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ORIGINAL PAPER

Post-transcriptional and Epigenetic Arms of RNA Silencing: A Defense Machinery of Naturally Tolerant Tomato Plant Against *Tomato Leaf Curl New Delhi Virus*

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Abstract Tomato leaf curl disease (ToLCD), caused by strains of Tomato leaf curl virus, is major constraint to tomato production globally. The present study was aimed to understand the mechanisms of ToLCD tolerance in a naturally tolerant tomato cultivar through post-transcriptional and DNA methylation-specific RNA silencing. We evaluated the distribution of virus-derived short-interfering RNAs (siRNAs) throughout the *Tomato leaf curl New Delhi virus* (ToLCNDV) genome along with DNA methylation patterns in intergenic (IR) and Rep (AC1) regions in two tomato cultivars differing in their ToLCNDV tolerance. The methylation pattern was correlated by expression analysis of key methyltransferases genes. In the tolerant cultivar, higher accumulation of viral IR-specific 24-nucleotides (nt) siRNA and AC1-specific 21-nt siRNA were found. Higher methylation levels were observed in various regions of IR. Additionally, AC1 region which facilitates binding of plant nuclear proteins was hypermethylated. DNA methylation in the key regulating region may control the expression of *AC1*, *AC2*, and *AC3* genes. Components of RNA silencing and DNA methylation machinery were found to be differentially expressed in both the cultivar of tomato at 21 dpi. Thus, we infer that both viral DNA methylation and siRNA-mediated RNA degradation play an important role in conferring tolerance against *Tomato leaf curl New Delhi virus*. Due to the inability to achieve field resistance in transgenic tomato by deploying the viral genes, targeting the viral genomic regions through RNAi technology reported here could offer an alternate defense strategy for generating transgenics to prevent yield loss.

Keywords DNA methylation · Epigenetics · Methyltransferase genes · Short-interfering RNA · Tomato · *Tomato leaf curl New Delhi virus*

Abbreviations

AC1	Replication-associated protein
AGO	Argonaute proteins
CMT3	Chromomethylase
DCL	Dicer-like proteins
DRM1/2	Domain rearrangement methyltransferase 1/2
PTGS	Post-transcriptional gene silencing
siRNAs	Small interfering RNA
TGS	Transcriptional gene silencing
ToLCNDV	*Tomato leaf curl New Delhi virus*

Introduction

Plants have developed complex gene regulatory mechanisms to cope with various environmental stresses. Recently, it was shown that DNA methylation, chromatin modulations, and small RNA-based mechanisms also contribute in regulating gene expression in response to these stresses (Boykoa and Kovalchuk 2011; Uthup et al. 2011; Grativol et al. 2012; Pattanayak et al. 2013; Sahu et al. 2013a,b). Plants have utilized the small RNA-based defense mechanisms to encounter RNA viruses and DNA virus transcripts (Muthamilarasan and Prasad 2013). This mechanism involves the production of small interfering RNAs (siRNAs) which leads to post-transcriptional gene silencing (PTGS) as well as transcriptional gene silencing (TGS; Seemanpillai et al. 2003; Bian et al. 2006). Both PTGS and TGS are initiated by the production of dsRNA from viral genome which involves the host RNA-dependent RNA polymerase (RDR). These dsRNAs are then processed by Dicer enzyme to produce siRNAs (21-nucleotides (nt) and 24-nt siRNAs) which are responsible for

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silencing viral components (Xie et al. 2004; Raja et al. 2008). Among them, 24-nt siRNAs leads to chromatin remodeling and transcriptional repression through DNA or histone methylation (Raja et al. 2010). In numerous cases, the accumulation of siRNA corresponding to different genomic regions from Geminivirus was associated with tolerance/resistance in such infected plants (Chellappan et al. 2004; Yadav et al. 2009; Sahu et al. 2010; Sahu et al. 2012; Sharma et al. 2013). In this context, analysis of the siRNA accumulation derived from different parts of the Geminivirus genome is an important approach towards understanding the host defense response to Geminivirus infection.

These aforementioned siRNA (21 nt and 22 nt) of varied sizes are incorporated into RNA-induced silencing complex (RISC) which with the help of Argonaute (AGO) protein cleaves the target viral mRNA, complementary to the siRNA. Contrastingly, AGO4, a component of RNA-induced transcriptional silencing complex (RITS), are involved in TGS which induces chromatin modification of the target viral DNA. This RNA-dependent methylation pathway involves several host methylation pathway genes which are responsible for methylation of the viral genome and its maintenance, causing transcription inhibition (Raja et al. 2010).

Recently, more specific outcomes have been reported stating that small RNA-directed methylation leads to TGS against the Geminiviruses (Raja et al. 2010). An altered replication of *Tomato golden mosaic virus* (TGMV) and *African cassava mosaic virus* was observed in protoplasts when transfected DNA was methylated before inoculation (Brough et al. 1992; Ermak et al. 1993). The mutants of *Arabidopsis* (defective in genes encoding cytosine methyltransferases, methyl cycle enzymes, and Dicer-like proteins) developed more severe viral infection than wild type (Raja et al. 2008). In another study, *Tomato leaf curl virus* (ToLCV) infection resulted in the silencing of the virus-derived transgene, but the virus escaped this defense mechanism due to the differential methylation of the viral and host plant DNA (Bian et al. 2006). Increased methylation of viral DNA has also been observed following recovery of watermelon plant from the *Cucurbit leaf crumple virus* infection (Hagen et al. 2008). A similar study also highlighted the recovery of pepper plants from *Pepper golden mosaic virus* by increased DNA methylation (Rodriguez-Negrete et al. 2009). Therefore, inter-relationship between PTGS and TGS seems to play a crucial part during plant defense against viruses (Csorba et al. 2009).

Member of the "*Geminiviridae*" family are non-enveloped viruses, containing circular single-stranded DNA (ssDNA; ~2.7 kb), mono- or bi-partite genome encapsidated inside twinned quasi-icosahedral unit. These viruses are continuously evolving to generate highly virulent strains via point mutations and recombinations (Nawaz-ul-Rehman and Fauquet 2009). Based on genome organization and biological properties, *Geminiviridae* has been classified into seven genera (i.e., *Becurtovirus*, *Begomovirus*, *Curtovirus*, *Eragrovirus*, *Mastrevirus*, *Topocuvirus*, and *Turncurtovirus*; ICTV 2012; Sahu et al. 2013a). In bipartite Geminiviruses, the genomes have two circular ssDNA, referred as DNA-A and DNA-B. Component DNA-A primarily encodes four proteins from the complementary-sense strand (AC1, AC2, AC3, and AC4) which are necessary for viral DNA replication and transcription and two proteins from the virion strand (AV1 and AV2). DNA B encodes two proteins (BC1 and BV1) required for intra- and intercellular movement of the viruses. Bidirectional promoters are located in the intergenic regions (IR) of both the genomes. A common region (CR) of about 200 nucleotides, which is required for replication initiation, is located within the IR of both the components. AC1 (Rep) is required for the initiation of virus replication (Nash et al. 2011), whereas AC2 and AC4 proteins acts as a suppressor of host-mediated gene silencing (Bisaro 2006). Among them, *Begomoviruses* are transmitted by white flies (*Bemisia* sp.) and have bipartite genome. ToLCV, a whitefly (*Bemisia tabaci* Genn.)-transmitted virus of tomato, is one of the most important constraints to tomato production in the tropical and subtropical areas of South/Southeast Asia (Green and Kalloo 1994 Czosnek and Laterrot 1997; Chakraborty 2008). This disease is caused by strains of *Tomato leaf curl New Delhi virus* (ToLCNDV), a species of the genus *Begomovirus*, family Geminiviridae.

In our previous study, a tomato cultivar H-88-78-1 showed delayed lower infectivity, late symptom appearance, reduced viral loads, and less symptom severity at 21 days post-inoculation (dpi) in comparison to Punjab Chhuhara (Sahu et al. 2010). The lower level of viral DNA accumulation in tolerant tomato genotypes/cultivars was attributed to an enhanced siRNA generation upon viral infection (Sahu et al. 2010). Hence, we aimed to evaluate the capability of these cultivars in activating the TGS/PTGS and the mode of DNA methylation against ToLCNDV. In this report, we compared the distribution of virus-derived siRNAs along the viral genome in a set of previously reported tolerant and susceptible tomato cultivars (Sahu et al. 2010). We had also demonstrated a higher accumulation of IR- and *AC1*-specific siRNA along with the corresponding DNA methylation in the viral genome. Further, the effect of this siRNA-dependent DNA methylation on the transcription of various viral transcripts was examined. The types of methylation corresponding to various methyltransferase genes were also investigated.

