA profiling were used in the analysis. The 21–24mer vsiRNAs obtained after categorization (as 'high' and 'very high expression') were used in the target prediction analysis. The vsiRNA profile was used as a query in small RNA target prediction module psRobot (Wu et al., 2012) (<http://omicslab.genetics.ac.cn/psRobot/>). For the target prediction analysis, the psRobot algorithm was used and was run locally owing to the relatively large number of vsiRNA and the anticipated quantum of output. To run the local version of the module, computational biology support from Queensland Facility for Advanced Bioinformatics (QFAB) Brisbane was sought. Tomato transcriptome from Solgenomics, release ITAG2.3 built on the SL2.40 assembly (source: ftp://ftp.solgenomics.net/tomato_genome/annotation/ITAG2.4.release/) was used as targets for the study. Various parameters of target prediction that we used were a) penalty threshold score-2.5 b) maximal number of permitted gaps-0 and c) positions after which gaps are permitted-1. The sRNA target prediction algorithm, psRobot, identifies target transcripts for query small RNAs based on sequence complementarity using modified Smith-Waterman algorithm (Wu et al., 2012). The algorithm also provides information regarding multiplicity of small RNA target sites and a score for the predicted target mRNAs.

It is pertinent to mention that the lower the psRobot score, greater the chance that the mRNA would be a target for the cognate sRNA.

2.3. Target transcripts and KEGG pathway annotation

Pathway mapping of the psRobot resultant transcripts was performed using KEGG Mapper – Search Pathway interface present in KEGG pathway tools (http://www.genome.jp/kegg/tool/map_pathway1.html). The target tomato transcripts were used as input query after converting transcript names to corresponding Entrez Gene IDs through BioMart-Ensembl and searching against the *Solanum lycopersicum* database in KEGG pathway analysis.

2.4. KEGG pathway enrichment and statistical analysis

KEGG pathway enrichment studies were performed for the target transcripts to ascertain which of the pathways are overrepresented in the vsiRNA-transcriptome interaction. Statistical analysis of KEGG pathway enrichment was performed using R version 3.1.0. *p*-values were calculated by performing cumulative hypergeometric probability (*p*-hyper function) analysis. Multiple hypothesis *p*-value correction was done using the Benjamini and Hochberg (1995) method (Benjamini and Hochberg, 1995).

2.5. Tomato cultivars and bioassays

TSWV was maintained on *N. benthamiana* under controlled conditions[25/18 °C (day/night)] in a greenhouse. The virus culture was used for inoculating TSWV resistant and susceptible cultivars of tomato (*S. lycopersicum*) cv. Red Defender (+Sw-5) and cv. Marglobe (−Sw-5), respectively (Mandal et al., 2006; Stevens et al., 1992). Plants were grown from seeds under greenhouse conditions at 26 °C with 16 h day and 8 h night for about 20 days until fully opened lower leaves were manually inoculated with an extract from TSWV-infected *N. benthamiana*. Homogenization of virus-infected tissue was done by grinding in 0.01 M sodium phosphate buffer (pH 7.0) containing 0.4% β-mercaptoethanol and the homogenate was used as the inoculum. Virus-inoculated and buffer-inoculated plants (mock-inoculated control plants) were maintained at 25/18 °C (day/night).

2.6. RNA extraction and relative quantification of host gene(s) expression

RNA extraction was done at 17 days post-inoculation (dpi), when the newly developed, upper leaves started to show symptoms associated with TSWV infection. This time point was taken based on our previous study on differential expression of small RNAs in TSWV infected tomato (Mitter et al., 2013). ELISA was performed using a commercially available kit (Agdia Inc., Elkhart, IN, USA) to confirm the presence of TSWV. Systemically infected leaves and leaves from buffer-inoculated plants were harvested and flash frozen in liquid Nitrogen. In order to avoid sampling variations, infected leaves of at least 5 plants were pooled for RNA extraction. Total RNA was extracted by using RNAeasy plant minikit (Qiagen) and treated with 5 U of RNase free DNase I (Invitrogen, Carlsbad, CA, USA) for 30 min at 25 °C. The integrity of the RNA was tested by 17% denaturing polyacrylamide gel electrophoresis, RNA concentration measured using Nano drop before performing qRT PCR. Presence of virus infection was also tested by performing RT-PCR using N gene-specific primers (Pappu et al., 2008). About 2 μg of RNA was reverse transcribed and qRT-PCR performed to ascertain the relative expression levels of some of the predicted target transcripts. The details of the target transcripts, primer sequences (designed using Biosearch Technologies <https://www.biosearchtech.com/>) used for qRT-PCR analysis are presented in

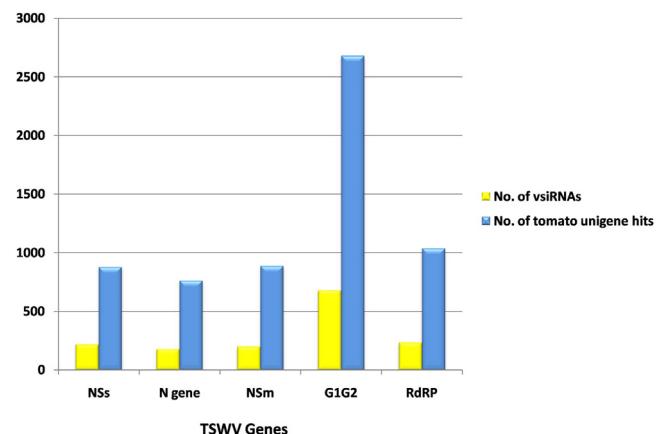


Fig. 2. Number of high frequency vsiRNAs mapped to tomato spotted wilt virus encoded genes and their respective number of target tomato genes.

Table 1. qRT-PCR was performed using Stratagene Mx3000P QPCR System according to the manufacturer's instructions. The reaction conditions for qRT-PCR were as follows: 5 min at 94 °C for denaturation, followed by 42 cycles of 10 s at 94 °C for denaturation, 20 s at 55 °C for annealing and 30 s at 72 °C for extension. The data on fluorescence was collected at 78 °C further to avoid any non-specific amplification and melting curve was performed from 70 to 95 °C (held for 1 s per 0.1 °C increase) with a final extension at 72 °C for 5 min. Fold changes in the expression of host mRNAs were calculated as follows:

$$\text{Fold change (FC)}$$

$$= \frac{2^{-(\text{Ct Challenged} - \text{Ct unchallenged})}}{2^{-(\text{Ct Challenged REF} - \text{Ct unchallenged REF})}}$$

Where unchallenged refers to gene expression data obtained from mock-inoculated control plants, REF is expressional changes of tomato ubiquitin gene-[gi|19396|emb| X58253.1 |(Catoni et al., 2009)].

