Research Article

Tobacco *RDR1* affects the expression of defence related genes in *Nicotiana benthamiana* during geminivirus infection

Abstract: Plants protect themselves from the invading viruses through RNA silencing. RNA dependent RNA polymerase-1 (RDR1) is one of the crucial protein of the RNA silencing pathway, which is induced after infection with viruses. Here, we used the transgenic *N. benthamiana* plant with overexpressed tobacco *NtRDR1* gene and found that these plants show reduced susceptibility towards Tomato leaf curl virus (ToLCV) infection compared with the wild-type *N. benthamiana*. To understand the reason for such reduced susceptibility, we generated high-definition sRNA cDNA libraries form ToLCV infected wild-type *N. benthamiana* and *NtRDR1* overexpressing lines of *N. benthamiana* and carried out next-generation sequencing. We found that during ToLCV infection majority of siRNAs generated from the host genome are of 24 nucleotide (nt) class while viral siRNAs are of 21-22 nt class, indicating that transcriptional gene silencing (TGS) is the major pathway for silencing of host genes while viral genes are silenced, predominantly, by post transcriptional gene silencing (PTGS) pathways. We further explored the changes in the expression of various defense-related genes which might be linked with the reduced susceptibility of *NtRDR1 N. benthamiana* plants. In addition, we showed that RDR1 negatively regulates *RDR6* expression in uninfected plants and ToLCV induces RDR6 expression during infection.

Keywords: Geminivirus; RDR1; RDR6; *Nicotiana benthamiana;* plant-defense; Next-generation sequencing; small RNA; *Nicotiana tabacum;* High-definition adapters; Tomato leaf curl virus

1. Introduction

In plants, the phenomena of RNA silencing, also known as gene silencing or RNA interference (RNAi) in animals, protects plants from invading viruses and viroids (Fire *et al.*, 1998, Goldbach *et al.*, 2003, Carbonell & Daros, 2017, Prakash *et al.*, 2017). The trigger for RNA silencing is the presence of double-stranded RNA (dsRNA), which are recognized by specific Dicer-like (DCL) proteins, and are cleaved into small RNAs (sRNAs) of 21-24 nucleotide (nt) (Mette *et al.*, 2000, Carmell & Hannon, 2004, Xie *et al.*, 2004). These sRNAs can be either micro-RNA (miRNA) or small-interfering RNA (siRNA) (Lai, 2003, Xie *et al.*, 2004). sRNAs generated by DCLs are further recruited by Argonaute (AGO) proteins and mediate the silencing of RNA transcripts or the genomic DNA by post-transcriptional gene silencing (PTGS) or transcriptional gene silencing (TGS), respectively (Henderson *et al.*, 2006, Ghildiyal & Zamore, 2009).

RNA dependent RNA polymerases (RDRs) are crucial enzymes which increase the number of substrate for DCLs by converting small- and single-stranded RNAs into long-dsRNA in primer-dependent or independent manner, leading to the amplification of the silencing signal (Vaucheret, 2006, Szittya *et al.*, 2010, Devert *et al.*, 2015). *Arabidopsis thaliana (A. thaliana)* genome encodes for six RDR proteins (AtRDR1-6) with varied functions (Wassenegger & Krczal, 2006). Based on the phylogenetic analysis, RDRs have been divided into three clades, viz., *RDR*α, *RDR*β and *RDR*γ, however, plants possess only two of them, *RDR*α and *RDR*γ (Zong *et al.*, 2009). AtRDR1, 2 and 6 belongs to *RDR*α while RDR3, 4 and 5 belongs to *RDR*γ clade. Functional characterization of RDR3, 4 and 5 and yet elusive. However, the role of RDR1, 2 and 6 have been implicated in providing resistance against viruses through PTGS and TGS (Chapman & Carrington, 2007, Searle *et al.*, 2010, Wang *et al.*, 2010, Melnyk *et al.*, 2011, Qin *et al.*, 2012, Lewsey *et al.*, 2016). Out of the six RDRs, the expression of RDR1 induces upon infection with viruses, viroids, exogenous application of salicylic acid (SA) and jasmonic acid (JA) (Zabel *et al.*, 1974, Dorssers *et al.*, 1984, Khan *et al.*, 1986, Xie *et al.*, 2001, Pandey & Baldwin, 2007, Hunter *et al.*, 2013, Hunter *et al.*, 2016). The extent of RNA silencing is affected when the accumulation of *RDR6* is inhibited in *Nicotiana benthamiana (N. benthamiana)* which lacks a functional RDR1 (Yang *et al.*, 2004, Qu *et al.*, 2005, Schwach *et al.*, 2005). Antiviral role of RDR1 and RDR6 has been implicated in several studies in *A. thaliana, N. benthamiana and N. tabacum* (Dalmay *et al.*, 2000, Mourrain *et al.*, 2000, Xie *et al.*, 2001, Yu *et al.*,