Materials and Methods

Plant Materials, Virus Inoculation, and Tissue Harvest

Tomato cultivars identified as a ToLCNDV tolerant (cv. H-88-78-1) and susceptible (cv. Punjab Chhuhara) in our previous report were selected for the present study (Sahu et al. 2010). In

the present study, we have selected these tomato cultivars (namely H-88-78-1 and Punjab Chhuhara) differing in ToLCNDV tolerance. The origin of H-88-78-1 is unknown. H-88-78-1 is a resistant line, and it has indeterminate growth. Punjab Chhuhara is a variety developed by Punjab Agriculture University, Ludhiana, India, from a cross between a line introduced from Israel and Shimla Gold. Punjab Chhuhara is a determinate variety, fruits are pear shaped and it is highly susceptible to ToLCV (Kalloo et al. 2000). Tomato seeds were germinated at 22 °C in the growth chamber at 75 % relative humidity with 16/8 h (light/dark) photoperiod. Thirty plants of both cultivars were agro-inoculated at the stem nodes of two-leaf stage with DNA-A and DNA-B (1:1) as described previously (Sahu et al. 2010) and were arranged in three biological replicates consisting of 10 plants each. *Agrobacterium tumefaciens* (strain GV3101) harboring only pCAMBIA 2301 was used for mock inoculation. At 21 dpi, two infected systemic compound leaves (each having three leaflets) emerging third from the point of inoculation were pooled for nucleic acid isolation.

Preparation of ToLCNDV Genomic Fragments and Labeling of siRNA

Twenty sets of primers were designed to amplify 10 fragments each of DNA-A (~275 bp each) and DNA-B (267 bp each) so as to cover the entire genomic length of ToLCNDV (Supplementary Table 1). Small-RNAs were isolated and radiolabeled following the method described previously (Rodriguez-Negrete et al. 2009). In brief, low-molecular-weight RNAs isolated from both ToLCNDV and mock-inoculated systemic leaves of cv. H-88-78-1 and cv. Punjab Chhuhara were separated by resolving on denaturing polyacrylamide gel (PAGE; 15 %). The region within a range of 21–4 nucleotides (nt) was excised, crushed, and suspended in elution buffer (80 % formamide, 40 mM PIPES pH 6.4, 1 mM EDTA, and 400 mM NaCl) with overnight shaking. The eluted products obtained were precipitated with 1 volume of isopropanol and 1 μl of 20 mg/ml glycogen. The pellets were suspended in Tris-HCl (20 mM, pH 7.5) and sodium acetate (300 mM). Reprecipitation was performed with 3 volumes of ice-cold ethanol and centrifuged at 14,000 rpm for 20 min. Pellets were air dried and dissolved in nuclease-free water to obtain low-molecular weight RNA. For radiolabeling, 1 μg of siRNA was subjected to de-phosphorylation with (10 U/μl) alkaline phosphatase (New England Biolabs, USA) and further labeled with γ^{32}P-ATP in the presence of (10 U/μl) T4 DNA polynucleotide kinase (New England Biolabs, USA). The blots containing DNA fragments were hybridized with labeled siRNA at 42 °C overnight. The images were scanned using PhosphorImager (Typhoon-9210, GE Healthcare, USA). In order to obtain the real quantification, we designed probes specific to tomato snRNA U6 (oligonucleotide 5′-TCATCCTTGCGCAGGGGCCA-3′). Quantification of the signals was performed by Quantity One image analysis software (Bio-Rad, USA).

Detection of Virus-Specific siRNAs

Virus-specific siRNAs were detected by northern blotting. About 2 μg of low-molecular-weight RNA isolated from the ToLCNDV and mock-inoculated tomato plants were denatured at 65 °C in loading dye and resolved on denaturing PAGE (15 %). Separated RNAs were transferred to nylon membrane according to a procedure described elsewhere (Sambrook and Russell 2001). Viral genome-derived A1, A2, A7, and A8 DNA fragments (amplified using the primers mentioned in Supplementary Table 1) were used as probes, which were radiolabeled with α^{32}P-dCTP using DNA labeling kit (New England Biolabs, USA). Hybridization with the radiolabeled probes was performed overnight at 42 °C. Ethidium bromide stained 21- and 24-nt long synthetic oligonucleotides were used as standard size markers.

Bisulfite Sequencing

Bisulfite sequencing process was carried out according to the procedure described previously (Rodriguez-Negrete et al. 2009). Total DNA isolated from the infected tissues was digested with (20 U/μl) *Sac*I endonuclease, followed by (20 U/μl) Proteinase K digestion. Bisulfite conversion of genomic DNA was carried out using EZ DNA Methylation-Gold kit (Zymo Research, USA) following the manufacturer's instructions. Virion strand-specific primers were designed from intergenic region (IR) of DNA-A and AC1 region (covering the parts of A7 and A8; position 1462–2045) to amplify both the methylated and nonmethylated viral DNA (Supplementary Table 2). PCR amplification was performed with (5 U/μl) hot start Taq polymerase (Zymo Taq; Zymo Research, USA). The amplified products were cloned into pGEM-T Easy vector (Promega, USA) and transformed to DH5α *Escherichia coli*-competent cells. To evaluate the effectiveness of technique and rate of conversion of nonmethylated cytosines, plasmid having viral DNA was pooled with the nonvirus-infected genomic DNA from both the cultivars. This pooled sample was further used as control to check conversion rate in each particular sample. Sequencing was performed with an automated sequencer (ABI Sequencer, version No.3770, Applied Biosystems, USA). CyMATE software was used for the identification and analysis of pattern-specific methylation at CG, CHG, and CHH depending upon their position in the clones (Foerster et al 2010; http://www.cymate.org/cymate.html). For this, a reference ToLCNDV sequence which can be described as the master genomic sequence without any bisulfite treatment was used.

Detection of siRNA from Corresponding Methylated Region

Amplification of the intergenic region of DNA-A (IR-A) corresponding to the targeted methylated region, three sets of primers were used to amplify three fragments (~60 bp each) corresponding to IR-A. Similarly, *AC1* gene (corresponding to the A7 and A8) was also subjected for intensive mapping. The sequence of all the primers and their positions in the viral genome are listed in Supplementary Table 3. PCR was carried out using the viral genomic DNA clone as the template and (5 U/μl) Taq DNA polymerase (Sigma Aldrich, USA). siRNA labeling was performed as described in previous section.

Northern Blot Analysis

Total RNA were isolated from the mock and 21-dpi samples of both H-88-78-1 and Punjab Chhuhara using Trizol reagent as described elsewhere (Sahu et al. 2010). About 25 μg of total RNA was electrophoresed on 1.2 % denaturing formaldehyde agarose gel in 1× MOPS running buffer and transferred to positively charged nylon membrane (Amersham Bioscience, USA). For the study of viral AC2- and AC3-specific mRNA accumulation, PCR amplification was carried out using specific primer pairs mentioned in Supplementary Table 4. For study of *Dicer-like* protein 3 (DCL3), *Dicer-like* protein 4 (DCL4), *Argonaute 1* (AGO1), Domain Rearrangement Methyltransferase-1 (DRM1), and Chromomethylase-3 (CMT3), primers were designed (Supplementary Table 5) from the sequence retrieved from SGN tomato genome database (http://solgenomics.net/organism/Solanum_lycopersicum/genome). Probes labeling, hybridization, and image analysis was done as described in earlier sections.

Southern Blot Analysis

Total DNA from the 21-dpi-old ToLCNDV-infected tomato of both the cultivars were isolated by the cetyltrimethylammonium bromide (CTAB) method (Porebski et al. 1997). Five microgram DNA was electrophoresed on 1 % agarose gel in TBE [Tris-borate EDTA; 45 mM Tris-borate, 1 mM EDTA (pH 8)]. Membrane transfer and radiolabeling of probe was done according to the method described by Sahu et al. (2010).

Statistical Analysis

For statistical comparisons of bisulfite-treated DNA molecules of IR and AC1 region at symmetric and asymmetric sites, individual unpaired *t* tests were performed by using the Graphpad Instant software (http://www.graphpad.com/scientific-software/instat/).

Results and Discussion

Distribution Pattern of ToLCNDV-Derived siRNA in Tolerant and Susceptible Cultivars

The sources of siRNA population within the ToLCNDV genome were determined using 20 PCR-amplified fragments, covering the entire DNA-A and DNA-B components (Fig. 1a, d), from ToLCNDV-inoculated cv. H-88-78-1 and cv. Punjab Chhuhara at 21 dpi. Before proceeding to the quantification, we were interested to check the level of tomato small nuclear RNA (snRNA) during ToLCNDV infection in tomato. This experiment reveals that there was no observable change in the expression pattern of snRNA in both the cultivar while comparing control, mock, and infected conditions. This indicates that viral infection dose does not interfere with host small RNA expression (Supplementary Fig. 1). The quantification of the siRNA blot showed that most of the siRNAs were targeted against IR (part A1) and *AC1* (part A7 and A8) regions of DNA-A in the tolerant cv. H-88-78-1 upon ToLCNDV infection (Fig. 1a, b). Considerable amount of siRNAs were also generated against the overlapping regions of *AV2* and *AV3* (part A2) in cv. H-88-78-1. On the other hand, no such differential pattern in level of siRNA was detected in susceptible cv. upon ToLCNDV infection (Fig. 1c). Distribution of siRNA in DNA-B was also analyzed following the similar strategy as DNA-A. As CR of DNA-A and DNA-B are similar in structure and sequence, small RNAs derived from DNA-A could hybridize on DNA-B IR. Interestingly, we observed remarkable difference in hybridization signals between the susceptible and tolerant cultivars. A plausible explanation of this outcome may be the difference in the targeted region or the time-dependent expression of both A and B components of virus. In case of MYMIV infection in soybean, a similar observation was highlighted where the difference in the IR was due to the selective generation of siRNA from the stem-loop region (Yadav and Chattopadhyay 2011). Here, the majority of the siRNAs were targeted against the IR and the regions adjoining *BV1* and *BC1* in both the cultivars (Fig. 1d–f). However, no considerable difference was observed between the siRNAs derived from the DNA-B genome in tolerant and susceptible cultivar of tomato. The result suggested that DNA-A-specific *IR* and *AC1*-related transcripts might have independent regulatory mechanism governing ToLCNDV tolerance. No such differences in DNA-B derived siRNA were observed in tolerant and susceptible cultivar.

