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Identification and validation of a virus-inducible ta-siRNA-generating TAS4 locus in tomato

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Trans-acting small interfering RNAs (ta-siRNAs) are a class of endogenous small RNA, associated with post-transcriptional gene silencing. Their biogenesis requires an initial microRNA (miRNA)-mediated cleavage of precursor RNA. Around 20 different ta-siRNA-producing loci (TASs), whose sequences are conserved, are reported in plants. In tomato, two TAS gene families have been identified, which are found to target auxin response factor gene and bacterial spot disease resistance protein Bs4 gene. Using high-throughput computational and experimental approach, we identified a new locus-producing ta-siRNA in tomato. We have also identified the putative miRNA regulating the production of ta-siRNA from this locus. The ta-siRNAs generated from *TAS4* were up-regulated upon infection with a DNA virus. The potential targets of ta-siRNAs were predicted to be variety of proteins including MYB transcription factors and cell cycle regulators for some of the ta-siRNAs produced.

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1. Introduction

Small RNAs have been classified into three main categories: short interfering RNAs (siRNAs), microRNAs (miRNAs), and piwi-interacting RNAs (piRNAs), based on their origins, structure, associated effector proteins and biological roles (Ketting 2011), which are important in regulating gene expression. Trans-acting siRNA (ta-siRNA) are a class of endogenous siRNA, which are produced from *tas* loci that act in post-transcriptional RNA silencing pathways to silence target RNA transcripts having sequence complementarity. The biogenesis of ta-siRNA starts with cleavage of its precursor by miRNA, followed by RDR6-mediated production of double-stranded RNA. This is then subsequently diced by DCL4 to produce phased ta-siRNA (Peragine *et al.* 2004; Allen *et al.* 2005). Ta-siRNAs are functionally similar to siRNAs in that they regulate the expression of genes to which they have significant complementarity (Yoshikawa *et al.* 2005). In tomato, four *tas* loci, produced by two gene families *tas3* (Yifhar *et al.* 2012) and *tas5* (Li *et al.* 2012) respectively, have been identified. *Tas 1/2/4* and the miRNAs regulating these gene families have not been reported in tomato yet. The ta-siRNAs produced from miR390-TAS3 regulate genes coding for auxin responsive factors (Williams *et al.* 2005; Fahlgren *et al.* 2006; Xia *et al.* 2012) and those made from *tas5* locus potentially target the resistance gene *Bs4*, a tomato gene conferring resistance to bacterial spot disease (Li *et al.* 2012).

Geminiviruses are single-stranded plant-infecting DNA viruses belonging to the Family Geminiviridae, whose members have characteristic twinned icosahedral particles. Geminiviruses are transmitted by the polyphagous insect whitefly (*Bemisia tabaci*) and infect a variety of crops, both monocot and dicot, causing severe yield losses, mainly in the tropical and sub-tropical regions of the World. The genus Begomovirus has the largest number of

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members within Geminiviridae, which can contain either a single (monopartite) or two (bipartite) DNA molecules as the genome (Borah and Dasgupta 2012). Monopartite genomes are homologous to the DNA-A of bipartite geminivirus, and all the viral factors required for encapsidation, viral replication, transmission, and systemic spread are encoded on this genome component. In bipartite begomoviruses, DNA-B encodes for proteins responsible for the viral movement (Ahmed *et al.* 1991). *Tomato leaf curl New Delhi virus* (ToLCNDV) is a bipartite begomovirus infecting tomato, mainly in the Indian subcontinent and causes upward curling of leaves and swelling of veins, resulting in heavy losses to tomato production (Sohrab *et al.* 2003). Tomato-infecting begomoviruses bring about a number of changes in its host ranging from the activation of DNA replication machinery in differentiated cells (Hanley-Bowdoin *et al.* 1999) to subversion of plant defence responses (Jackel *et al.* 2014). Virus infection in plants is one of the primary causes of changes in the pattern of small RNAs of the host. The changes mediated by ToLCNDV in tomato microRNAs have already been reported (Pradhan *et al.* 2015). However, there is no report yet of any virus-inducible *tas* locus in plants.

In this article, we describe a *TAS4* locus in tomato, which was indicated while comparing the small RNA deep sequencing data of healthy tomato plants and of tomato plants infected with ToLCNDV for known *TAS* loci. ToLCNDV was found to induce the expression of *TAS4*. In other plants, miR828 is responsible for initiation of TAS4 phasing, and we found miR828 to be present in tomato. We also identified the phased siRNA produced from the *TAS4* locus as a result of the initial cleavage of TAS-mRNA by miR828, based on small RNA deep sequencing data of tomato (Pusa Ruby). We show the ToLCNDV-inducible nature of *TAS4* by using both *in silico* methods and by Northern analysis.