2003, Qu *et al.*, 2005, Schwach *et al.*, 2005, Wang *et al.*, 2010, Rakhshandehroo *et al.*, 2017). Expression of *RDR6* is affected when the *RDR1* expression is inhibited (Rakhshandehroo *et al.*, 2009). During infection with virus, RDR1 is involved in the production of virus-associated siRNA (vasiRNA) from the endogenous loci of the host, targeting host's transcripts (Cao *et al.*, 2014). Report suggests that, *RDR1* down-regulated lines of tobacco shows susceptibility towards Potato virus Y (PVY) infection and reduced expression of a few defence related genes, including Myb transcription factor (TF) (Rakhshandehroo *et al.*, 2009, Rakhshandehroo *et al.*, 2012). Interestingly, report suggests the presence of binding sites for Myb family of TFs on the promoter of RDR1 of various plant species (Prakash & Chakraborty, 2019).

Tomato leaf curl viruses (ToLCVs), belonging to geminiviridae family and begomovirus genus, are a major constraint for the production of tomato, and estimated to cause yield loss of $140 million in Florida, USA, annually (Moffat, 1999). NGS of sRNAs is becoming very popular to understand plant physiology under biotic and abiotic stresses. RNA sequencing is being widely used as a tool for the discovery of new viruses as well as to find the differentially expressed genes in the host plants during virus infection (Kamitani *et al.*, 2016). With the biotic stress point of view, for the first time, high-resolution sRNA map of geminivirus was constructed in 2011 by deep sequencing (Yang *et al.*, 2011). Generation of sRNA libraries using high definition (HD) adapters has been shown to be efficient in reducing the ligation bias of sRNAs with the adapter sequences (Xu *et al.*, 2015).

Recently, Basu et al reported that *NtRDR1* overexpressing lines of *N benthamiana* shows reduced susceptibility against ToLCV infection compared with the wild-type plants (Basu *et al.*, 2018). Therefore, to understand the cause of reduced susceptibility of *NtRDR1* transgenic plants against ToLCV infection, we have performed NGS of small RNA cDNA-libraries, generated using HD adapters, from ToLCV infected wild-type *N. benthamiana* and *NtRDR1* overexpressing lines of *N. benthamiana.* We have shown here that expression of many defence related genes are affected which might be linked with the reduced susceptibility of *NtRDR1* overexpressing lines of *N. benthamiana*.

2. Materials and Methods

Source of Seeds, Plant growth conditions, Agro-infection and Sample collection

Seeds of wild-type *N. benthamiana* was procured from Central Tobacco Research Institute (CTRI), Andhra Pradesh, India. Transgenic seeds of *NtRDR1 N. benthamiana* were received from Professor Hui Shan Guo, Chinese Academy of Sciences, Beijing, China. Seedlings were grown for 16 hours of daylight in a growth room at a constant 22°C temperature.