Recently, high-resolution sRNA map for a monopartite begomoviruses, *Tomato yellow leaf curl China virus* (TYLCCNV) was generated via next-generation sequencing approach (Yang et al. 2011). The comparison of the "hot spots" for siRNA generation in both monopartite (TYLCCV+β) and bipartite (ToLCNDV) revealed that in both the cases the production of V-sRNAs was higher in 3′

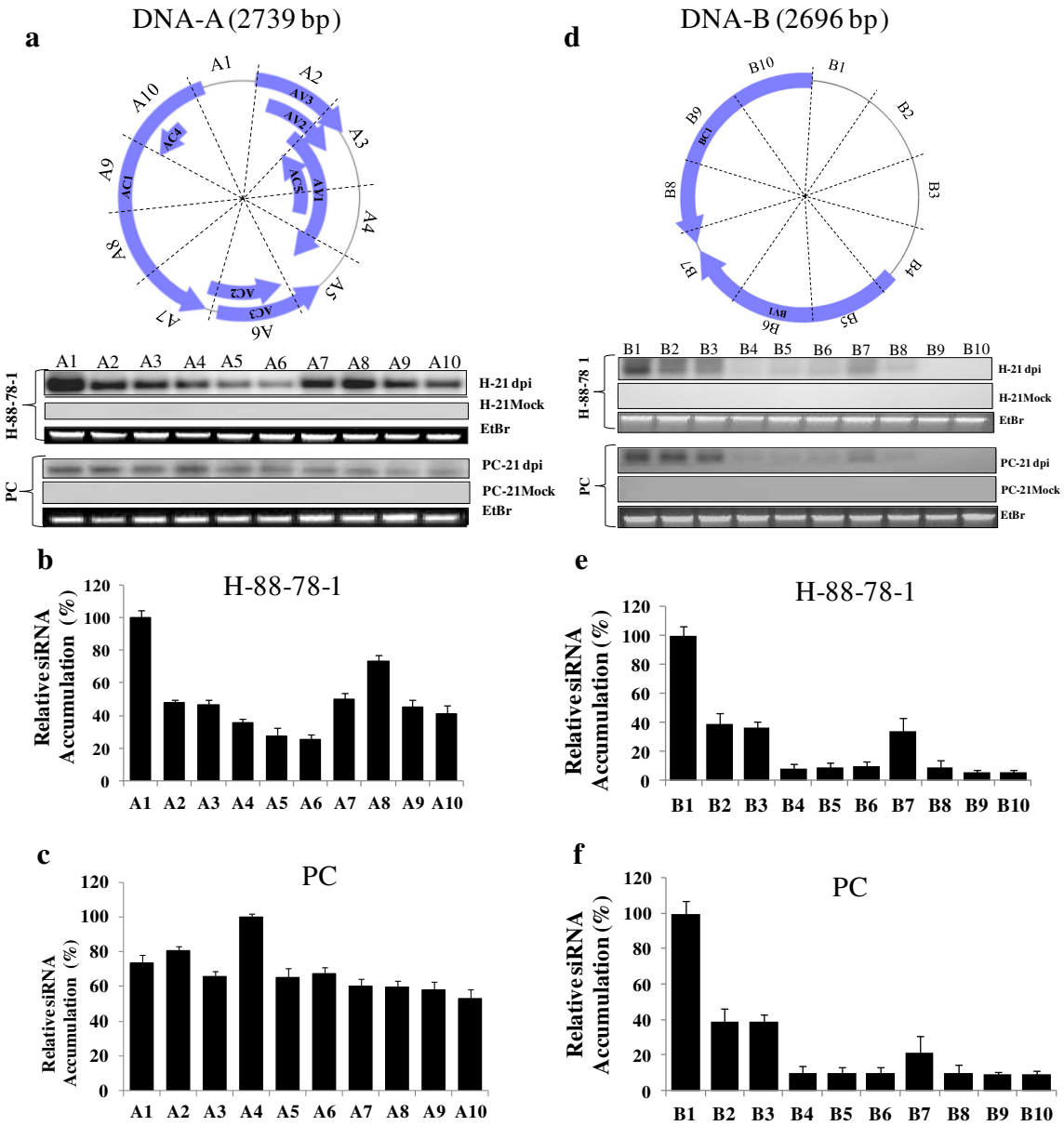


Fig. 1 Distribution pattern of ToLCNDV specific siRNAs along the DNA-A and DNA-B components of ToLCNDV in cv. H-88-78-1 and cv. Punjab Chhuhara. **a** Division of the ToLCNDV DNA-A components. Reverse northern hybridization was performed to explore siRNA distribution 5′-end-labeled siRNA isolated from ToLCNDV-infected and mock-inoculated systemic tissues of both cultivars at 21 dpi. **b** The relative percent intensities of the signals in ToLCNDV-inoculated cv. H-88-78-1. The highest intensity value was scored as 100 %. **c** The relative percent intensities of the signals in ToLCNDV-inoculated cv. Punjab Chhuhara. The highest intensity value was scored as 100 %. **d** Division of the ToLCNDV DNA-B components. Reverse northern hybridization was performed to survey siRNA distribution by 5′-end-labeled siRNA isolated from ToLCNDV-infected and mock-inoculated systemic tissue of both cultivars at 21 dpi. **e** Relative percent accumulations of DNA-B-specific siRNA in cv. H-88-78-1. **f** Relative percent accumulations of DNA-B-specific siRNA in cv. Punjab Chhuhara. For equivalent loading, ethidium bromide-stained DNA gels have shown. All experiments were performed in three biological replicates. Standard deviations are shown as *bar graph*. *AC1* replication-associated protein, *AC2* transcription activator protein, *AC3* replication enhancer protein, *AV1* coat protein, *AV2* pre-coat protein, *BV1* nuclear shuttle protein, and *BC1* movement protein

terminal of AC1 and in the overlapping regions of AC2 and AC3. Although as observed in ToLCNDV, majority of siRNAs were generated from the IR region, interestingly in TYLCCV (-β) IR region was least targeted. Another study on Geminiviruses highlighted the similar results in *African cassava mosaic virus* infection (isolate from Cameroon), where early viral genes such as AC1 and AC2 were selectively targeted (Chellappan et al. 2004). In contrast to these observations, the late gene (BC1) encoded by the DNA-B component of *East African cassava mosaic Cameroon virus* was the targeted region of the siRNA generation. Thus, the mode of action to activate RNA silencing may vary in case of Geminiviruses.