2. Materials and methods

2.1 *Growth conditions of plants and virus inoculation*

Tomato (cultivar Pusa Ruby) plants were grown at 25±2°C under 16 h/8 h light/dark conditions in green house. For virus inoculation, agrobacterium cells LBA4404, harbouring ToLCNDV DNA-A and DNA-B (Pratap *et al.* 2011) were grown till OD_{600} of 1.0 and centrifuged to collect the cells, which were then re-suspended in an equal volume of MES buffer containing 200 μM acetosyringone. It was kept at 28°C for 1 h and around 4 mL of the mixture was infiltrated according to a previously described protocol (Singh *et al.* 2014) on the abaxial surface of the top leaf of tomato plants in the six-leaf stage.

2.2 *Computational prediction of miR-828 and tas4 locus in tomato*

To detect miR828, its sequence was searched in the genomic sequence of tomato variety Pusa Ruby. Bowtie, which is a tool for mapping short reads onto a genome (Langmead *et al.* 2009) was used to map the miR828, in the tomato genome. About 300 nucleotides flanking the sequence of miR828 was retrieved from the tomato genome and the secondary structure of precursor was obtained using RNA-fold with default parameters (Lorenz *et al.* 2011). The passenger strand was retrieved based on secondary structure. The presence of the mature and the passenger strand sequence of miR828 was checked in the tomato Sol-genomics database (Fei *et al.* 2011) and sRNA deep sequencing library derived from healthy control file (HT) and ToLCNDV-infected (IT) tomato leaves (obtained from Gene Expression Omnibus (GEO) with the accession no. GSE53253 (*http://www.ncbi.nlm.nih.gov*). Similar analysis was performed in *Cucumber mosaic virus* (CMV)-infected tomato plants (accession no. GSM1372424, *http://www.ncbi.nlm.nih.gov*). The target of miR828 was searched against unigene library of tomato genome using Tomato Functional Genomics Database (Fei *et al.* 2011). The presence of consequent phased 21-nt siRNAs from both the strands was searched in HT and IT files as well as sol-genomics database (Fei *et al.* 2011). Targets of 21-nt siRNAs were predicted against Tomato ITAG cDNA v2.3 library in Tomato Functional Genomics Database (TFGD) using default parameters.

2.3 *Northern hybridization*

Total RNA was isolated from tomato plants (Pusa Ruby) inoculated with ToLCNDV, 30 days post-inoculation (dpi), from newly emerged leaves and from mock-inoculated plants. TRIZOL reagent (Invitrogen) was used for the RNA isolation step, following the manufacturer's protocol. About 100 μg of total RNA was resolved in 12% denaturing urea polyacrylamide gel. RNAs were transferred to nylon membrane (Hybond N, Amersham) by electro-blotting at 0.8 Amp for 40 min using Trans-Blot SDsemi-dryelectrophoretictransfercell (Bio-Rad). The membranes were cross-linked by a UV cross linker. Hybridization was carried out at 37°C using hybridization incubator (Techne) for 12 hours. The reverse compliment oligonucleotide of the siRNA to be detected (table 1) and miR828 were synthesized commercially (SIGMA-Aldrich) and used as a probe. Each probe was end-labelled with T4 polynucleotide kinase (Fermentas) using 4000 Ci/mmol [γ-^{32}P]ATP (PerkinElmer Life Sciences, USA) followed by purification through a G25 column (GE Healthcare Life Sciences, UK) according to

Table 1. Candidate siRNAs and their abundance, identified from novel TAS4 locus in Healthy (HT) and ToLCNDV-infected (IT) Tomato Small RNA Libraries