Infectious *Agrobacterium tumefaciens (A. tumefaciens)* strain EHA105 harbouring ToLCV was inoculated in wild-type *N. benthamiana* and *NtRDR1 N. benthamiana* as described previously (Kumari *et al.*, 2010). Systemic leaves were collected from three biological replicates at 21 days post-inoculation (dpi), immediately kept in liquid N2 and kept at -80°C until isolation of total RNA.

Total RNA isolation for sRNA library preparation

Total RNA from the leaf samples was isolated by TRIzol reagent (Invitrogen) as per the manufacturer's instruction. mirVana miRNA isolation kitTM (Ambion) was used for the purification of the total RNA as per the manufacturer's instruction. After elution of the RNA, ethanol precipitation was performed for the concentrating total RNA. For ethanol precipitation, 3 volume of absolute ethanol, 0.1 volume of 3M sodium acetate and 25 ug/mL glycogen (Ambion) were added in the MCT containing total RNA and incubated at -20°C overnight. MCT containing the mixture was centrifuged at 15000´g for 15 min at 4°C. The pellet was washed with 80% ethanol followed by air-drying of the samples at room temperature for 5 minutes. RNase/DNase free water was added to the dried precipitate. Thermo Scientific Nanodrop 2000 was used to determine the concentration of RNA and stored at -80°C.

Generation of sRNA cDNA library using HD adapters and Next Generation Sequencing

sRNA cDNA-libraries were prepared as described previously by using HD adapters (Billmeier & Xu, 2017). sRNA libraries were generated from the total RNA isolated from wild type wild-type *N. benthamiana* and *NtRDR1 N. benthamiana.* All of the libraries were generated in triplicates. Illumina HiSeq 2500 platform (50 bp, single-end) at BaseClear, Netherland (www.baseclear.nl) was used for sequencing of all the libraries.

Bioinformatic analysis of small RNA sequences

Raw FASTQ files were converted to FASTA format. Reads containing unassigned nucleotides were excluded. The 3' adapter was trimmed by using first 8 nt of the 3' HiSeq 2500 adapter (TGGAATTC). The high definition reads were mapped (no mismatch allowed) to the *N. benthamiana* genome using PatMaN (Prufer *et al.*, 2008). The high definition signatures (four assigned degenerate nucleotides at the ligating ends) of the reads were also trimmed. The latest set of plant miRNAs were downloaded from miRBase (v21) (Kozomara & Griffiths-Jones, 2014). Read per million approach was used for the normalization of the sRNA libraries (Mortazavi *et al.*, 2008) . To identify differentially expressed sRNA, we added offset of 10 to normalised counts before calculating log-fold change between different conditions. This was done to correct for low expression level counts, to avoid false positive results. sRNA greater than two-fold change in expression were considered differentially expressed. Principal component analysis (PCA) was done using DESeq2 package of R (Love *et al.*, 2014). Target prediction was done by psRNATarget server (Dai *et al.*, 2018).

Construction of Heat map

To generate heat map of differentially expressed sRNAs, Multi Experimental Viewer (MeV 4.9.0) software was used. For clustering, Euclidean distance was used as distance matrix, and complete linkage clustering was used as linkage method.

Target Prediction

Targets of the differentially expressed sRNAs of 21-24 nt were predicted by psRNATarget server (Dai *et al.,* 2018). Targets of sRNAs were predicted against "*N. benthamiana*, transcript, Niben, 101" cDNA library. Following parameters were used for the target prediction: Expectation-0, penalty for G:U pair-0.5, penalty for other mismatches-1, extra weight in seed region-1.5, seed region-2 to13 nt, number of mismatches allowed in seed region-2, HSP size-19, buldge (gap) was allowed, penalty for extending gap-0.5 and translation inhibition range-10 to 11 nt.