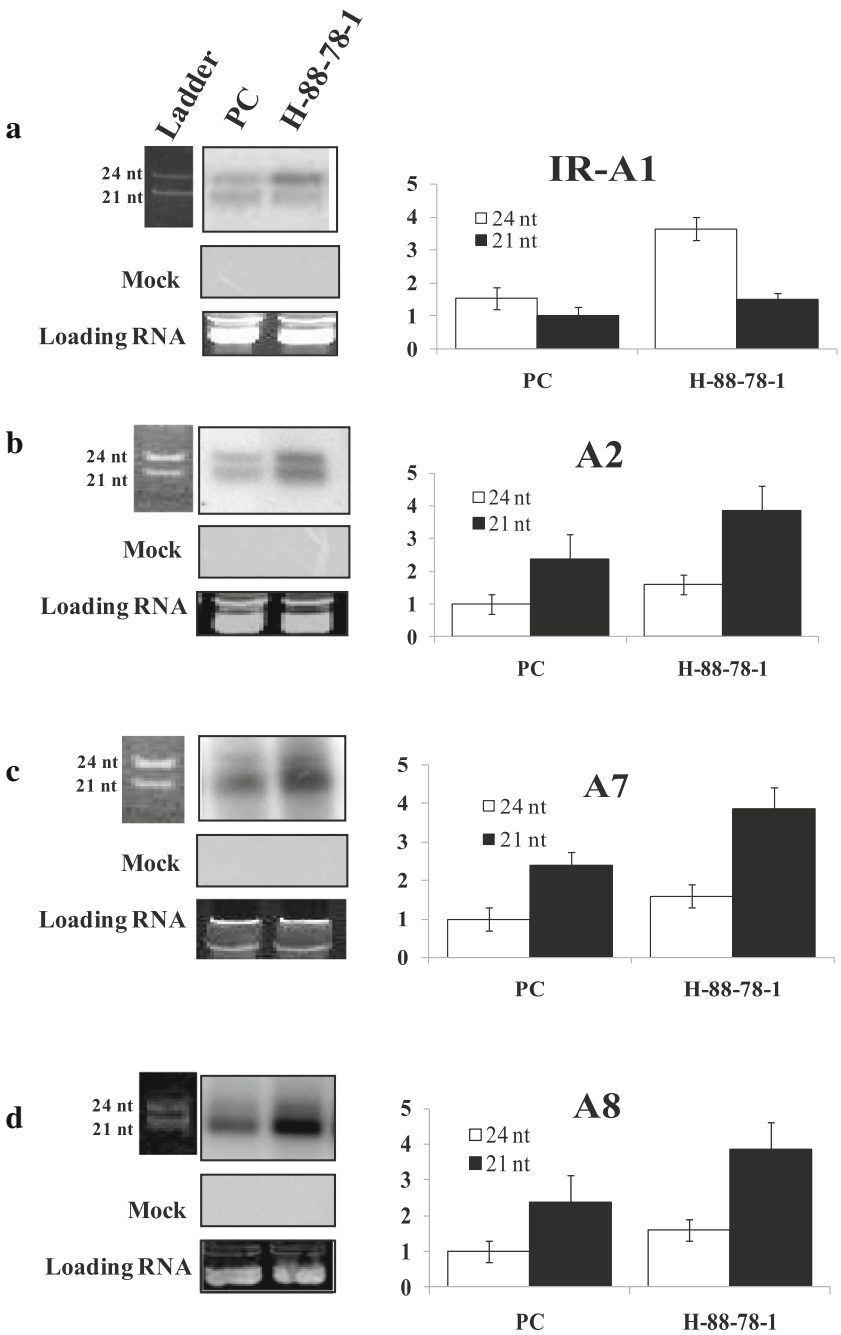


Fig. 2 Nature and comparative level of the ToLCNDV-specific siRNAs produced by tolerant and susceptible cultivars during ToLCNDV infection at 21 dpi. Low molecular weight RNA isolated from the systemic leaves of cv. H-88-78-1 and cv. Punjab Chhuhara were separated on a 15 % denaturing PAGE gel and blotted to a nylon membrane. IR-A-, A2-, A7-, and A8-specific amplified products were used as probes for the radiolabeling. Size and relative quantities of **a** IR A1, **b** A2, **c** A7, and **d** A8 have been shown. Mock sample were also hybridized with the region-specific radio-labeled probes. Equivalent loading is shown by ethidium bromide-stained gel. *Bar diagram* shows relative signal intensities of the different classes of siRNAs. Standard size ladder of 21- and 24-nt is shown in the figure. Each experiment was performed in three biological replicates for significance of obtained results

Comparative Concentration and Nature of ToLCNDV-Derived siRNA

The above observation suggested the possibility of siRNA-mediated defense in response to ToLCNDV infection in tolerant cv. H-88-78-1. In order to monitor the correlation between the siRNAs appearance with PTGS and TGS, we attempted to investigate the nature of siRNAs produced. Hence, we selected the fragments A1, A2, A7, and A8 producing more siRNA in the tolerant tomato cultivar (H-88-78-1) at 21 dpi (Fig. 2a–d). The region A1 (corresponding to IR) in the tolerant cultivar was found to produce higher (~3.5-fold) 24-nt siRNA, while the amount of 21 nt was at comparatively lower level (Fig. 2a). In contrast, the susceptible cv. Punjab Chhuhara had lower level of 24-nt siRNA. As evident from these results, accumulation of 24-nt siRNA in cv. H-88-78-1 is correlated to the activation of TGS, this imparts ToLCNDV tolerance. Further, accumulation of PTGS-specific 21-nt siRNAs corresponding to A2 (*AV3*) and A7 and A8 (*AC1*) were also much higher in the tolerant plants

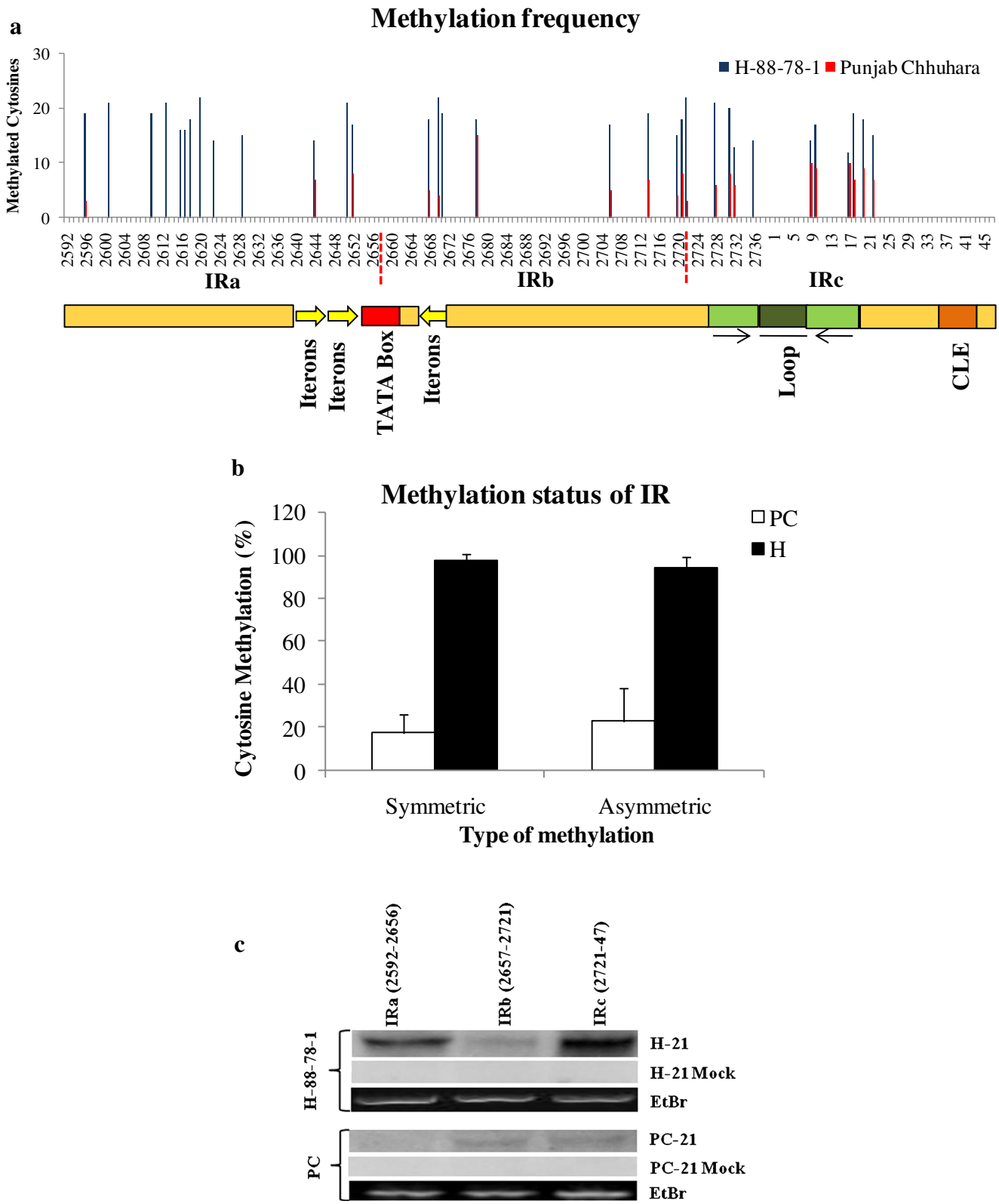


Fig. 3 Distribution of methylated cytosines along the intergenic region (IR) of ToLCNDV genomic component DNA A. **a** *Bar graph* representing the position and methylation frequency of cytosine along the IR. **b** Level of symmetric and asymmetric cytosines in the IR of DNA-A. *Bar* graph representing the percentage of asymmetric and symmetric methylated cytosines, along the IR region in cv. H-88-78-1 and cv. Punjab Chhuhara, calculated using average and standard deviation obtained from five randomly selected clones. **c** Validation of the methylated region with the profiling of siRNA derived from the corresponding length in cv. H-88-78-1 and cv. Punjab Chhuhara. IR was divided into three parts termed as IRa, IRb, and IRc

Table 1 Rates of cytosine methylation on ToLCNDV intergenic region (IR) and replication-associated protein (AC1) regions

Regions	Number of cytosine			Plants	Cytosine methylation (%)	
	Asymmetric	Symmetric	Total		Symmetric	Asymmetric
IR (2592-29)	24	8	32	PC-I	17.5	24.1
				H-I	97.5	95.83
Rep (1798-2026)	53	11	64	PC-I	1.8	7.92
				H-I	58.18	54.34

PC-1 Punjab Chhuhara infected with ToLCNDV; *H-I* H-88-78-1 infected with ToLCNDV

(Fig. 2b–d). They accumulated ~2.5-fold higher amount of 21- to 22-nt than to 24-nt siRNA (Fig. 2b–d). Accretion of both 21- (~4-fold) and 24-nt siRNAs (~1.5-fold) analogous to the open-reading frame (ORF) of the genes (part A7 and A8; Fig. 2c, d) made us to anticipate that, principally, PTGS may contribute to the tolerant behavior of cv. H-88-78-1. These observations are supported by our previous studies where we showed that less abundance of viral replicative intermediate in tolerant cultivar may have a correlation with a relatively higher accumulation of virus-specific siRNAs (Sahu et al. 2010, 2012).