Statistical calculations for qRT-PCR experimental data were inferred from three biological replicates as described in comparative CT method and MIQE guidelines (Schmittgen and Livak, 2008; Bustin et al., 2009).

2.7. Target mRNA cleavage validation

TSWV-derived siRNA mediated cleavage of target tomato genes and the subsequent accumulation of cleaved mRNA were ascertained by 5' Rapid amplification of cDNA Ends (5' RACE) technique using SMARTer® RACE 5'/3' kit (Clonetech, CA, USA). Total RNA extracted after 17 days post-inoculation (17 dpi), was used in RACE experiments. The gene specific primers (GSPs) used for 5'RACE for some of the target tomato transcripts are presented in Table 2.

3. Results

3.1. vsiRNA copy number threshold and siRNA targets

High copy number vsiRNAs derived from TSWV-infected tomato were mapped to their respective gene or ORF loci in the viral genome (Fig. 2). It was found that vsiRNAs derived from the ORF encoding glycoproteins (G_N/G_C) were relatively high in number (678 distinct vsiRNAs), followed by vsiRNAs (234) derived from the ORF encoding RNA dependent RNA polymerase (RdRp). Genes encoding structural proteins, NSs and NSm, yielded 217 and 200 positive vsiRNAs, respectively. When the vsiRNAs numbers were normalized to the unit length of the gene (number of vsiRNAs/kbp)

Table 1

Selected tomato transcripts and primer sequences used in qRT-PCR validation of vsiRNA-induced down regulation of target host transcripts.

S.No	<i>Solanum lycopersicum</i> ID	psRobot score	Target transcripts	Primers
1	Solyc07g022790.2.1	0.2	Calcium-transporting ATPase	F: 5' TCC TGA TAC TCT TGT GCC TGT CC 3' R: 5'CCA ATA GCT GTG GCA GGC AAT C 3'
2	Solyc12g017800.1.1	0.8	Cc-nbs-lrr, resistance protein	F: 5'CCC AAA CCT GAA GTG CTC AAA GCG 3' R: 5' GCT TCC CAC CCT ACC AGA CAA TCG 3'
3	Solyc04g050960.2.1	1.0	Chromatin remodeling complex subunit	F: 5'CAG TTG CTA AGA GTT GCT CAG TGG 3' R: 5' TCT CAC CTC CGT TGG TTG TCT C 3'
4	Solyc04g011430.2.1	1.2	Ubiquitin-conjugating enzyme 13 E2	F: 5'TGC TAA CCT GGA AGC TGC TAA GG 3' R: 5'ACC GTC TTA CAC AAC GAC TGA CC 3'
5	Solyc03g031700.2.1	1.5	ABC transporter FeS assembly protein SufB	F:TGT CCA GTG CAG ACA ATG CCA AG R: AGT GTT GGC AGC AGC AGT ATC AC
6	Solyc01g102300.2.1	1.5	BHLH transcription factor	F: 5' ACT TAT ACC CAA CTG CAA CAA GGC 3' R: 5' GCT GCA TAG GGA ACA TCA TTG GC 3'
7	Solyc11g008560.1.1	1.5	AP2-like ethylene-responsive transcription factor	F: 5' GGA AGA GCA GTG GTT TCT CTC GTG 3' R: 5' ATC TTC CAT GCT GAT GAT GCC TTG 3'
8	Solyc02g072000.2.1	1.5	Heat stress transcription factor A3	F: 5' AGG ATC ACC AAG GAC TTG AAT TGC 3' R: 5' TGG TCT TCT GAC GGT GTT CTG C 3'
9	Solyc05g018230.2.1	1.5	High-affinity glucose transporter	F: 5' TTG GTA GCG GTT GCT GTT ACT C 3' R: 5' CCA AGA CCT TCC ATA TGC GAC AAC 3'
10	Solyc05g052500.2.1	1.8	Dehydration-responsive family protein	F: 5' AGA GTG CTC AAA CAT GTT CTC CTG 3' R: 5' GGG CCT CTT GAA CTT TCC TTT CTG 3'

Table 2

Tomato genes and corresponding gene specific primer sequences (GSPs) used in vsiRNA mediated mRNA cleavage validation of target transcripts.

S. No	Tomato target genes	Gene specific primers (5' to 3')
1	Solyc07g022790.2.1 Calcium-transporting ATPase (CTA)	GSP CTA: GTACAAACGGGCCCTTAAT
2	Solyc12g017800.1.1 Cc-nbs-lrr, resistance protein (cc-nbs-lrr)	GSP cc-nbs-lrr: TTGTGAAATGAGGCGATCAA
3	Solyc04g050960.2.1 Chromatin remodeling complex (ChrRMC)	GSP ChrRMC: AGAGAGAGGCCCTTGGAAAC
4	Solyc04g011430.2.1 Ubiquitin-conjugating enzyme (UCE)	GSP UCE: GGATGCCAGATCTCTGTGGT
5	Solyc03g031700.2.1 ABC transporter (ABCT)	GSP ABCT: CACAGTGTGATCTACGAGTGA
6	Solyc01g102300.2.1 BHLH transcription factor (BHLH-TF)	GSP BHLH-TF: AGTCCTTGACCAAGGATGTGC
7	Solyc02g072000.2.1 Heat stress transcription factor (HS-TF)	GSP HS-TF: CTCCCCATTTTCAGGGTCAA
8	Solyc05g018230.2.1 High-affinity glucose transporter (HAGT)	GSP HAGT: GCAATGAGGGCAGTGAAGAT
9	Solyc05g052500.2.1 Dehydration-responsive family protein (DR)	GSP DR: CATGCCTCAATCACACAG
10	Solyc11g008560.1.1 ethylene-responsive transcription factor (ER-TF)	GSP ER-TF: CCAGTCCACCTATGCCCTGT

length of respective gene), it was found that the siRNAs from N and NSm were comparable with 229 and 220 vsiRNAs/kbp, respectively. In case of RdRP gene, the normalized number of vsiRNA was least at 27, whereas normalised vsiRNAs derived from NSs (154) and G_N/G_C (199) were moderate. Accordingly, the corresponding vsiRNA hits on the tomato transcriptome also varied with viral genome loci. The G_N/G_C -derived vsiRNAs were found to be targeting 2678 transcripts followed by 1035 transcripts targeted by RdRP-derived vsiRNAs. The least number of tomato transcript hits was exhibited by vsiRNAs derived from nucleocapsid gene (N) with 761 putative targets. Comparison of number of target sites on the host transcriptome for vsiRNAs derived from NSm and NSs genes revealed that the former recorded 887 potential target sites on the tomato transcriptome, while NSs displayed slightly lower number of target sites (877)

(Fig. 2). Despite the least number of vsiRNAs derived from RdRP and a moderate number of vsiRNAs from G_N/G_C , these vsiRNAs exhibited relatively high number of tomato transcriptome targets.