Total RNA isolation for Real-Time Quantitative Reverse Transcription PCR (qRT-PCR)

Total RNA from the leaf samples (collected at 21 dpi) was isolated by TRIzol reagent (Invitrogen) as per the manufacturer's instruction. Concentration and quality of RNA were checked by Thermo Scientific Nanodrop 2000. Before cDNA preparation, DNase treatment was given to total RNA. For cDNA synthesis, 1.0 µg of DNase treated RNA, 1.0 µl of 1.0 µg/µl oligo dT were mixed with the required amount of nuclease-free water (considering the total volume of final mixture 20.0µl) and incubated at 72℃ for 10 minutes followed by snap chilling (10 minutes) to remove secondary structures. After that 5X reaction buffer, 2.0 µl of 10 mM dNTPs, 2.0 µl of 25 mM $MgCl_2$, 1.0 µl of 200U/µl reverse transcriptase (Thermo Scientific RevertAid H minus) and 0.5 µl of 40U/µl RiboLock RNase inhibitor was added to the mixture. Reverse transcription of total RNA was performed in the thermal cycler (Applied Biosystem 2720) at 42℃ for 60 min, followed by heat inactivation of reverse transcriptase at 72℃ for 10 minutes. Relative expression of various transcripts was checked by qRT-PCR (Illumina EcoTM Real-Time PCR System). PowerUpTM SYBRTM Green Master Mix was used for relative quantification of the transcripts. Tubulin was used as an internal control (reference gene). Prism 8 (GraphPad) was used for plotting individual graphs.

3. Results

Transgenic *NtRDR1 N. benthamiana* shows reduced susceptibility against ToLCV infection

Both wild-type as well as *NtRDR1* transgenic plants, infected with ToLCV showed mild leaf curling, venal chlorosis and stunted growth at 7-9 dpi. However, ToLCV infected *NtRDR1* transgenic *N. benthamiana* showed reduced symptoms starting at 18-20 dpi compared to that of wild-type plants. The transgenic *NtRDR1 N. benthamiana* mock plants did not show any phenotypic difference when compared with wild-type mock plants (Fig. 1).

24 nucleotide siRNAs are predominantly produced from the host genome while 21-22 nucleotide siRNAs are the major class of sRNAs generated from the ToLCV genome

sRNA deep sequencing was performed by generating sRNA cDNA libraries using HD adapters to reduce the ligation bias. Deep sequencing data suggests that most of the redundant as well as non-redundant host reads of mock-inoculated and ToLCV infected, wild-type and *NtRDR1* transgenic lines, belong to 24 nucleotide siRNAs class (Fig. 2 A, B, C & D). However, 21-22 nucleotide siRNAs were the most abundant class of sRNA arising

from the ToLCV genome (Fig. 2 E). There was no significant difference in the siRNA population in between wild-type and transgenic lines either in mock or in ToLCV infected plants, suggesting that, as such, there is no effect in the generation of different size-classes of sRNAs in presence and absence of RDR1.

We analyzed the presence of specific nucleotide at 5' terminus of viral siRNA (vsiRNA) to understand the involvement of AGO protein in vsiRNA sorting. In both wild-type as well as *NtRDR1* transgenic lines, there was a slight bias for 'G' at the 5' terminus of the 21 and 22 nt vsiRNAs (Fig. 3A, B and 4A, B). Bias was also found for 'A' at the 5' terminus of the 24 nt vsiRNAs in both wild-type as well as *NtRDR1* transgenic lines (Fig. 3 D and 4D).

Differential expression analysis of siRNAs and prediction of siRNA target transcripts

The sRNA reads, obtained after the deep sequencing, were subjected to the differential expression analysis. siRNA expression values were normalized against total reads and expression change were calculated using offset-fold change method (Mohorianu *et al.*, 2011). The combination of plants used for the differential expression analysis are shown in the table 1.

Table 1: List of pair of plants for which the differential expression analysis was performed.