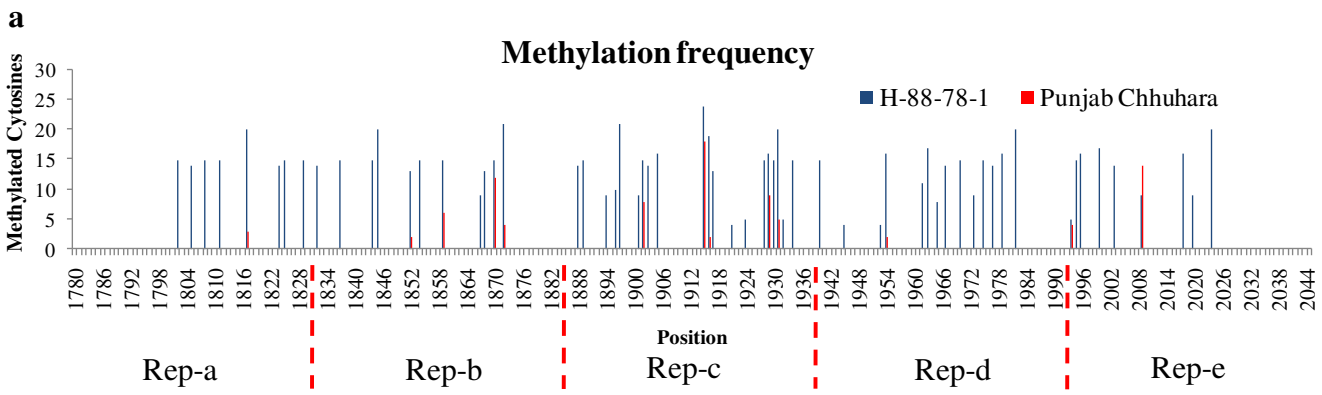
In addition to PTGS, TGS has also been observed in the present investigation. Various studies have associated siRNAs with Geminiviruses, reporting the role of 21- to 24-nt siRNAs against coding and intergenic regions (Akbergenov et al. 2006; Yadav and Chattopadhyay 2011). These regulatory small RNAs are generated via various pathways which entail numerous evolutionarily conserved protein families. Each silencing pathway is characterized by the involvement of distinct set of silencing factors leading to the diversity in the process (Brodersen and Voinnet 2006; Xie and Qi 2008). Genetic data also indicate that 24 nt and considerable fraction of the 21- to 22-nt viral siRNAs were produced by the Dicer-like proteins (DCL3 and DCL2) as diverse silencing pathways involved during Geminivirus–plant interactions. Considering these reports, it may be concluded that the noncoding regions may be targeted by the TGS system, while the viral gene ORF may be targeted by the PTGS system for piloting the degradation of viral transcripts.

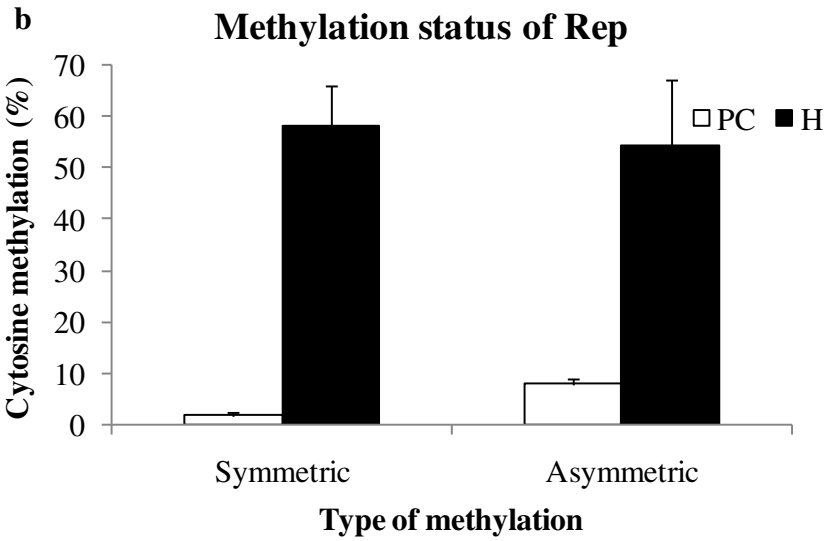
Dissection of Methylated Cytosines in IR and AC1

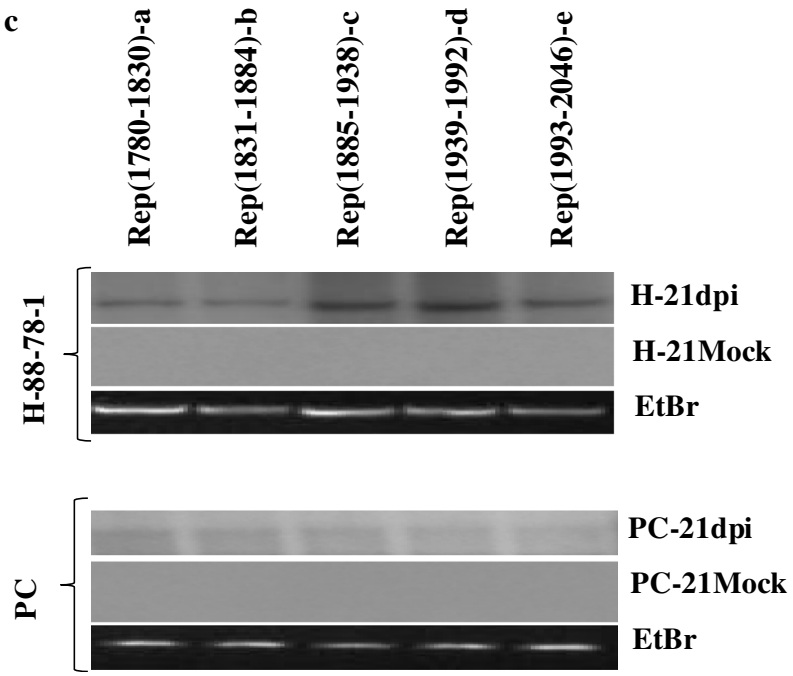
In a similar study, it was found that in the plant tissues showing symptom recovery after viral infection has a higher proportion of methylated viral DNA (Rodriguez-Negrete et al. 2009). Another study revealed that, the *Mungbean yellow mosaic India virus* (MYMIV)-resistant soybean variety has higher level of IR-specific DNA methylation (Yadav and Chattopadhyay 2011). Cytosine methylation analysis of the ToLCV IR has also highlighted the role of DNA methylation in transgenic tobacco (Bian et al. 2006). Based on these studies, we aimed to find a link between the existence of 24-nt (in IR) and 21- to 22-nt (in *AC1*) siRNAs, and the pattern of DNA methylation at IR and *AC1* regions. For this, bisulfite sequencing was performed to identify the methylated cytosines (Cm) in the virion strands corresponding to the IR and AC1 gene (covering part A7 and A8) of DNA-A. The IR and *AC1* fragments were amplified using the bisulfite-treated DNA, isolated from ToLCNDV-inoculated systemic tissues of cv. H-88-78-1 and cv. Punjab Chhuhara at 21 dpi. DNAs from a set of 25 ToLCNDV-infected plants were pooled from both the cultivars. These pooled DNA samples of both tomato cultivars were further subjected to the bisulfite conversion, used for PCR amplification and cloning of the amplified fragments. A total of 24 clones from viral DNA isolated from each cultivar were sequenced, and the sequence analysis showed that a significant difference in cytosine methylation patterns of the viral DNA was obtained from two contrasting cultivars.