ta-siRNA	Strand	Normalised abundance in healthy(HT) file$ (1×10^{-7})	Normalised abundance in infected(IT) file$ (1×10^{-7})	Presence of read in TFGD*
Positive				
D1(+)	TTGAGCAAGAAAGTCAGAGTT	absent	53	yes
D2(+)	AGTAAGACGATGAAGGTACGA	20	36	yes
D3(+)	GGATGATGTTGTTTTAACCAA	absent	absent	yes
D4(+)	CCTCAACCTCGGACCTTCATC	4	absent	Yes
D5(+)	TATAACGTCAATCATTACGCA	absent	403	Yes
D6(+)	CGACGAAGCGTTCATCACCTC	absent	83	Yes
D7(+)	TATTTCACATAATATTTCACT	absent	absent	No
D8(+)	CAAAGAAAATTTTGTGCATGG	absent	absent	Yes
D9(+)	CCCAAAGACAACATGAAGTTT	absent	absent	Yes
D10(+)	GTTAATTGGAACATTCATTTG	absent	absent	No
Negative				
D1(−)	CTCTGACTTTCTTGCTCAAAT	absent	absent	No
D2(−)	GTACCTTCATCGTCTTACTAA	359	418	Yes
D3(−)	GGTTAAAACAACATCATCCTC	4	18	Yes
D4(−)	TGAAGGTCCGAGGTTGAGGTT	161	252	Yes
D5(−)	CGTAATGATTGACGTTATAGA	absent	absent	No
D6(−)	GGTGATGAACGCTTCGTCGTG	4	27	Yes
D7(−)	TGAAATATTATGTGAAATAGA	absent	absent	No
D8(−)	ATGCACAAAATTTTCTTTGAG	absent	27	Yes
D9(−)	ACTTCATGTTGTCTTTGGGCC	absent	18	Yes
D10(−)	AATGAATGTTCCAATTAACAA	absent	absent	No

$ Files obtained from Gene Expression Omnibus (GEO) with accession no. GSE53253.

*TFGD: Tomato Functional Genomic Database.

the supplier's protocol. The membranes were autoradiographed using a TYPHOON phosphor imager (GE Healthcare Life Sciences, UK).

3. Results

3.1 *Detection of miR828 in tomato*

In a search for loci producing tasiRNA in tomato, we scanned the genome for phased clusters of ~21-nt reads using PERL script. With this procedure, along with the previously known TAS loci we found additional locus, *TAS4*, mapping on *Solanumly copersicum* genome version 2.5, chromosome 5, between 3809600 and 3810050. Because miRNA-directed cleavage sets the phase for production of tasiRNAs (Allen *et al.* 2005) we searched for miRNA complementary sites upstream and downstream of the *TAS4* predicted locus and identified a single miR828 complementary site on chromosome number 10 at position 3437989 and 3737941 respectively on the antisense strand and both mature (AGATACTCATTTGGGTAAGAAG) and star (TCTTGCTCAAATGAGTATTCCA) sequences of tomato miR828 was further found to be present in infected file using Bowtie similarity search. The mature miRNA are formed by cleavage of precursor miRNA (pre-miRNA) by the action of dicer or dicer-like proteins (Allen *et al.* 2005). Only one precursor form of miR828 was found in tomato. When compared with previously reported miR828 sequences, the mature miRNA sequence from tomato was identical to ppe-miR828b (accession no. MI0021619) and mdm-miR828a (accession no. MI0023133) reported from peach and apple respectively, but showed single nucleotide difference with ath-miR828 reported from arabidopsis (accession no. MI0005384) and vvi-miR828b reported from grapevine (accession no.MI0007972; figure1A). To check the predicted secondary structure of the pre-miR828, the most likely structure of the sequence was