3.2. Tomato transcriptome targets

Small RNA target prediction analysis was carried out using TSWV-derived siRNAs as query and tomato transcripts as targets in psRobot (Fig. 3). A total of 11898 putative target sites on tomato transcriptome were identified that had the potential for interaction with the 2450 unique TSWV-derived siRNAs. The potential target transcripts, when categorized, were found to be involved in encoding various types of cellular proteins. Considering the enormity of vsiRNA targets obtained from this analysis, transcripts that attracted at least 25 vsiRNA hits or higher were distributed

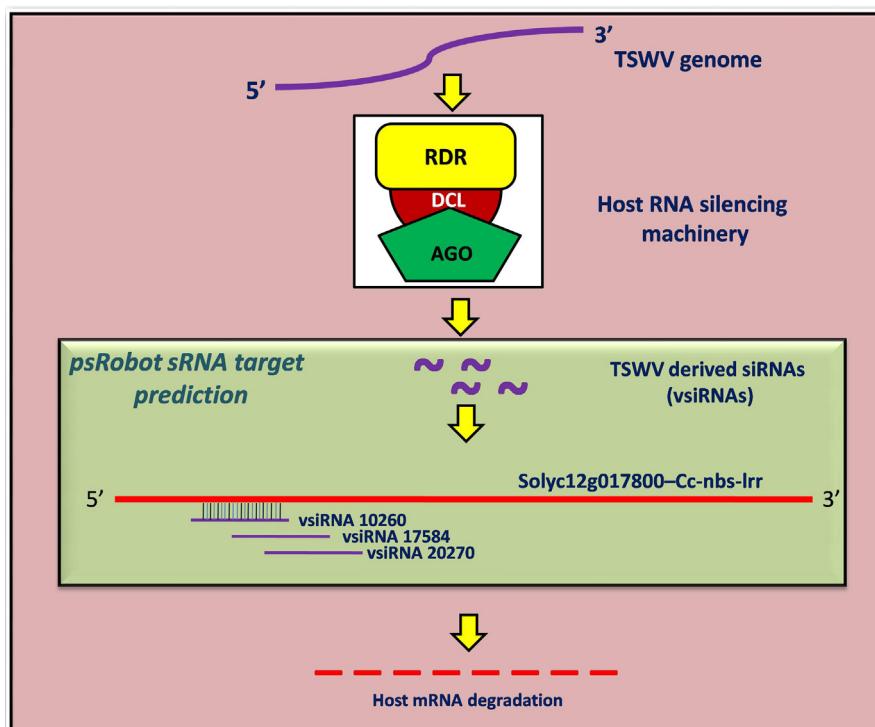


Fig. 3. Work flow depicting generation of tomato spotted wild virus vsiRNAs and their use in psRobot to identify potential tomato target genes. The figure depicts tomato transcript (*Solyc12g017800*) encoding for cc-nbs-lrr resistance protein as potential target for TSWV derived vsiRNAs. Multiple TSWV-derived vsiRNAs were found to be targeting the transcript (*Solyc12g017800*). Note that the transcript was identified as a potential target for TSWV vsiRNA. However, our results show that the gene was found to be downregulated only in cv Marglobe whereas expression analysis of cc-nbs-lrr mRNA obtained from cv Red Defender revealed its upregulation. Despite the multiple targeting by TSWV vsiRNAs the cc-nbs-lrr transcript was not downregulated in the cv Red Defender.

into functional classes (Fig. 4). The classification was based on the functional categories in psRobot output using the International Tomato Annotation Group (ITAG2.3) annotation. Functional categorization of potential target transcripts revealed that host enzymes are largely targeted by vsiRNAs. Among the host-derived enzymes, receptor-like kinases, cytochrome P450, and Ulp1 protease family are represented as targets (Fig. 4). Considering the number of genes in the group with potential target sites for the vsiRNAs, the enzymes category was followed by transcription factors (TFs), wherein hormone responsive TFs were found to be targeted (Fig. 4). Since TFs are master regulators in the gene regulatory pathways, implications of TFs being targets are enormous since it can disrupt the gene regulatory networks of the host. Proteins associated with membranes such as transporters (ABC transporters, importins) or those involved in resistance signalling pathways (TIR nbs resistance protein) were also found to be targets of vsiRNAs. Cellular structural proteins including kinesins and ribosomal proteins were also found to be potential targets among the host transcripts. All these potential targets imply that all aspects of the cellular functioning were potentially targeted by TSWV-derived vsiRNAs (Fig. 4).

3.3. KEGG pathway analysis

Considering the large number of target regions in the tomato cDNA dataset, there exists remarkable multiplicity in target sites for the vsiRNAs as each of them potentially could target many transcripts. However, the Kyoto Encyclopaedia of Genes and Genomes (KEGG) (<http://www.genome.jp/kegg/>) is a valuable genomic resource that aids in deciphering the cellular functions using the data generated from high-throughput experimental strategies. All the putative tomato target transcripts which are based on ITAG 2.3 annotations were converted into Entrez Gene IDs for querying KEGG pathway. The analysis was performed consider-

ing only those genes that were mapped to Entrez Gene IDs and were annotated in at least one KEGG pathway ($n = 957$), against a background of all Entrez IDs having KEGG pathways mapped ($n = 4409$). Target transcripts that were assigned with Entrez Gene IDs were categorized according to the classification enumerated in the recent KEGG release (Release 70.1, June 1, 2014). Among the 34727 total ITAG 2.3 cDNA IDs, 20656 were converted to Entrez Gene IDs, of which 4409 have KEGG pathway annotations. Out of the 5946 ITAG2.3 transcripts (4584 Entrez Gene IDs) having vsiRNA targets, 957 were annotated in KEGG pathways. Pathways that exhibited at least 20 vsiRNA hits were shown in Fig. 5. The highest number of hits was found in basic metabolism as 35 basic metabolic pathways had multiple (>20) vsiRNA targets (Fig. 5).