IR contained 32 cytosines, out of which all (100 %) showed methylation at any of the cytosines in H-88-78-1, whereas only 21 (65.6 %) were methylated in Punjab Chhuhara (Fig. 3a). Alignments of randomly selected five clones from bisulfite-treated samples were shown in the Supplementary Fig. 2. An alignment of all 24 IR clones of bisulfite-treated viral DNA isolated from both the cultivars are also shown in Supplementary Fig. 3. Further, we aimed to characterize the type of cytosine methylation (symmetric and asymmetric) using average and standard deviation obtained from five randomly selected clones (clone numbers 1–5) in both the

Fig. 4 Distribution of methylated cytosines along the replication-associated protein (AC1; 1780-2045) of ToLCNDV genomic component DNA-A in ToLCNDV-tolerant and susceptible cultivar of tomato. **a** *Bar graph* representing the position and frequency of methylated cytosine in locus 1780–2045. A total of 24 individual clones were sequenced and subjected to the methylation pattern recognition from each samples. **b** Level of symmetric and asymmetric cytosines in the AC1 of DNA-A. *Bar graph* representing the percentage of asymmetric and symmetric methylated cytosines, along the AC1 region in cv. H-88-78-1 and cv. Punjab Chhuhara, calculated using average and standard deviation obtained from five randomly selected clones. **c** Validation of the methylated region with the profiling of siRNA derived from the corresponding length in cv. H-88-78-1 and cv. Punjab Chhuhara. AC1 (1780-2045) was divided into five parts termed as Rep (1780-2045)-a, Rep (1780-2045)-b, Rep (1780-2045)-c, Rep (1780-2045)-d, and Rep (1780-2045)-e


a
Methylation frequency
Methylated Cytosines
H-88-78-1
Punjab Chhuhara
Position
Rep-a
Rep-b
Rep-c
Rep-d
Rep-e


b
Methylation status of Rep
Cytosine methylation (%)
PC
H
Symmetric
Asymmetric
Type of methylation


c
Rep(1780-1830)-a
Rep(1831-1884)-b
Rep(1885-1938)-c
Rep(1939-1992)-d
Rep(1993-2046)-e
H-88-78-1
H-21dpi
H-21Mock
EtBr
PC
PC-21dpi
PC-21Mock
EtBr

cultivars (Table 1). Sequence analysis revealed that the cytosines were methylated in both the symmetric (CG/CNG) and asymmetric (CHH) positions. Higher level of cytosine methylation at symmetric (97.5 %) and asymmetric (95.8 %) sites were observed in tolerant cv. H-88-78-1 than susceptible cv. Punjab Chhuhara (17.5 and 24.1 %, respectively; Fig. 3b). These difference in the levels of cytosine methylation between the two cultivars were statistically significant for both symmetric (P=0.046) and asymmetric (P=0.0182) sites. A region centered on iterons and stem-loop illustrated a relatively high proportion of methylation along with the upstream of iterons in tolerant cv. than the susceptible cv. A similar kind of observation was previously surveyed within the *C4* gene of ToLCV DNA from *Nicotiana benthamiana* (Bian et al. 2006).

The role of the symmetry of the CpG site in stable maintenance of methylation patterns after DNA replication is well known (Holliday and Pugh 1975). On other hand, asymmetric methylation has been shown to involve in the gene silencing (Paszkowski and Whitham 2001). In the present study, higher level of asymmetric methylation in IR and rep region was observed. The *cis*-acting elements of IR play an important role in controlling the viral DNA replication and gene expression. *AC1* (*AL1* in some Geminiviruses), binds to CR sequences upstream of the omnipresent motif 'TAATATTAC' (Fontes et al. 1992, 1994a, b). This motif is involved in the initiation of rolling circle replication (Saunders et al. 1991; Stanley 1995). We hypothesized that asymmetric methylation in IR region may have hindered these replication-related processes during virus multiplication, while the asymmetric methylation in the *Rep* gene can be correlated to the gene silencing. Recent study in Geminivirus *Rep* gene has also revealed its active involvement in disrupting the methylation-specific machinery and promoting TGS (Rodríguez-Negrete et al. 2013). Methylation pattern obtained in the IR was further validated by generation of siRNA in corresponding region (Fig. 3c). IR was further divided in three PCR fragments, each of ~60 bp covering the entire length (Supplementary Table 3). It was observed that, the fragment corresponding to the loop, iterons and upstream of iterons-containing region had relatively higher siRNA production in the resistant cultivar (Fig. 3c).

The region covering *AC1* ORF (1462–2045) was also cloned using bisulfite-treated total DNA from ToLCNDV-inoculated samples. A similar strategy was applied by taking 24 individual clones from each sample of the tolerant and susceptible cvs. Sequences were aligned and further subjected to the methylation pattern recognition (Supplementary Figs. 4 and 5). Alignments of randomly selected five clones from bisulfite-treated samples were shown in the Supplementary Fig. 4. Cytosine methylation was found to be evenly distributed in the length of *AC1* (1780-2045; Supplementary Fig. 4; Fig. 4a). In cv. H-88-78-1, this region contained a total of 64 cytosines, of which 62 (96.8 %) showed methylation at any of the cytosines, while in cv. Punjab Chhuhara only 13 (20.3 %) of cytosines were methylated (Supplementary Figs. 4, 5; Fig. 4a). The levels of methylation at symmetric and asymmetric cytosines were examined in the AC1 (1780–2045) with a similar strategy used for IR (Table 1; Fig. 4b). We observed that the H-88-78-1 has relatively higher level of asymmetric cytosine (54.3 %) in comparison to Punjab Chhuhara (7.9 %). The observation was statistically significant with the P=0.0157. However, variation in the level of symmetric cytosine methylation between Punjab Chhuhara (1.8 %) and H-88-78-1 (58.2 %) was not statistically significant (Table 1; Fig. 4b).

The viral DNA isolated from the tolerant cv. had higher level of symmetric (CG/CNG) and asymmetric (CHH) type methylation in comparison to viral DNA isolated from susceptible cultivar (Fig. 4b). siRNA mapping of *AC1* locus (1780–2045) corresponding to the methylated region were also validated, and it was observed that the c and d region produced much higher level of siRNA (Fig. 4c). The hypermethylation of this locus may have an important role in providing tolerance against the ToLCNDV. However, this study needs further investigation to conclude the role of ToLCNDV *AC1* (1780–2045) locus.

The observed methylation of *AC1* ORF (1780–2045) motivated us to discover its importance in the host defense against Geminivirus. Increasing evidence of cytosine methylation in ORF of various genes has indicated their plausible role in triggering defense against Geminiviruses (Bian et al. 2006). AC1 protein has numerous functions related with guiding the replication complex (Fontes et al. 1992; Thommes et al. 1993), unwinding of replicating templates (Gorbalenya et al. 1990), and DNA-nicking (Koonin and Ilyina 1992). Furthermore, the AC1 protein bound to its own promoter (Fontes et al. 1992; Thommes et al. 1993) and its target sequences were also involved in AC1-dependent transcription repression (Sunter et al. 1993; Eagle et al. 1994). A fascinating observation from our study revealed that the cytosine near a conserved binding site ('AACGTCATC' within the TGMV *AL-1629* promotor) of *AC1* gene was methylated (Supplementary Fig. 4). It was revealed that this region was required for the binding of various plant nuclear proteins such as ATF (involved in expression of *Adenovirus E1a*-inducible gene; Tu and Sunter 2007), *TGA2* (a basic leucine zipper protein interacts with SA; Lin and Green 1988) and *Myb* factors (involved in apoptosis and cell proliferation; Araki et al. 2004). This unveiled the fact that both methylation and the phenomenon involving binding of the host nuclear factor may have some complex role in providing defense against the Geminivirus. However, conserved binding sites located within *AC1* region (upstream of the *AC2* and *AC3* in case of ToLCNDV) also suggested that universal machinery regulates the expression of the genes involved in replication and suppression of host defense. Therefore, it indicates the role of methylation in a particular DNA stretch for controlling gene transcription.

Expression Pattern of *AC1*, *AC2*, and *AC3* Gene Components of ToLCNDV

Hypermethylation of viral genome prompted us to evaluate the efficiency of the transcription of *AC1*, *AC2*, and *AC3* genes in the tolerant and susceptible cvs. Thus, the expression of *AC1* (*Rep*), *AC2* (*TrAP*), and *AC3* (*REn*) transcripts were evaluated at 21 dpi (Fig. 5a, b). They showed lower accumulation in tolerant cv. H-88-78-1 in comparison to the susceptible cv. Punjab Chhuhara. While the expression of *AC2* and *AC3* showed more than 60 % reduction in cv. H-88-78-1, *AC1* expression reduced up to ~80 % (Fig. 5a, b). *AC1* (*Rep*) is a replication-associated protein gene and *AC2* (*TrAP*) is a transactivator protein that controls late-gene expression, involved in RNAi suppression whereas *AC3* functions in enhancement of replication. These observations lead us to postulate that hypermethylation may regulate the expression of *AC1*, *AC2*, and *AC3* so as to reduce late-gene expression along with RNAi suppression.