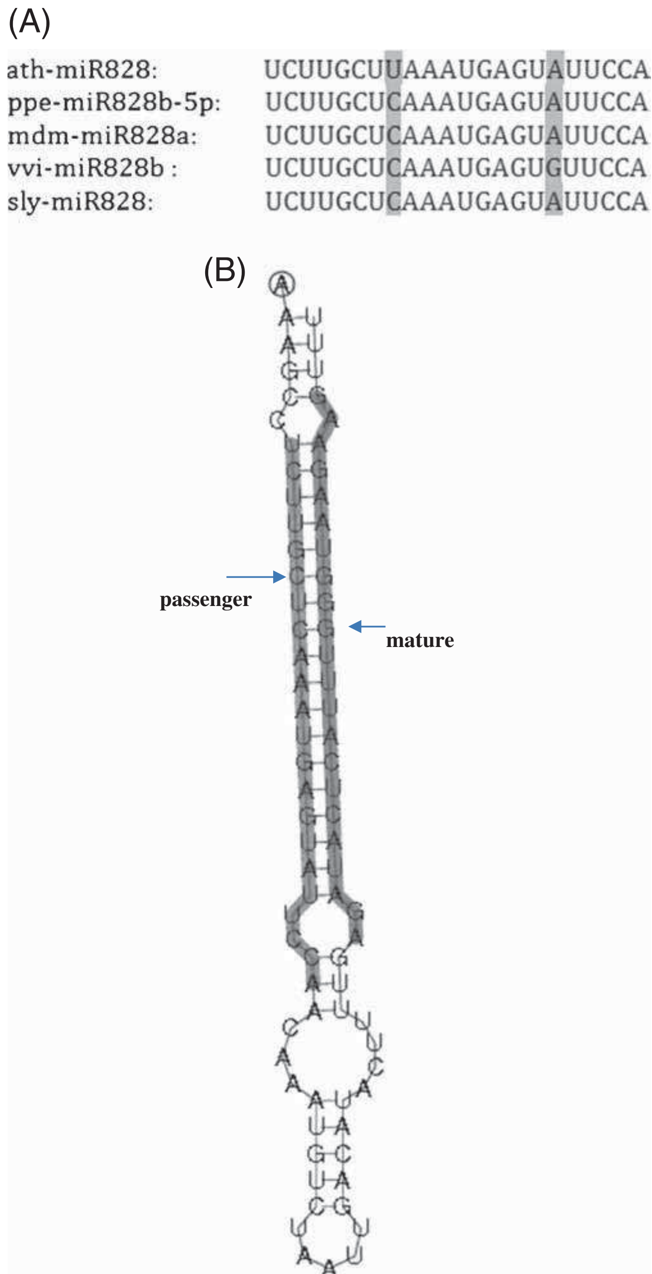


Figure 1. Sequence and structure of miR828. (**A**) Comparison of miR828 sequences in Arabidopsis, peach, apple, grapevine and tomato and (**B**) structure of tomato miR828, indicating the passenger and mature sequences.

predicted and it folded into a stem loop secondary structure with free energy of 84.74 kcal/mol (figure 1B), which is considered to represent an energetically stable structure. To detect mature miR828 in publicly available tomato small RNA databases, all miRNA sequences were retrieved from miR-base (Griffiths-Jones *et al.* 2008). The presence of miR828 mature and passenger strand was then checked in both HT and IT Pusa Ruby tomato files (as mentioned above). MiR828 mature and passenger strand was present in the IT file with normalized abundance (12×10^{-7} and 6×10^{-7} respectively), but was absent in HT files. The presence of both mature and passenger strands was also checked in the Tomato Functional Genomics database (Fei *et al.* 2011), which contains the small RNA sequencing reads of tomato under various growth conditions and from various tissues, in which both mature and passenger stands were detected. To determine whether miR828 is induced upon viruses other than ToLCNDV, *in silico* analysis was performed using deep sequencing RNA data of tomato plant infected with CMV. The analysis indicated that miR828 was not induced in tomato after CMV infection.

Following agroinoculation of cloned ToLCNDVDNAs in tomato plants, symptoms typical of virus infection started to appear at 21 days post-inoculation (dpi), including upward curling of leaves and stunting of growth (figure 2). Mock-inoculated plants showed no symptoms and appeared no different from uninoculated plants. To detect the presence of miR828 in tomato plant, northern blot analysis was performed using total RNA from leaves of four individual mock-inoculated and ToLCNDV-inoculated tomato plants at 30 dpi. The accumulation of miR828 could be detected in most plants, and was seen to be induced approximately three-fold in ToLCNDV-inoculated plants. Figure 3A

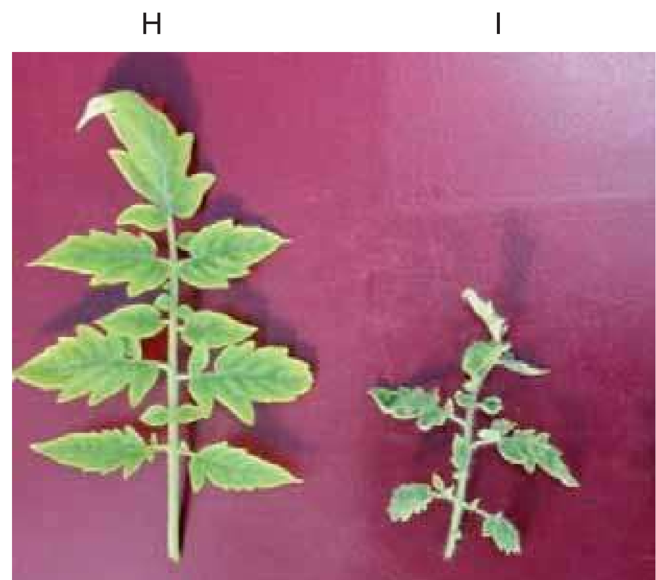


Figure 2. Tomato leaf mock-inoculated (H) and inoculated (I) with ToLCNDV at 21 days post-inoculation.