Among the targets, tomato transcripts that encode enzymes involved in 'biosynthesis of secondary metabolites' showed many potential vsiRNA cross-reaction sites (461 hits), followed by tomato transcripts encoding enzymes involved in biosynthesis of amino acids, starch and sucrose metabolism, and carbon metabolism (108, 105, 104 hits, respectively) for the vsiRNAs. More than two-thirds (27 out of 35) of the highly represented basic metabolic pathways attracted less than or equal to 50 vsiRNA hits. However, the least hit pathways are very diverse that included metabolism-related to amino acids, carbohydrate, energy, nucleotides, lipids, other secondary metabolite synthesis, metabolism of terpenoids, polyketides, and metabolism of cofactors and vitamins. Metabolic pathways are followed by basal genetic information processing pathway with multiple hits by vsiRNAs. Within the pathway for genetic information system, protein processing in endoplasmic reticulum, ribosomes, spliceosomes, RNA transport and ubiquitin-mediated proteolysis exhibited more than 50 vsiRNA hits each. Cellular processing activities such as peroxisome, endocytosis and phagosome followed the genetic information pathway with 60, 55 and 31 hits, respectively.

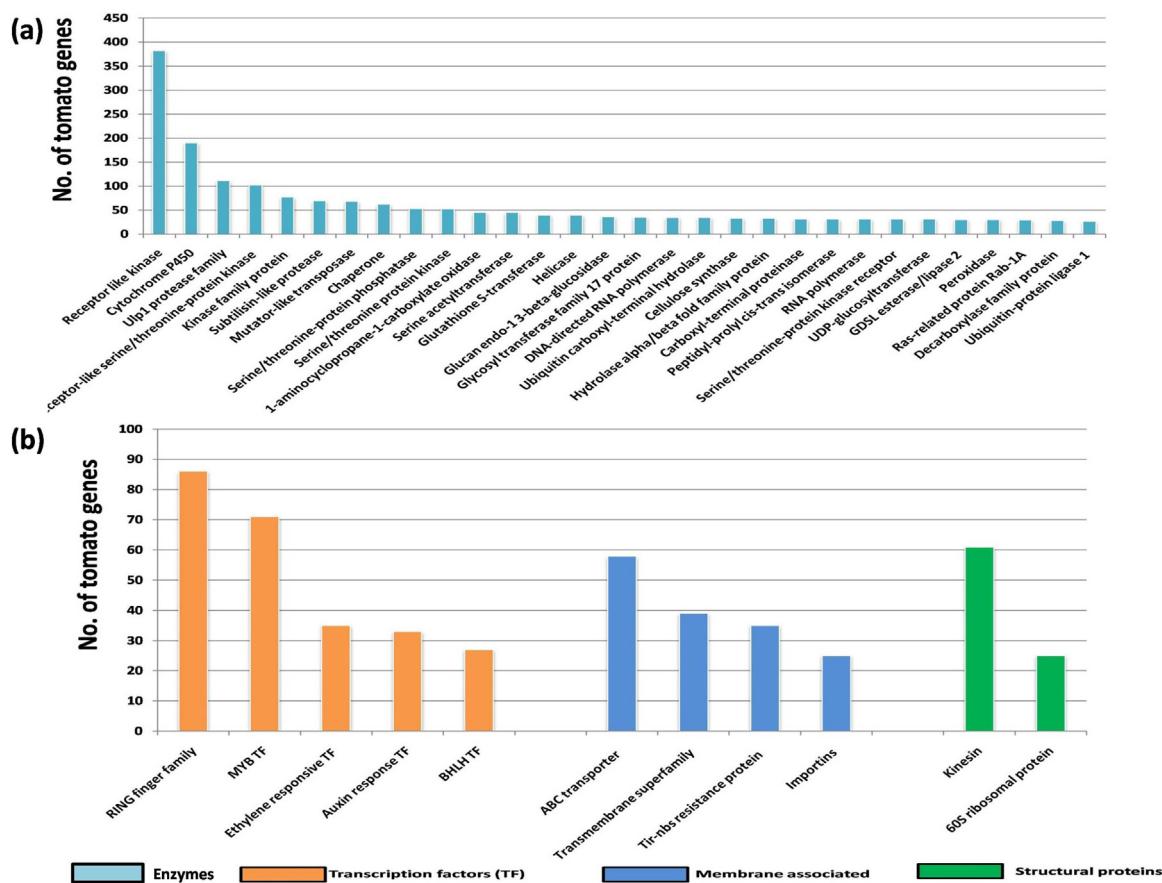


Fig. 4. Number of vsiRNA targets in tomato transcriptome with ITAG2.3 functional annotations. (a) enzymes, and (b) transcription factors, membrane associated and structural proteins. Only targets with a minimum number of 25 hits by different vsiRNAs are depicted.

The signalling system under environmental information processing showed 122 vsiRNA targets on the plant hormone signal transduction pathway followed by phosphatidyl inositol signalling pathway with 33 target transcripts. Besides the cellular processes, whole organismal systems involved in plant-pathogen interactions and circadian rhythm were also found to be potential targets for TSWV-derived siRNA mediated gene silencing as they showed 73 and 27 vsiRNA hits, respectively.

3.4. KEGG pathway enrichment

The KEGG pathway enrichment analysis was performed to identify any pathways or genes that were over-represented than expected in the vsiRNA-transcriptome interaction. The list of pathways that were found to be enriched statistically (p -value < 0.05) are presented in Table 3. Multiple hypothesis correction using Benjamini-Hochberg procedure was carried out in order to avoid the false discovery rate. After correction analysis, we obtained statistically insignificant p -values for many of the KEGG pathways. Despite the statistically insignificant p -values after multiple hypothesis correction, the predicted interactome is biologically noteworthy because the sRNA-mediated binding and degradation of complementary mRNAs has been proven in many instances. It is apparent that the general metabolic pathways involved in lipid metabolism, starch sucrose metabolism, riboflavin metabolism are enriched. Among the pathways that are enriched but not related to metabolism include ABC transporters, circadian rhythm- plant, and aminoacyl tRNA biosynthesis which belong to environmental information processing, organismal systems and genetic information processing categories, respectively. It implies that besides

metabolic pathways, cross-silencing effects of vsiRNAs potentially transcend other aspects of host cellular functioning. However, when the KEGG pathway enrichment studies were carried out only with high copy number vsiRNA (> 1000) target transcripts, it revealed that the terms involved in basic metabolism such as steroid biosynthesis, citrate cycle (TCA cycle), and nitrogen metabolism were found to be enriched. The enriched terms also involved intracellular transport and catabolic processing in peroxisome. Thus, overall, it is interesting to observe that tomato transcripts that are involved in metabolic pathways are the potential targets for vsiRNAs-based cross silencing.