Transcription of the Geminivirus genome is a complex process as it generates multiple overlapping polycistronic mRNAs. In begomoviruses, the leftward AC1 transcription unit has a single major start site which serve as a dicistronic mRNA for both the AC1 and AC4 proteins (Mullineaux et al. 1993; Shivaprasad et al. 2005). As observed in various cases of begomoviruses, another separate transcript was mapped which covered the entire AC2 and AC3 genes (Hanley-Bowdoin et al. 1988; Sunter et al. 1989; Frischmuth et al. 1991; Mullineaux et al. 1993). AC3 was found to be transcribed as a second cistron under a strong AC2 promoter in case of *Tomato leaf curl virus* (Mullineaux et al. 1993). Thus, results obtained from our study also highlighted that the hypermethylation of CR may have a correlation with lower transcription of AC1 ORF. This effect was further observed in the case of AC2 and AC3 transcription. As AC2/3 transcription is under the influence of strong AC2 promoter, we assume that the hypermethylation in this promoter may hinder the transcription of AC2 and AC3. Thus, the reduction observed on expression was almost similar in case of AC2 and AC3 (Fig. 5a, b). Measurement of viral DNA copy number was also evaluated from the representative samples of leaf tissue used for viral gene expression analysis. This indicated that the lower level (>50 % reduction) of viral DNA load in H-88-78-1 at 21 dpi has a correlation with the reduced level of viral

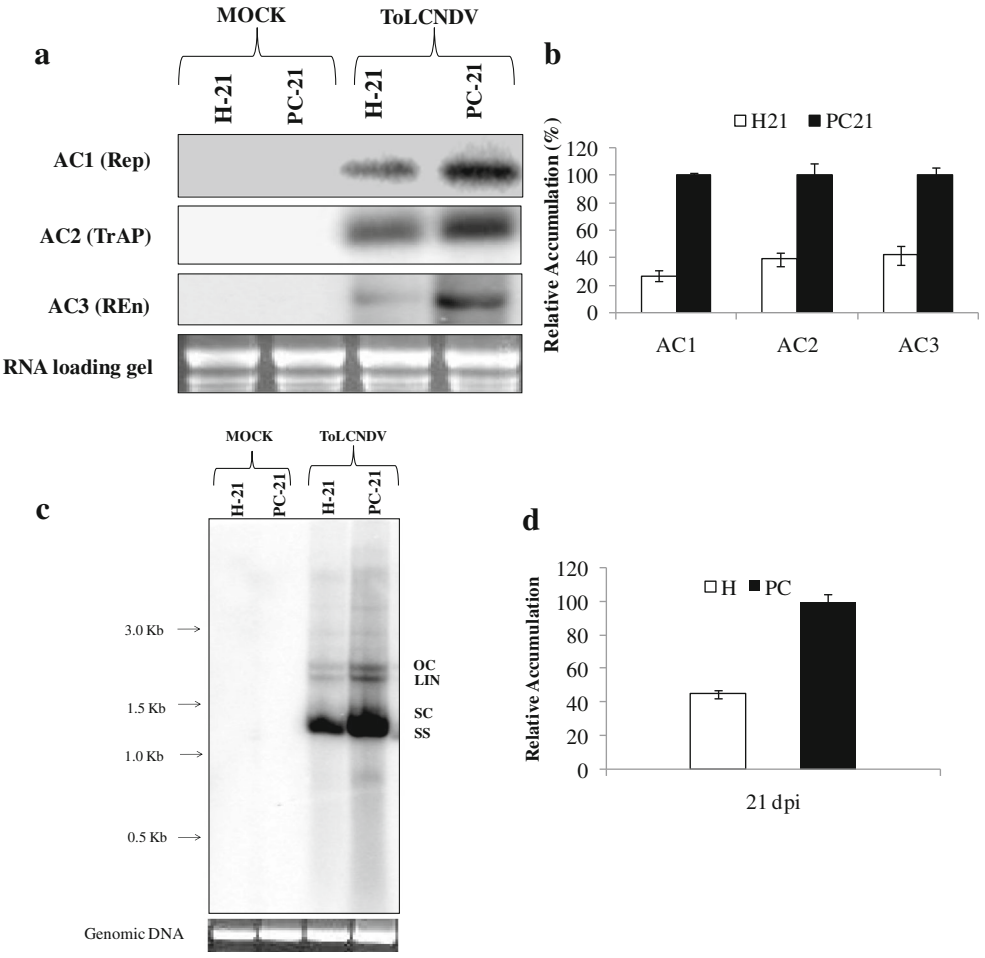


Fig. 5 Expression of *AC1*, *AC2*, and *AC3* in cv. H-88-78-1 and cv. Punjab Chhuhara. **a** Northern hybridization shows accumulation of the of *AC1*, *AC2*, and *AC3*. **b** Relative accumulation of *AC1*, *AC2*, and *AC3* in tolerant and susceptible cultivar at 21 dpi. **c** and **d** Southern blot analysis of samples collected for expression analysis. Mock- and ToLCNDV-treated samples of cv. H-88-78-1 and Punjab Chhuhara at 21 dpi were subjected to viral DNA accumulation analysis. Coat protein fragments were used as a probe for viral DNA accumulation study. Viral replicative forms are shown as open circular (*OC*), linear (*Lin*), super coiled (*SC*), and single strand (*SS*). *Bars* indicate the standard deviations (±SD). *AC1* replication-associated protein, *AC2* transcription activator protein, *AC3* replication enhancer protein

mRNAs. Thus, this suggests the correlation the between viral DNA accumulation and the expression level of viral *AC1*, *AC2*, and *AC3* genes (Fig. 5c, d).

Expression Pattern of Genes Involved in the siRNA-Mediated DNA Methylation Process

As stated earlier, the production of siRNAs leads to the activation of gene silencing process. This process involve complex network of various key genes which regulates mRNA degradation as well as DNA methylation process. We further aim to evaluate the relative expression of these genes in cv. H-88-78-1 and cv. Punjab Chhuhara at 21 dpi (Fig. 6a, b). It was found that *DCL3* and *DCL4* showed relatively higher (≥1.8-fold) expression in H-88-78-1 while compared to Punjab Chhuhara (Fig. 6a, b). On the other hand, *AGO1* which is a component of RISC complex showed relatively unchanged expression (~1-fold) in H-88-78-1 than Punjab Chhuhara (Fig. 6a, b). The role of genes involved in DNA and histone methylation has been well documented (Bender 2004; Chan et al. 2005). Among them, methyltransferase (*MET1*) were identified as key enzymes in maintaining CG-type methylation, whereas others like Domain rearrangement methyltransferase (*DRM1/2*) and chromomethylase (*CMT3*) were shown to be important for methylation at non-CG sites (Raja et al. 2008). In cv. H-88-78-1, level of *DRM1* was found to be upregulated by ~2.5-fold during ToLCNDV infection, whereas Punjab Chhuhara showed reduced expression level (Fig. 6a, b). Tomato cv. H-88-78-1 showed higher expression (~1.5-fold) in level of *CMT3*, while comparing with the mock control (Fig. 6a, b). Based on these results, it may be suggested that the change in the level of these key methylation maintenance enzymes may probably be correlated with siRNA generation and cytosine methylation. In our previous study, we have found several differentially expressed genes such as histone h2b and AGO4-2 in H-88-78-1 (Sahu et al. 2010), thus predicting their plausible involvement in the process of siRNA-directed silencing pathway in tolerant tomato cultivar.

Based on our observations and the published literature, we propose a mechanism that governs the tolerant characteristics of tomato cv. H-88-78-1 (Fig. 7). The ToLCNDV infection initiated both TGS and PTGS pathways which was evident by the generation of 21- to 24-nt siRNA. The 21-nt siRNAs activated the corresponding mRNA degradation which may result in the reduced *AC2* and *AC3* expression. Further, TGS activated siRNA-mediated DNA methylation of the key promoter elements which may alter the viral DNA replication in tolerant cultivar. Activation of DNA methylation maintaining enzymes also emphasized the strong DNA methylation-mediated tolerance against ToLCNDV.

Thus we infer that both lower transcription rates and siRNA mediated RNA degradation govern the tolerance in cv. H-88-78-1 which together may lead to reduced infection and that these effects are manifested somewhat differently at different viral genome regions. Ideally, it may result in the stronger reduction in level of viral RNA than observed. A better explanation of this outcome may be that the process of siRNA-mediated methylation depends upon various factors and conditions. In this study, we have found that not all the cytosines on all the DNA molecules were methylated and, therefore, the RNA level is dependent on degree of methylation of a particular DNA molecule. Another reason may be the dynamics of RISC assembly during virus infection. One of the active components of RISC complex, i.e., AGO1 showed