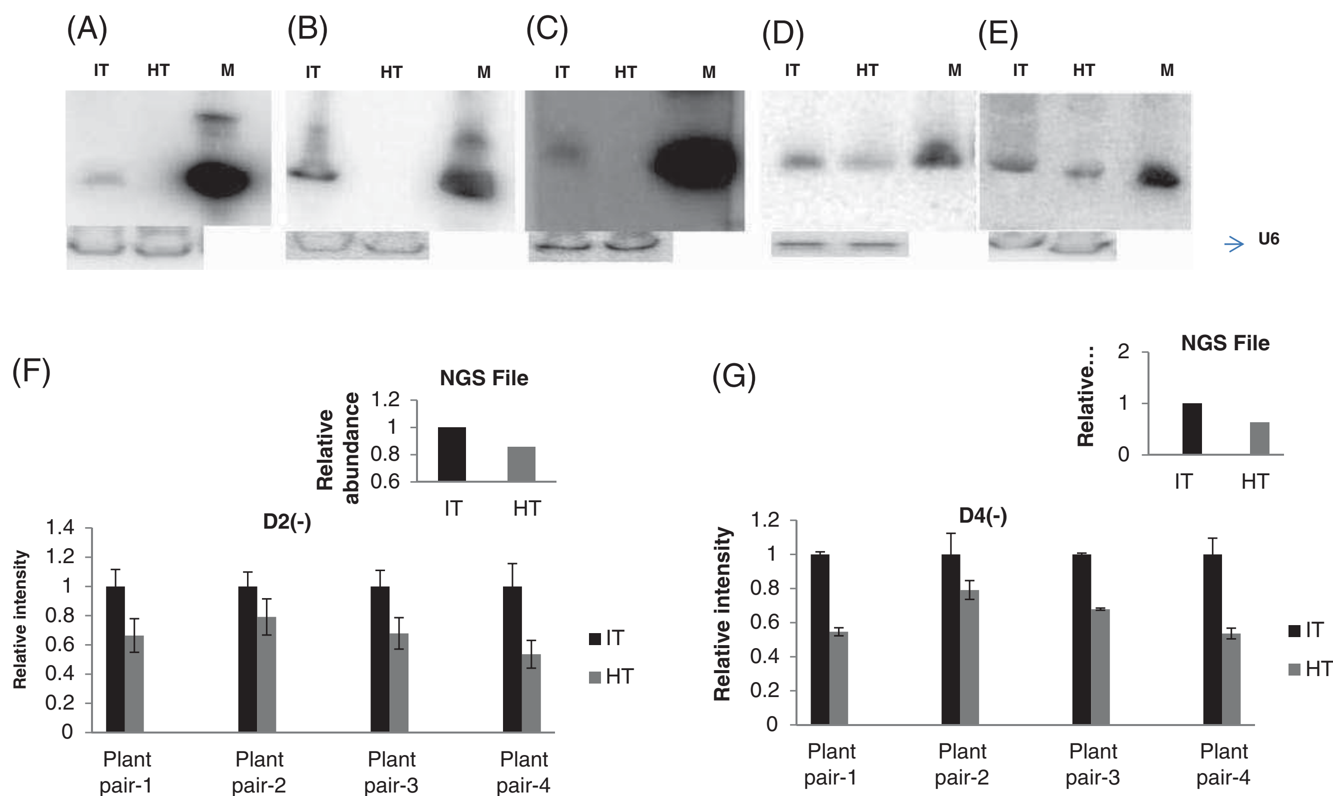


Figure 3. MiRNA and ta-siRNA related to TAS4 locus of tomato. (**A**) Northern blot for the detection of miR828 mature sequence in mock-inoculated and ToLCNDV-inoculated tomato plant with total RNA collected from leaf sample. IT and HT represent ToLCNDV-infected and mock-inoculated plant respectively. Small RNAs were detected using specific oligonucleotide probes given in table 2. (**B–E**) Northern blot for validation of ta-siRNAD5(+), D6(+), D2(−), D4(−) respectively from mock-inoculated (HT) and ToLCNDV-inoculated (IT) tomato leaf samples at 30 dpi. (**A–E**) Lanes marked M represent 21 nucleotide size marker and the loading controls are represented by small RNAs showing U6 RNA in the lower portions. U6 RNA was visualized by stripping the signal and probing the same blot. (**F**) Bar diagram showing relative intensities of siRNAD2(−) and (**G**) siRNA D4(−) in mock inoculated (HT) and ToLCNDV-infected (IT) tomato plants. Each intensity value was normalized with U6 loading control and the normalized value was set as '1' for each of infected samples. The experiment was repeated for each plant-pair and standard deviations are shown above the bar. The relative values derived from the NGS data are shown as insets for the sake of comparison.