3.5. Expression levels of selected targets transcripts

Inoculated leaves of tomato cultivars Red Defender and Mar-globe were assessed for TSWV infection through RT-PCR and ELISA. The presence of TSWV in infected plants was confirmed by RT-PCR amplification of the N-gene, whereas no amplification was seen for RNA from mock-inoculated plant (Fig. 6). Among the multitude of vsiRNA targets from tomato transcripts, 10 were selected to study their expression levels following TSWV infection. vsiRNA target region was found at the 5' end of five target transcripts, 3' end of two transcripts and in middle of the gene for three targets. The selection of the transcripts was based on the output of psRobot program, wherein those target genes with lowest score value (lower the score better the chances of being a target) and representing various molecular functions were chosen for the expression analysis. Relative expression changes of the target transcripts were normalized to the expression levels of host ubiquitin gene. The expression fold change values of the transcripts from the

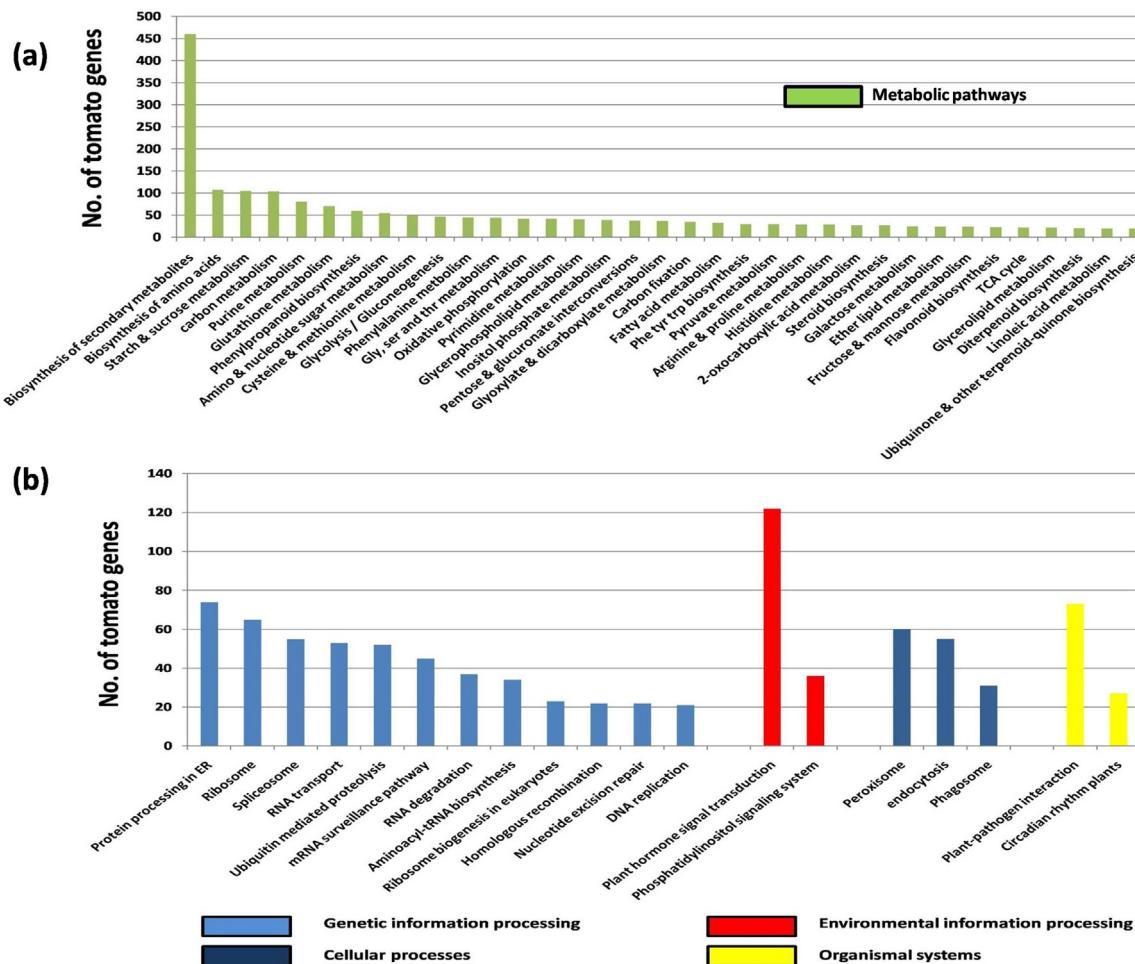


Fig. 5. Number of vsiRNA targets in tomato transcriptome annotated to KEGG pathway. (a) metabolic pathways, and (b) genetic information, environmental information processing, organismal systems and cellular processes pathways. Only targets with a minimum number of 20 hits by different vsiRNAs are depicted.

Table 3

KEGG pathway enrichment studies on the target tomato transcripts (metabolic pathways with *p*-value less than 0.05 alone are presented).

Metabolic pathways enriched	P-value	P-value after Multiple hypothesis correction
Ether lipid metabolism	0.002041	0.13597
Starch and sucrose metabolism	0.002706	0.13597
Riboflavin metabolism	0.00471	0.13597
ABC transporters	0.004786	0.13597
Phenylalanine, tyrosine and tryptophan biosynthesis	0.005353	0.13597
Metabolic pathways	0.012925	0.23921
Histidine metabolism	0.013185	0.23921
Circadian rhythm plant	0.016325	0.251357
Purine metabolism	0.017813	0.251357
Steroid biosynthesis	0.02628	0.333753
Glutathione metabolism	0.035758	0.405565
Ubiquinone and other terpenoid quinone biosynthesis	0.038435	0.405565
Aminoacyl tRNA biosynthesis	0.041514	0.405565

two tomato cultivars revealed their differential regulation. None of the studied transcripts from cv. Marglobe showed up regulation, and all were down regulated as their fold change in expression varied from one fold (chromatin remodelling complex protein) to 20 fold (high affinity glucose transporter) (Fig. 7). However, the expression levels of transcripts from cv. Red Defender revealed differential regulation as three among the studied transcripts showed up regulation (transcript encoding Cc-nbs-lrr, resistance protein, AP2-like ethylene-responsive transcription factor, and heat stress transcription factor A3).