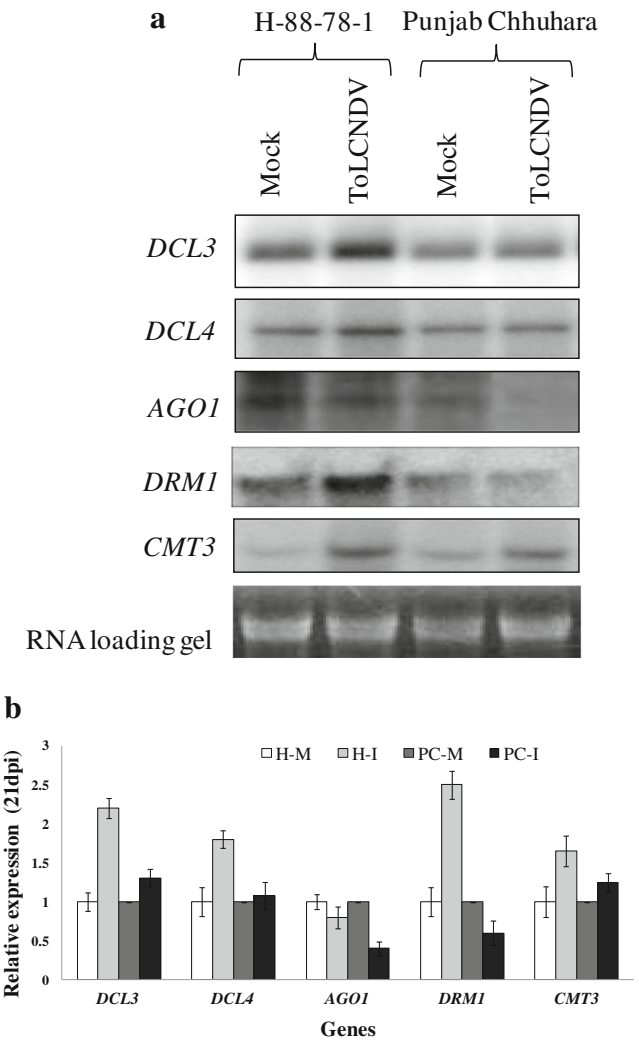


Fig. 6 Expression of gene involved in siRNA-mediated DNA methylation and RNA degradation in cv. H-88-78-1 and cv. Punjab Chhuhara. **a** Northern hybridization shows accumulation of the *Dicer-like protein* 3 (*DCL3*), *Dicer-like protein* 4 (*DCL4*), *Argonaute 1* (*AGO1*), *Domain Rearrangement Methyltransferase-1* (*DRM1*), and *Chromomethylase-3* (*CMT3*) in tolerant and susceptible cultivar at 21 dpi. **b** Relative accumulation of the genes in tolerant and susceptible cultivar at 21 dpi. *Bar* represents the standard deviation (±SD). *HM* mock-inoculated H-88-78-1, *HI* ToLCNDV inoculated H-88-78-1, *PC-M* mock-inoculated Punjab Chhuhara, *PC-I* ToLCNDV-inoculated Punjab Chhuhara

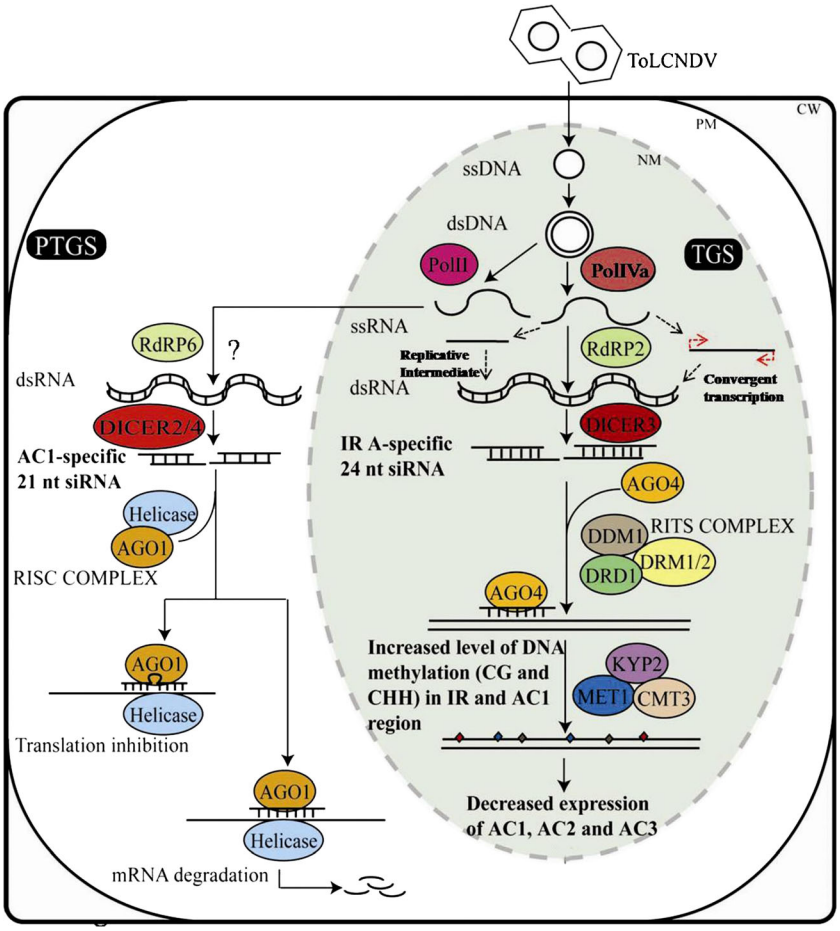


Fig. 7 Schematic diagram explaining antiviral silencing pathways in tolerant variety of Tomato (cv. H-88-78-1). The gene silencing is initiated by production of dsRNA via *RDR6* (in cytoplasm) and *RDR2* (in nucleus). The cytoplasmic events include processing of ds RNA into 21-nucleotide siRNA by DICER 2/4. These resulting siRNA are incorporated into RISC complex which unwinds the siRNA and uses one strand of the pair as the guide to target mRNA. Depending upon the complementarily AGO1, either slices the target mRNA or inhibits its translation. In nucleus, the dsRNA are cleaved by DICER3 to produce 24-nucleotide siRNA which are recruited to AGO4 containing RITS complex, responsible for chromatin remodeling, guided by siRNA. This RNA-directed DNA methylation requires activity methylation maintaining genes such as: *DRM* (domain rearranged methyltransferase; causes *de novo* methylation at CHH), *DRD* (makes DNA accessible to RNA signals), *CMT* (maintains the methylation at CNG), *MET* (for maintenance of CG methylation), and *KYP2* (DNA methylation maintenance at CHH and H3K9 methylation). *Bold fonts* indicate the major outcomes in this study of siRNA mediated methylation. *AC1* replication-associated protein, *AC2* transcription activator protein, *AC3* replication enhancer protein, *AGO* Agronaute, *CMT* chromomethylase, *CW* cell wall, *DDM* decrease in DNA methylation; *DRD* defective in RNA-directed DNA methylation, *DRM* domain-rearranged methyltransferase, *IR* intergenic region, *KYP2* kryptonite, *MET* methyltransferase, *NM* nuclear membrane, *nt* nucleotide, *PM* plasma membrane, *Pol* polymerase, *PTGS* post-transcriptional gene silencing, *RDR* RNA-dependent RNA polymerase, *RISC* RNA-induced silencing complex, *RITS* RNA-induced transcriptional silencing complex, *siRNA* small interfering RNA, *TGS* transcriptional gene silencing, *ToLCNDV* Tomato leaf curl New Delhi virus

relatively minor reduced expression in the H-88-78-1 as compared to cv. Punjab Chhuhara at 21 dpi (Fig. 6a, b). This can be the rate-limiting factor for RNA degradation, therefore, responsible for the reduced transfer rate of viral siRNA to the target gene.

Conclusions

Studies on Geminivirus–host interaction are crucial for understanding the molecular and cellular biology of host defense, providing a basis for rational design of virus control strategies. Previously, several variations of the TYLCV *Rep* gene constructs were introduced into the tomato genome (Yang et al. 2004), but plants generated an array of responses to the virus, ranging from susceptibility to resistance for different constructs. At the present scenario, we need to explore the mode of action of different viral genome components along with the plant factors responsible for the counter defense strategy. Through the present study, several evidences are provided to support the assumption that our tolerant cultivar use small RNA-directed methylation as a defense against ToLCNDV. To our knowledge, we have, for the first time, analyzed complete ToLCNDV genome components and evaluated the

overall variation in the pattern of siRNA accumulation in a naturally tolerant and susceptible tomato cv. The detection of Geminivirus-specific siRNAs and the higher level of methylation in tolerant hosts suggest that in response to the ToLCNDV infection, plant activates the RNA-silencing process and suppresses the transcription of the viral gene. This study provides an insight into tolerance ToLCNDV-mediated by the IR- and *AC1*-derived siRNAs and the cytosine methylation in respective regions. This study may also be useful for generating transgenics with efficiency to not only combat against ToLCNDV infection but also to study diverse plant–Geminivirus interactions. In fact, even the susceptible cv. can be used as a model to study the repression of methylation. Newly developed tool such as use of synthetic and natural products (i.e., 5-Azacytidine, Decitabine, Zebularine, Procainamide, and Genistein) and targeted DNA methylation of genome (i.e., designing artificial zinc fingers or TALEs fused to a methyltransferase to target-specific genes) can be utilized to develop new traits in plants. Therefore, genetic manipulation of the genes regulating methylation, artificially targeted DNA methylation, and by targeting sequence-specific siRNAs may be exploited to enhance virus tolerance. Thus, by taking advantage of available tools and present study, we should look forward for exciting developments of ToLCNDV-tolerant tomato plants in the near future.

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