S. No.	Differential expression in between plants
1.	Wild-type *N. benthamiana* vs ToLCV_*N. benthamiana*
2.	*NtRDR1 N. benthamiana* vs ToLCV_*NtRDR1 N. benthamiana*
3.	ToLCV_*NtRDR1 N. benthamiana* vs ToLCV_wild-type *N. benthamiana* (Host aligned sRNAs)
4.	ToLCV_*NtRDR1 N. benthamiana* vs ToLCV_wild-type *N. benthamiana* (ToLCV aligned sRNAs)

Principle component analysis (PCA) suggested that the sRNA reads from biological replicates of the specific plants (same treatment) clustered together, while the different plants (different treatment) clustered separately. (Fig. 5). Several siRNAs were found to be differentially expressed in all of the different combinations (Fig. 6 A-D, Table S1.1-S1.4). Targets of the differentially expressed siRNAs of 21-24 nt were predicted by psRNATarget server (Dai *et al.*, 2018) (Table S2).

RDR1 overexpression leads to increased accumulation of many defense-related genes

All of the differentially expressed sRNAs of 21-24 nucleotide range were checked for their target transcripts (Table S2). Many sRNAs were predicted to target mRNA transcript of various genes. A summary of total number of differentially expressed sRNAs and its targets are listed in the table S3. We checked the expressions of nine genes based on the target prediction data of differentially expressed sRNAs.

Expression of transcripts of *Constitutively Photomorphogenic-9 (COP-9) signalosome (CSN) complex subunit-7*, *Pentatricopeptide repeat containing protein (PPRP)*, *Laccase-3*, *Glutathione peroxidase-1 (GPX-1)*, *Universal stress protein (USP) A like protein* and *Heat shock transcription factor B4 (HSTF-B4)* was enhanced in the *NtRDR1* overexpressing lines of *N. benthamiana* compared with the wild-type *N. benthamiana* (Fig. 7 A-F). Surprisingly, the expression of all of these genes were significantly reduced during ToLCV infection in *NtRDR1* transgenic lines.

In addition to these observations, during ToLCV infection, the expression of *Laccases-3* is found to be significantly reduced in both, wild-type *N. benthamiana* as well as *NtRDR1* transgenic lines compared with the mock plants (Fig. 7C). Moreover, the expression of *USP A-like protein* was reduced in mock-inoculated as well as ToLCV infected wild-type *N. benthamiana* compared with the mock-inoculated and infected *NtRDR1* lines (Fig. 7E).

We also observed a significant reduction in the transcript accumulation of *Auxin response factor-18*, *WRKY-6* and *Short chain dehydrogenase reductase-3a* during ToLCV infection in *NtRDR1* transgenic lines compared with the mock plants as well as ToLCV infected wild-type *N. benthamiana* plants (Fig. 7G-I).

RDR1 negatively regulates *RDR6* expression in uninfected plants, but positively regulate *RDR6* expression during ToLCV infection

Expression of *RDR6* was significantly increased in the mock-inoculated wild-*type N. benthamiana* compared with the ToLCV infected wild-type *N. benthamiana* and mock-inoculated *NtRDR1* transgenic lines (Fig. 8). However, expression of *RDR6* was increased in *NtRDR1* transgenic lines during ToLCV infection compared with the mock-inoculated transgenic lines (Fig. 8).

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4. Discussion

N. benthamiana is widely used as a model organism for studying plant-pathogen interaction This plant is closely related to *N. benthamiana*, *S. lycopersicum* and *S. tuberosum*. Thus, components and mechanisms of various biochemical pathways in such plants are believed be conserved. *N. benthamiana* genome possesses n=19 chromosome (more than 3.5GB in size) and is sequenced (draft genome available at https://solgenomics.net/organism/Nicotiana_benthamiana/genome).