Multiple TSWV-derived siRNAs were found to be targeting a tomato mRNA (Solyc12g017800). Although, this transcript was found to be downregulated in cv. Marglobe, the transcript from cv. Red Defender showed upregulation. Similarly, two more transcripts encoding ER-TF(Solyc11g008560.1.1) and HS-TF (Solyc02g072000.2.1) were also found to be upregulated in cv. Red Defender. Considering the location of vsiRNA target sites in these mRNA, all the three upregulated transcripts in Red Defender, were found to have vsiRNA target regions at the 5' end of tomato genes. Eventhough host mRNA targets were selected based on psRobot scores (lower the score better the chance for downregulation), the

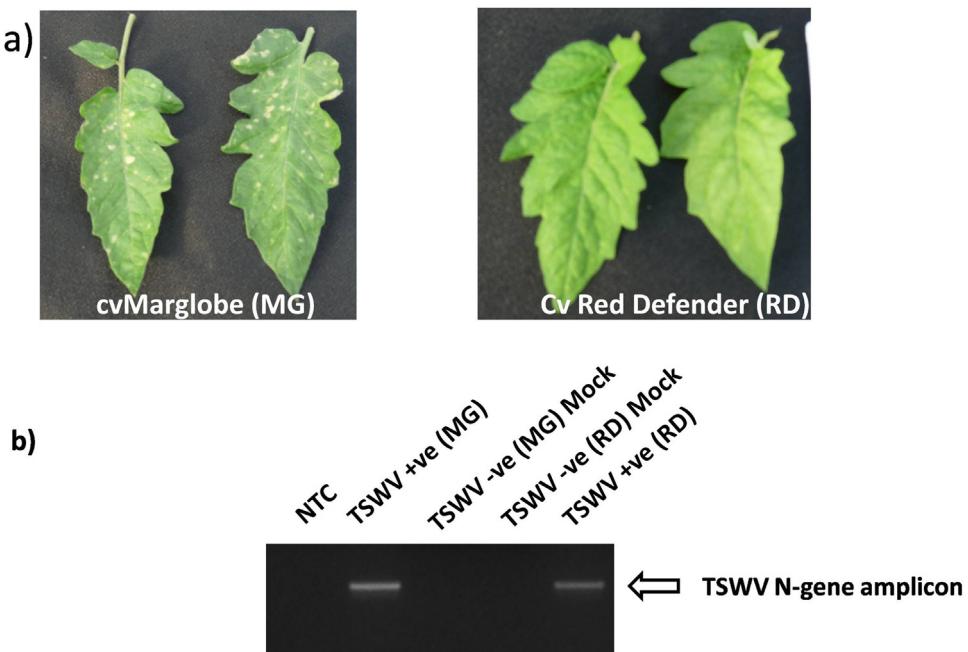


Fig. 6. Mechanical inoculation of tomato spotted wilt virus in tomato cultivars and RT-PCR detection.

a) Uninoculated, younger leaves of tomato cultivars mechanically inoculated with tomato spotted wilt virus and appearance of typical symptoms 17 days post inoculation (17dpi) in cultivar Marglobe whereas cultivar Red Defender showed no discernible symptoms associated with TSWV infection.
 b) RT-PCR amplification of tomato spotted wilt virus(TSWV) nucleocapsid gene (N-gene) with total RNA template extracted from mock and TSWV inoculated tomato leaves using sequence specific primers (NTC: No template negative control; TSWV +Ve: TSWV presence in inoculated plants; TSWV -ve: Absence of TSWV in mock-inoculated plants where RD: cv Red Defender; MG: cv Marglobe)

level of repression of host transcripts did not correlate with the scores (Table 1 and Fig. 7).

3.6. RACE validation of predicted targets

In this study we used 5' RACE technique to map the cleavage sites of the predicted target genes in tomato. All the target tomato genes were confirmed as real targets of TSWV derived vsiRNAs in the cv. Marglobe. In Marglobe, RACE analysis yielded amplicons of expected size for all ten tomato genes under study. However, among the target transcripts analyzed in cv. Red Defender, mRNAs corresponding to genes Solyc12g017800.1.1 (cc-nbs-lrr), Solyc11g008560.1.1 (ER-TF), Solyc02g072000.2.1 (HS-TF) did not show fragments of expected size in RACE PCR (Fig. 8). Sequence alignment of TSWV-derived siRNAs and target tomato mRNAs followed by RACE validation showed that in seven out of 10 tomato mRNAs studied, the sites of vsiRNA-guided cleavage in target mRNAs were found in the expected position i.e., between the tomato mRNA nucleotides pairing to vsiRNA residues 10 and 11 (Fig. 8). In tomato cv. Marglobe, the locations of vsiRNA-guided cleavage of three mRNAs (cc-nbs-lrr, HAGT, and ER-TF) were not on the expected locus, whereas in cv. Red Defender, mRNAs cc-nbs-lrr and ER-TF showed no cleavage at all (Fig. 8).

4. Discussion

Viruses, as obligate parasites, are dependent on the host plant's metabolism to successfully establish pathogenesis. In the process of viral infection, various host gene regulatory networks and ultimately the metabolic pathways are entirely perturbed (Hull, 2013). Here we examined the effect of highly frequent vsiRNAs derived from TSWV on tomato transcriptome. We analyzed population of high copy number 21–24nt vsiRNAs for its proclivity to target tomato transcriptome even though 24 nt siRNAs are not implicated in post transcriptional gene silencing (PTGS).

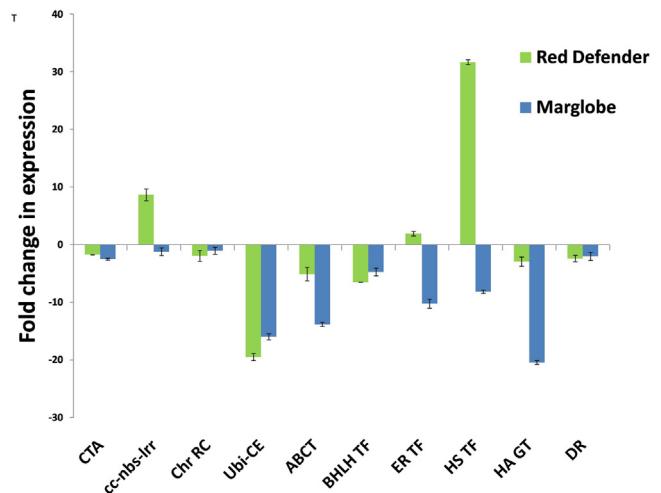


Fig. 7. Quantitative RT-PCR (qRT-PCR) validation of the relative expression levels of selected target transcripts from two different tomato cultivars- Red Defender and Marglobe. Y- axis indicates the signal intensity of each of the target transcript as normalized with reference to Ubiquitin gene. The selected target transcripts that showed propensity for down regulation in bioinformatics analysis are CTA- Calcium-transferring ATPase, cc-nbs-lrr- Cc-nbs-lrr, resistance protein, Chr RC- Chromatin remodelling complex subunit, Ubi-CE Ubiquitin-conjugating enzyme 13 E2, ABCT- ABC transporter FeS assembly protein SubfB, BHLH TF- BHLH transcription factor, ER TF- AP2-like ethylene-responsive transcription factor, HS TF- Heat stress transcription factor A3, HA GT- High-affinity glucose transporter, DR- Dehydration-responsive family protein. Error bars indicate the standard deviations of qRT-PCR signals.