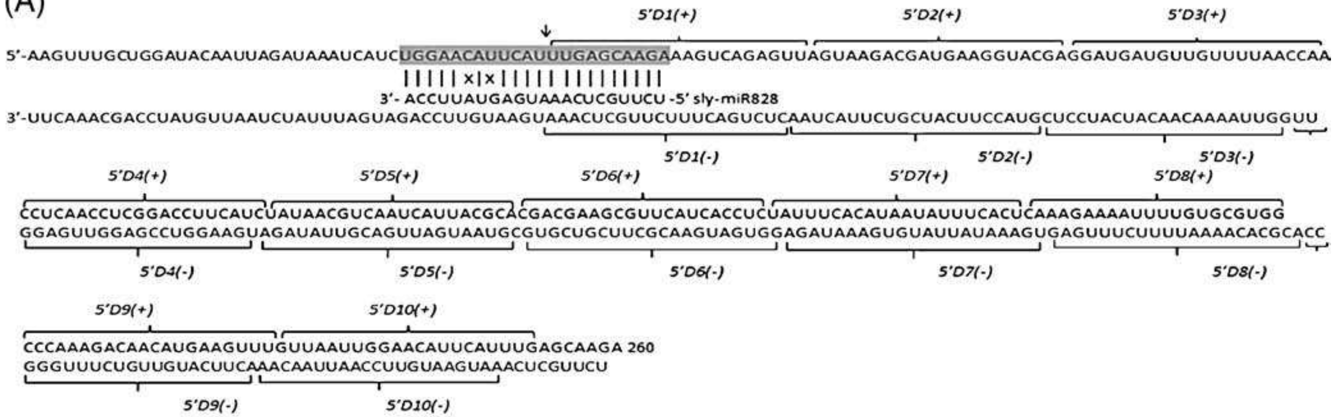
shows one representative result, where miR828 did not accumulate in the healthy sample but accumulated to a low level in the inoculated sample.

3.2 *miR828 regulates TAS4 in tomato*

It is known that miRNA cleaves target TAS-mRNA between 10th and 11th nucleotide from its 5′ site, as measured from the sequences of the first ta-siRNA generated post-cleavage (Allen and Howell 2010). If miR828 cleaves *tas4* transcript, the expected cleavage products (ta-siRNAs) should be detectable. The nucleotide sequence of the predicted TAS4 locus having miRNA828 binding site (grey region) is shown in Fig 4A. The expected cleavage site of the TAS4 mRNA by miR828 is shown by arrow and the subsequent 21-nt phased siRNAs from both positive (+) and negative (−) strand are shown by the brackets. Indeed, the *tas4*-derived ta-siRNA sequences could be detected using computational methods. Table 1 gives their sequences and their abundance.

The tomato miR828 target was similar to the target sequences in apple, peach, arabidopsis and grapevine, where TAS4 is known to be present (figure 4B).The generation of consequent phased 21-nt siRNAs, a characteristic feature of ta-siRNA, from both the strands was searched in HT as well as IT files and also in the sol-genomics database. The ta-siRNAs from the predicted TAS4 locus could be located in HT and IT files (table 1).

The ta-siRNAs accumulation was also examined using Northern analysis. Ta-siRNAs D5(+), D6(+), D2(−) and D4(−) were visible as distinct bands of approximately 21



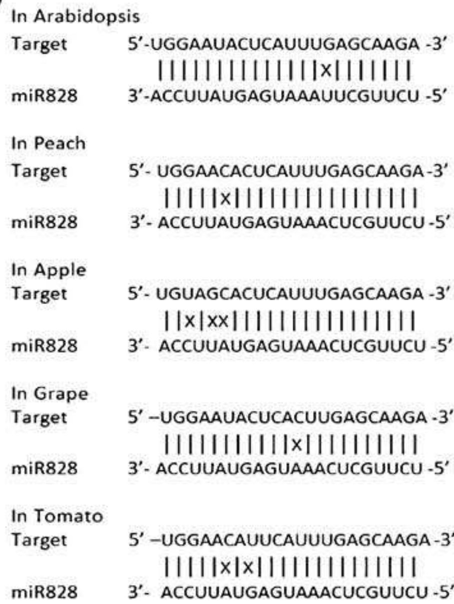


Figure 4. Structure and comparison of targets of *TAS4.* (**A**) Diagrammatic representation of TAS4, the 21 nt phased ta-siRNAs relative to miR828 cleavage site are indicated by brackets; (+) and (−) represent sense and antisense strand respectively arrow shows the cutting site of miRNA, highlighted region represents target of miRNA828 in which 'X' shows mismatch nucleotides. (**B**) miR828 target comparison in different plants, where '|' and 'X' shows matched and mismatched nucleotides respectively.