Although, *N. benthamiana* has the gene coding for *RDR1*, one of the antiviral protein, but it is naturally mutant and not functional, perhaps, making the plant susceptible to virus infection (Yang *et al.*, 2004). Expression of *RDR1* is induced after virus infection and exogenous application of SA in *N. tabacum* (White, 1979, Xie *et al.*, 2001, Alamillo *et al.*, 2006). Report suggests that RDR1, but not RDR6 of the plants, functions in the generation of siRNAs against the viral genome as a defence strategy (Xie *et al.*, 2001, Rakhshandehroo *et al.*, 2009). RDR1 also acts as a defense protein against geminivirus infection and attenuates symptoms by enhancing the methylation of the viral genome (Basu *et al.*, 2018).

In the present study, it has been found that ToLCV infection induces symptoms in both wild-type as well as transgenic *NtRDR1 N. benthamiana* at 7-9 dpi. However, at around 20 dpi, *NtRDR1* transgenic lines were found to show reduced symptoms, suggesting the antiviral function of *NtRDR1* in *N. benthamiana*. At 20 dpi, ToLCV infected wild-type *N. benthamiana* plants shows more stunted grown as compared to ToLCV infected *NtRDR1 N. benthamiana* (Fig 7.1). However, there was no difference in the phenotype of mock-inoculated wild-type and transgenic plants.

NGS analysis revealed that the sRNAs generated from the host genome were most abundant in 24 nt species of sRNAs. However, the sRNAs generated from the ToLCV genome were predominantly of 21-22 nt in size. This suggests that DCL2 and DCL4 functions as antiviral DCLs, functioning in the antiviral silencing pathway in *N. benthamiana*. While DCL3 is involved in the production of sRNAs from the host genome. DCL2 and 4 are known to function mostly in the PTGS while DCL3 functions in the TGS (Prakash *et al.*, 2017), implicating that probably, host sRNAs would silence the transposons, repetitive elements and other genes of the host genome while vsiRNAs would cleave viral transcripts post-transcriptionally.

Recruitment of vsiRNA into specific AGO is determined by the 5'terminal nucleotide of vsiRNA (Takeda *et al.*, 2008). A bias for A at 5' terminus of the vsiRNA leads to the sorting of such vsiRNAs with AGO2 and AGO4. While U and C at 5' terminus of the vsiRNA are responsible for londing into AGO1 and AGO5, respectively. So far, presence of G as the 5' terminal nucleotide of vsiRNAs has not been linked with the sorting with any AGO. Results from our study shows that 24 nt vsiRNAs from both wild-type as well as *NtRDR1* transgenic lines are predominated with A at the 5' terminus suggesting that, probably, AGO2/AGO4 might be involved in the sorting of such vsiRNAs.

Viruses hijack components of Ub-26S pathways for supporting its own replication by diverting Ub-26S proteiosome pathways to new targets such as the modification of AGO protein to suppress the gene silencing (Alcaide-Loridan & Jupin, 2012, Byun *et al.*, 2014, Verchot, 2016). Through NGS analysis and target prediction of differentially expressed sRNAs, 4.7-fold higher expression of siRNAs that target the *subunit 7 of Cop9 signalosome complex*, was found in *NtRDR1 N. benthamiana* compared with the wild-type *N. benthamiana*. This was further validated with the qRT- PCR analysis. This study revealed that the expression of *subunit 7 of Cop 9 signalosome complex* is decreased in the ToLCV infected transgenic *N. benthamiana* but not in the ToLCV infected wild-type *N. benthamiana* suggesting the role of NtRDR1 in regulating, directly or indirectly, the expression of *subunit 7 of Cop 9 signalosome complex* during ToLCV infection. It is hypothesized that, probably, host is producing increased level of siRNAs for silencing the components of Ub-26S proteasome pathway so that ToLCV will not usurp the Ub-26S proteasome pathway for its benefit (Randow & Lehner, 2009, Alcaide-Loridan & Jupin, 2012).