The small non-coding RNAs derived from TSWV genome exhibited sequence complementarity to host transcriptome and hence exhibited potential to down regulate respective transcripts. Considering that the selected vsiRNAs have been mapped to the entire genome of TSWV, coupled with their high numbers, it is expected

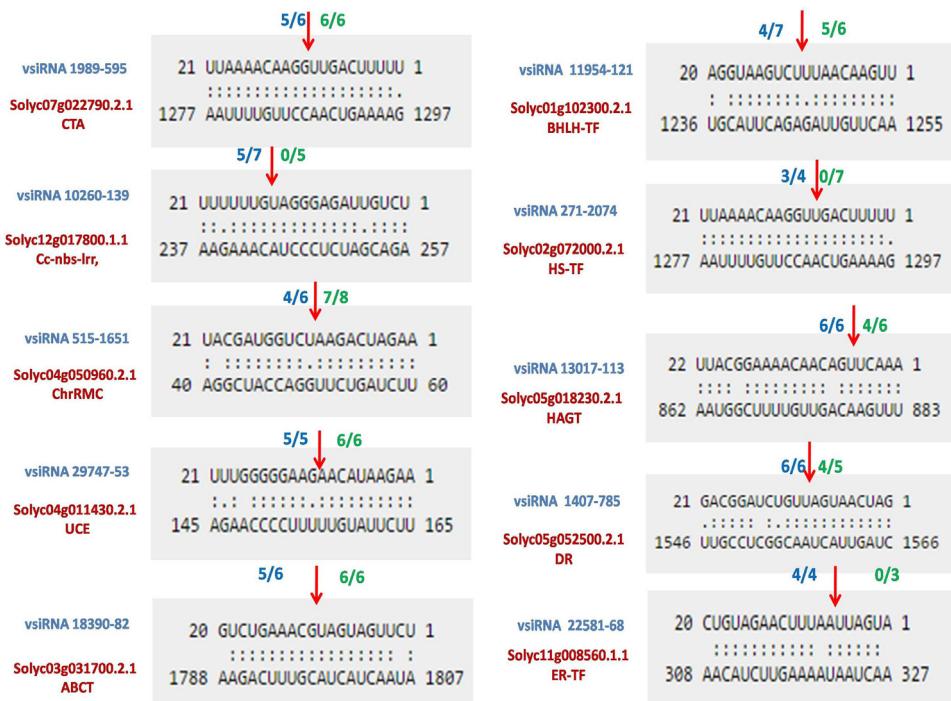


Fig. 8. Validation of vsiRNA directed cleavage of target transcript(s) by performing 5' RACE studies. Watson-crick base pairing of TSWV derived siRNA and tomato target transcripts. The point of cleavage of target mRNA is represented by a red arrow. Values besides arrow represent number of clones that confirmed the point of cleavage after sequencing the RACE products (Blue: cv. Marglobe and Green: cv. Red Defender). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

that many of the host mRNAs might have siRNA complementary binding sites. We used the small RNA target prediction algorithm, psRobot, to map vsiRNA targets on the complete transcriptome of tomato. The critical resource of tomato gene annotations (ITAG 2.3) was used to identify mRNAs that are potential targets for vsiRNA-induced silencing. Results showed that the tomato transcriptome displayed many potential targets as almost all the aspects of basal metabolic processes are affected. Among the various transcripts, those encoding enzymes are targeted profusely followed by transcription factors, membrane-associated and structural proteins. Further, basal metabolic pathways from carbon metabolism to secondary metabolite synthesis to ubiquinone or terpenoid biosynthesis are potential targets for downregulation mediated by TSWV derived siRNAs. Previous reports showed that genes involved in secondary metabolite biosynthesis are downregulated upon TSWV infection in tomato (Catoni et al., 2009) which is in accordance with our *in silico* observed repression. In view of the co-existence of vsiRNAs and potential mRNAs that exhibit sequence complementarity to TSWV derived siRNAs in the same cellular pool, cross reaction is possible. However, not all the transcripts are potential vsiRNA targets at a given point of time despite the constant presence of vsiRNAs in cytoplasm because of temporal differences in the expression of host mRNAs. Hence, it cannot be ruled out that only a subset of cellular mRNAs could be target for vsiRNAs at any given moment. Downregulation of target transcripts in tomato cv. Marglobe was confirmed by qRT-PCR and 5'RACE analysis suggesting a direct action of TSWV-derived siRNAs on tomato transcripts. In the absence of degradome sequencing analysis to identify end-products of sRNA-mediated target cleavage, unequivocal demonstration of vsiRNA-mediated off-target activity is pending. However, results of target transcript quantitation and mapping of cleavage sites in target transcripts using RACE-PCR demonstrated the high propensity for vsiRNA-mediated target cleavage.

Among the selected target transcripts, none was found to be over-expressed in cv. Marglobe compared to the control, whereas transcripts involved in biotic and abiotic stress gene regulation such as cc-nbs-lrr, ethylene responsive TF and heat responsive TF were found to be upregulated in cv. Red Defender. The cv. Red Defender carries the TSWV resistant gene *Sw-5* that confers resistance to tomato against TSWV and the resistance manifests as hypersensitive reaction (HR). Findings in the present study also suggest that genes involved in secondary metabolite synthesis such as polyamines could be potential targets for vsiRNAs. The molecular connection between HR and polyamine synthesis was previously reported (Takahashi et al., 2004), however, the roles of vsiRNAs in this molecular network remain to be elucidated. Despite potentially being targeted by multiple vsiRNAs, the cc-nbs-lrr transcript remained upregulated in the cv. Red Defender. Similarly, heat stress transcription factor (HS-TF) and ethylene-responsive transcription factor (ER-TF), though potentially targeted by 10 and 5 TSWV-derived siRNAs respectively, remained upregulated. Interestingly, all the three upregulated potential target genes showed vsiRNA target regions at the 5' end of tomato transcripts. Furthermore, seven of the target tomato mRNAs showed vsiRNA-guided cleavage points between position complementary to 10th and 11th nucleotides of vsiRNA (Elbashir et al., 2001; Shimura et al., 2011; Miozzi et al., 2013). Strikingly, two upregulated target mRNAs in cv. Red Defender displayed cleavage sites beyond the expected locus (Fig. 8). In addition, differential processing of vsiRNAs by RNA silencing machinery of tomato cultivars could influence the observed differential dynamics of vsiRNA-mediated gene regulation. Thus, besides sequence complementarity, factors such as the position of the vsiRNA target in mRNA, secondary structural features of the target mRNA, differential processing of viral RNAs into siRNAs could also play a significant role in small RNA-targeted gene silencing pathways. It is possible that genes might be regulated at multiple levels other than post-transcriptional level. Our findings also warrant investigations into the molecular cross-talk between

abiotic stress signalling and biotic stress regulation in tomato transcriptome.