nucleotides (figure 3B–E).For each Northern, corresponding reverse complement sequence of tasiRNA sequence was used (table 2). The comparative intensities of the signals showed up-regulation of D2(−) and D4(−) in infected samples whereas siRNA D5(+) and D6(+) were not detected in virus-infected samples. To determine whether the inducibility of TAS4-derived tasiRNAs by ToLCNDV was reproducible, the Northern analysis was repeated in four individual mock-inoculated and ToLCNDV-inoculated samples. The comparative abundance of D2(−) and D4(−) are shown in figures 3F and 3G respectively, which varied, but showed approximately two-fold increase in the infected samples. The TAS4-derived ta-siRNAs were not found to be up-

Table 2. Sequence of oligonucleotides used as probes

Oligo name	Sequence (5′-3′)
miRNA828	CTTCTTACCCAAATGAGTATCT
D5(+)	TGCGTAATGATTGACGTTATA
D6(+)	GAGGTGATGAACGCTTCGTCG
D2(−)	TTAGTAAGACGATGAAGGTAC
D4(−)	AACCTCAACCTCGGACCTTCA

regulated, when small RNA data of CMV-infected tomato was analysed.

3.3 *Target prediction*

The targets of TAS4-associated ta-siRNA in tomato were predicted using Tomato Functional Genomics Database (Fei *et al.* 2011). A wide variety of targets, such as transporter proteins, F-box family proteins, auxin response factors, pentatripeptide repeat containing protein, calmodulin-binding transcription activator and LRR receptor like serine/threonine protein kinase were predicted. One of the siRNAs [D4(−)] targeted MYB gene transcript and other cell cycle regulating factors, such as S-phase cyclin A-associated protein, spindle and kinetochore associated protein 1, kinase family protein and kinesin-like protein as shown in table 3.

4. Discussion

Transcripts formed from TASloci have complementary sites for miRNA, which are essential for their maturation, one in the case of TAS1, TAS2 and TAS4 and two in the case of TAS3. Following cleavage by miRNA, phased siRNAs are produced, which are 21 nucleotides long. Although there could be several siRNAs produced from one locus, they may have varying abundance and out of the total siRNAs produced from one locus, only some may be functional

Table 3. Predicted target genes of tomato TAS4-derived ta-siRNAs

ta-siRNA	Target description	Score
TTGAGCAAGAAAGTCAGAGTT	S phase cyclin A-associated protein	2.5
	GPI inositol-deacylase	2.5
AGTAAGACGATGAAGGTACGA	RNA-binding protein	3
GGATGATGTTGTTTTAACCAA	Thiamine-repressible mitochondrial transporter THI74	2
	Receptor like kinase	2.5
CCTCAACCTCGGACCTTCATC	Subtilisin-like protease	3
	MYB transcription factor	3
TATAACGTCAATCATTACGCA	Calmodulin-binding transcription activator 1	3
TATTTCACATAATATTTCACT	LRR receptor-like serine/threonine-protein kinase	3
	Glutamate-gated kainate-type ion channel receptor subunit GluR5	3
CAAAGAAAATTTTGTGCATGG	Response regulator 10	2
	IST1 homolog	3
CCCAAAGACAACATGAAGTTT	Nodulin-like protein	3
GTTAATTGGAACATTCATTTG	Auxin response factor	3
	B3 domain-containing protein Os11g0197600	3
CTCTGACTTTCTTGCTCAAAT	ABC transporter G family member 15	3
	Structural maintenance of chromosomes family protein	3
GTACCTTCATCGTCTTACTAA	Oxidoreductase 2OG-Fe(II) oxygenase family	3
GGTTAAAACAACATCATCCTC	Transcription initiation factor TFIID subunit 10	2.5
	Phosphatidylinositol binding clathrin assembly protein	3
TGAAGGTCCGAGGTTGAGGTT	MYB transcription factor	2.5
	UDP-glucosyltransferase	2.5
CGTAATGATTGACGTTATAGA	Unknown Protein	2.5
TGAAATATTATGTGAAATAGA	Stearoyl-acyl carrier protein desaturase	2.5
	Glycosyltransferase-like protein	2.5
ATGCACAAAATTTTCTTTGAG	GPI transamidase component PIG-S-like	2
	Helicase-like protein	2.5
ACTTCATGTTGTCTTTGGGCC	Unknown Protein	3
AATGAATGTTCCAATTAACAA	F-box family protein	3
	Exostosin-like protein	3