To our knowledge, very few literatures are available regarding the role of lignin in plant-virus interaction, such as report suggests that the increased expression of genes involved in the lignin and SA biosynthesis pathway during a compatible host-virus interaction, further providing evidence that the increased lignin content is liked with the enhanced defence against plant viruses (Malinovsky *et al.*, 2014, Anjanappa *et al.*, 2017). Although, many literatures are available suggesting the crucial role of lignin in providing defense against fungi, bacteria and nematodes (Bellincampi *et al.*, 2014). Plant laccases are known to function in the lignin degradation pathway and decreased lignin content is associated with the increased accumulation of SA, jasmonic acid (JA) and abscisic

acid (ABA) in plants (Higuchi, 2004, Gallego-Giraldo *et al.*, 2011). Expression of the *Laccase-3* was found to be reduced drastically during ToLCV infection in wild-type as well as in *NtRDR1 N. benthamiana.* ToLCV infected plants produced enhanced level of siRNAs targeting *Laccase* so that, probably, the rate of lignin degradation would be limited, which would provide strength to the plant during ToLCV infection.

During virus infection, generation of reactive oxygen species (ROS) is increased in the host cells to restrict the virus movement up to ceratin cells (Hernandez *et al.*, 2016). To reduce the self-damage caused by ROS, host produces glutathione peroxidases (GPXs) which function in removing ROS content from the cells. Our study also showed that increased production of siRNAs targeting *GPX1* leading to reduced accumulation of *GPX1* in ToLCV infected wild-type *N. benthamiana* and *NtRDR1 N. benthamiana*. This decrease in the *GPX1* level would be associated with the increased accumulation of ROS so that the pathogen spread would be limited to certain cells/tissues. *NtRDR1* mock-inoculated plants accumulated significantly higher accumulation of *GPX1* compared to the wild-type mock-inoculated plants, suggesting that RDR1 regulates, directly or indirectly, the expression of GPX-1.

NtRDR1 transgenic plants showed enhanced expression of *USPA like protein* compared with the wild-type plants. In addition, we also found a significantly increased accumulation of *USPA like protein* in the *NtRDR1* overexpressing lines compared with the wild-type plants during ToLCV infection, which might be the reason for reduced ToLCV symptoms in the *NtRDR1* transgenic lines around 18-20 dpi.

WRKY 6 functions as a positive regulator of the immune response. In *N. attenuata*, WRKY6 is required for resistance against necrotrophs (Skibbe *et al.*, 2008). In pepper, WRKY6, which functions as an activator of WRKY40, provides resistance against fungal infection and tolerance against high temperature and high humidity (Cai *et al.*, 2015). Induced expression of *WRKY6* TF in transgenic *NtRDR1* plants suggests that these lines might show resistance towards insect pathogens and tolerance towards high temperature and high humidity (Skibbe *et al.*, 2008, Cai *et al.*, 2015). However, there was reduced accumulation of *WRKY6* transcripts in ToLCV infected *NtRDR1 N. benthamiana.* Since, WRKY6 functions in providing resistance to necrotrophic pathogens (probably by increased accumulation of JA), and JA signalling is antagonistic to SA signalling (Thaler *et al.*, 2012), therefore, reduced expression of *WRKY6* transcripts in ToLCV infected *NtRDR1 N. benthamiana* might be because of the activation of genes involved in SA and systemic acquired resistance (SAR) pathway. Role of SA and SAR has been implicated in providing defence to the host against viruses (Carr *et al.*, 2010).