Virus and viroid-derived siRNAs have been implicated in negative regulation of host mRNAs and in turn leading to the production of typical symptoms associated with disease in several instances (Catalano et al., 2012; Shimura et al., 2011; Smith et al., 2011; Navarro et al., 2012). However, earlier studies considered the vsiRNAs as pathogenicity determinants, and as such, the development of particular symptoms due to infection have been attributed to the off-target effects of vsiRNAs on the host transcriptome. Here, we presented the global transcriptome analysis of tomato with respect to TSWV-derived siRNAs. The findings presented here are consistent with the previous studies where it has been shown that virus or viroid-derived siRNAs effectively silence the host transcripts (Shimura et al., 2011; Smith et al., 2011; Angell and Baulcombe, 1997; Qi et al., 2009; Miozzi et al., 2013; Wang et al., 2016; Xia et al., 2016).

A study involving sugarcane mosaic virus (SCMV) and maize mRNAs revealed the cross-silencing of host transcriptome by SCMV-derived siRNAs (Xia et al., 2014). SCMV siRNAs were found to potentially target transcripts encoding proteins involved in biogenesis of ribosomes, biotic and abiotic stresses thus targeting various physiological pathways of the host (Xia et al., 2014). Similarly, vsiRNAs obtained from a crucifer-infecting isolate of tobacco mosaic virus (TMV-Cg) were predicted to target multiple host factors such as TFs, and proteins involved in RNA processing and defense (Qi et al., 2009). Direct evidence for vsiRNA-mediated host mRNA cleavage was obtained for target mRNAs encoding polyadenylation specificity factor and translocon-associated protein (Qi et al., 2009). vsiRNA populations derived from grapevine fleck virus (GFKV) and Grapevine rupestris stem pitting-associated virus (GRSPaV) were shown to target *Vitis vinifera* derived transcripts involved in ribosome biogenesis, biotic and abiotic stresses besides their role in plant's defense mechanism by targeting viral transcripts (Miozzi et al., 2013). The down regulation of tomato target genes (*SolWD40-repeat*) by pospiviroid-derived small RNAs was reported during viroid infection in tomato (Avina-Padilla et al., 2015).

Similarly, vsiRNA profiles of cotton leaf curl Multan virus (CLCu-MuV) and cotton leaf curl Multan betasatellite (CLCuMuB) from infected upland cotton plants, were characterized and potential vsiRNA target host genes were identified to be TFs involved in biotic and abiotic stresses (Wang et al., 2016). Furthermore, the role of vsiRNAs in promoting viral infection was also shown using virus induced gene silencing (VIGS) and 5' RACE analysis (Wang et al., 2016). Putative target genes for vsiRNAs produced during synergistic infection of maize chlorotic mottle virus (MCMV) and sugarcane mosaic virus (SCMV) in maize revealed that categories of host target genes were similar spanning metabolic process, transcription regulation, among others (Xia et al., 2016).

The study of viral genome-derived small RNAs provided important insights into virus-host interactions (Ramesh et al., 2015). The possibility of sRNA-mediated downregulation of cognate mRNA has been demonstrated with the use of 21nt virus-derived sequences as effector molecules of silencing in artificial miRNA (amiRNA)-mediated antiviral resistance (Niu et al., 2006; Fahim and Larkin, 2013). Furthermore, the entire edifice of functional genomics approach such as VIGS is based on the observation that the host mRNAs are silenced in *trans* by vsiRNAs derived from recombinant viruses (Becker and Lange, 2010). RACE studies further corroborated the qRT-PCR findings that the tomato-derived mRNAs were indeed cleaved due to the activity of TSWV-derived vsiRNAs. All the tomato target mRNAs extracted from the cv. Marglobe analyzed for vsiRNA-based cleavage, showed accumulation of specific products in the RACE experiments. However, the three differentially expressed genes in cv. Red Defender (cc-nbs-lrr, ER-TF and HS-TF) did not show accumulation of cleavage-specific prod-

ucts upon TSWV infection. Biological implication of TSWV-tomato interaction at the small RNA interface could be explained based on the expression pattern of the NBS-LRR class resistance genes. Expression of NBS-LRR genes was found to be controlled by *cis* and *trans* acting elements including secondary siRNAs and miRNAs (Shivaprasad et al., 2012). Among the three upregulated genes in cv. Red Defender, expression levels of nbs-lrr mRNA could be a major factor that determines the ability of tomato to resist TSWV infection. Functional genomics approach such as VIGS could be useful for investigating the biological significance of nbs-lrr class genes and to substantiate their role in TSWV resistance.

We propose that resistance or susceptibility of tomato to TSWV infection hinges on the cultivar's ability to overcome vsiRNA-mediated silencing of NBS-LRR gene. Besides, RNA silencing and R-gene mediated resistance mechanism that functions on the principle of hypersensitive reaction, it is possible that the activation of stress responsive gene signalling pathways would also play a major role in conferring TSWV resistance in tomato. Thus it appears that resistance to TSWV in tomato works at multiple levels of gene regulatory pathways. Further, we speculate that an effective molecular cross-talk among the biotic and abiotic stress signalling components and TFs shifts the 'balance of power' in favor of the resistant host.

5. Conclusion

TSWV-derived vsiRNAs were found to target a wide range of host genes involved in various metabolic pathways. The predicted and validated interactome scenario presented here is the first report on the interaction between a tospovirus genome-derived siRNAs and tomato transcripts. The broad spectrum of host targets for vsiRNAs signifies the importance of vsiRNAs not only as pathogenicity determinants but also as major determinants of virus-host interactions. It highlights the significance of the role of vsiRNAs in viral replication, pathogenicity and in perturbing host machinery. It remains to be seen if the cross reaction potential of vsiRNAs with host transcripts is merely an act of fortuitous pairing and down regulation of cognate transcripts, or is a systematic counter-offensive strategy deployed by virus to shift the virus-host 'tug of war' toward the pathogen's favour. Furthermore, it is tempting to speculate that the differential behaviour of tomato cultivars to TSWV infection could be due to its ability to overcome vsiRNA-based off-target gene silencing of host transcripts especially NBS-LRR class R-genes.

Conflict of interest

The authors declare that they have no competing interests.

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