(Axtell *et al.* 2006; Howell *et al.* 2007). Ta-siRNA are known to be conserved among different species and the presence of PpTAS4, which produces phased siRNA in *Physcomitrella patens*, indicates the presence of ta-siRNA or related mechanism also in lower organisms (Talmor-Neiman *et al.* 2006; Hsieh *et al.* 2009). To date, only four TAS loci have been found in plants (Peragine *et al.* 2004; Vazquez *et al.* 2004; Allen *et al.* 2005; Gasciolli *et al.* 2005; Xie *et al.* 2005; Yoshikawa *et al.* 2005; Rajagopalan *et al.* 2006). Besides TAS loci, there are several genomic regions, namely, protein coding and retro-transposon loci that generate phased siRNAs (Howell *et al.* 2007). These ta-si-like siRNAs act not only *in trans* but also *in cis* to keep the transposition activities under control. In addition, several loci have been reported that produce phased siRNA without miRNA binding initiation (Heisel *et al.* 2008; Zhu *et al.* 2008; Johnson *et al.* 2009) although they involve DCL4, a protein required for ta-siRNA biogenesis, and hence, are not considered canonical ta-siRNA.

TAS1/TAS2 loci are known to involve miR173 and the resulting ta-siRNAs target pentatricopeptide repeat gene transcripts (Allen *et al.* 2005). TAS3 involves miR390, which is highly conserved across various plant species, and TAS3-derived ta-siRNAs target Auxin Response Factor (ARF) transcription factor family members (ARF2, ARF3/ETT, ARF4; Garcia *et al.* 2006). MiR828 is conserved among plants and is specifically involved in the regulation of MYB transcription factors, targeting both *myb113* and *tas4* transcripts. The resulting *TAS4* ta-siRNAs regulate MYB113 and related genes, including those coding for PAP1/MYB75, PAP2/MYB90, involved in biogenesis of anthocyanin (Rajagopalan *et al.* 2006). Both *TAS4* ta-siRNAs and miR828 are found to be up-regulated in shoot by phosphorus and nitrogen deficiency in stress (Rajagopalan *et al.* 2006). MiR828 is predicted to target several *MYB* gene transcripts (Rajagopalan *et al.* 2006). It has also been reported to affect fibre development in cotton (Guan *et al.* 2014) and has been documented to be wound-inducible and to regulate lignin accumulation in sweet potato, *Ipomeabatatas* (Lin *et al.* 2012a, b). However, the role for miR828 in *MYB* gene expression and biogenesis of ta-siRNAs in the *TAS4* family is poorly understood.

Certain miRNAs in tomato (sly-miR156a, 160a, 162, 172 and 482b) are up-regulated in case of ToLCNDV infection (Pradhan *et al.* 2015). In this study we present evidence for the up regulation of miR828 inToLCNDV-infected plants. In apple, arabidopsis, grapevine and peach, miR828 is known to be responsible for the regulation of TAS4 locus (Zhang *et al.* 2013). Recent studies indicate that miR828 is induced by sugar and attenuated in abscisic acid in *Arabidopsis* (Luo *et al.* 2011) and is induced following wounding in sweet potato leaves (Lin *et al.* 2012a, b).The results presented in this study showed up-regulation of phased ta-siRNA from the TAS4 locus in ToLCNDV-infected tomato as compared with healthy plants. The high expression of ta-siRNA in the infected file compared to the mock-infected one was perhaps due to the increased abundance or the activity of miR828 following virus infection. On the other hand, miR828 and ta-siRNA were not up-regulated when mock-inoculated plant was compared with CMV-infected plant, indicating that up-regulation of TAS4 ta-siRNAs could be part of the response of tomato plants towards infection with ToLCNDV. It would be interesting to explore the ToLCNDV-specific features behind the inducibility of miR828.

Plants have several mechanisms to fight against the invading viruses. A recent report indicates that viral DNA methylation and siRNA-mediated RNA degradation are two mechanisms in conferring tolerance against ToLCNDV (Sahu *et al.* 2014). Geminiviruses are known to manipulate host DNA replication machinery. For example, *Cabbage leaf curl virus* (CaLCuV) and *Tomato golden mosaic virus* (TGMV), both have been shown to influence the respective host transcriptomes (Ascencio-Ibañez *et al.* 2008). One recent report describes some of the novel miRNAs up-regulated in ToLCNDV infection and the target of the miRNA includes AP2/ERF, CC-NBS-LRR and TCP19 proteins (Pradhan *et al.* 2015). CC-NBS-LRR proteins, which play a crucial role in pathogen recognition, had earlier been reported as being target of TAS4-derived ta-siRNAs in other plant species (Rajagopalan *et al.* 2006), which supports our prediction of such proteins being the target of TAS4-derived ta-siRNAs in tomato. Virus defence of tomato plants definitely involves multi-layered mechanisms and the TAS4-derived ta-siRNAs of tomato might also be the major participants in such defence activities.

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