In plants, transacting siRNAs (tasiRNAs) are produced from the RNA polymerase II dependent *TAS1-4* transcripts. miRNA mediated cleavage of *TAS1-4* transcripts acts as a source for the generation of tasiRNA. Following the cleavage of *TAS* transcripts, RDR6 convert the remaining transcript into dsRNA. Finally, DCL4 cleaves such dsRNAs into 21 nt tasiRNAs in phased manner (Chen *et al.*, 2010). PPRP might be targeted by the tasiRNAs generated from the miRNA173-targeted *TAS1* and *TAS* 2 transcripts while ARF are targeted by the tasiRNAs generated from the miRNA390-targeted *TAS3* transcripts (Chen *et al.*, 2007, Howell *et al.*, 2007, Marin *et al.*, 2010). To our knowledge, there is no report suggesting the role of PPRP in plant virus infection. ToLCV infection in wild-type *N. benthamiana* triggers an increase in the transcript level of *HSFB4* and *PPRP*, suggesting response of host towards ToLCV infection. In contrast, the opposite trend was observed in *NtRDR1* lines, where, the expression of *HSFB4* and *PPRP* was high in mock-inoculated plants and reduced during ToLCV infection. Explanation of such change in the *HSFB4* and *PPRP* transcripts in transgenic lines needs further experiments. Probably, the induction of symptom appearance in the host plants infected with ToLCV might be because of the reduced expression of *ARF18* transcripts, since ARF18 is needed for the controlled growth and development of the plant (Huang *et al.*, 2016).

Because of the lack of information about SDRs in the plant pathogenesis, it is difficult to explain the reduced expression of *SDR3a* in ToLCV infected *NtRDR1* transgenic *N. benthamiana*.

Basu et al revealed that the expression of *RDR6* transcripts decreases after increase in *RDR1* expression (Basu *et al.*, 2018). The mock-inoculated wild-type *N. benthamiana* (which lack a functional *RDR1*, naturally) showed increased level of *RDR6* transcripts. This could be because of the absence of functional RDR1 protein in the wild-type plants. However, ToLCV infected wild-type *N. benthamiana* exhibited reduced accumulation of *RDR6* transcripts, suggesting that *RDR6* level is increased in the absence of functional RDR1 and decreased during ToLCV infection in *N. benthamiana.* Similarly, *RDR6* expression was drastically reduced in the *NtRDR1* mock-inoculated plants, however, there was a significant increase in the expression of *RDR6* in infected transgenic plants, suggesting ToLCV induces expression of *RDR6* in *NtRDR1 N. benthamiana*.

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5. Conclusions

Our study revealed that during ToLCV infection, size of the majority of host siRNAs is 24 nt while vsiRNAs are of 21-22 nt, implicating that host genes are silenced by TGS while the viral genes are silenced by PTGS pathway. In addition, we have made an attempt to understand the reason for the reduced susceptibility of *NtRDR1*

overexpressed lines of *N. benthamiana* plants. Here, we showed that the reduced expression of *subunit-7 of CSN complex* and *WRKY6*, and increased expression of *USPA like protein* in *NtRDR1* transgenic lines during ToLCV infection (compared with the ToLCV infected wild-type *N. benthamiana*) is linked with the reduced susceptibility of *NtRDR1 N. benthamiana* plants. We also showed that RDR1 negatively regulates *RDR6* expression in uninfected plants and ToLCV induces *RDR6* expression during infection.

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Conflicts of Interest: We declare that the authors have no competing financial interests.

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Figures

Fig. 1 Photograph showing wild-type and transgenic *NtRDR1 N. benthamiana* plants infected with ToLCV (30 dpi). Upper panel- A: wild type *N. benthamiana* (mock); B: wild type *N. benthamiana* infected with ToLCV; C: *NtRDR1 N. benthamiana* infected with ToLCV; D: NtRDR1 *N. benthamiana* (mock). Lower panel- enlarged view of B: wild type *N. benthamiana* infected with ToLCV and C: NtRDR1 *N. benthamiana* infected with ToLCV are shown.

Fig. 2 Size-class distribution of the (A) redundant and (B) non-redundant sRNAs reads form the sequenced cDNA sRNA libraries from mock-inoculated wild type and *NtRDR1* overexpressing lines of *N. benthamiana*. Size-class distribution of (C) *N. benthamiana* genome mapped redundant and (D) non-redundant reads, and (E) ToLCV genome mapped redundant reads, from ToLCV infected wild-type and *NtRDR1 N. benthamiana* cDNA-sRNA libraries. Data is shown from the reads obtained after NGS of the three biological replicates.

Fig. 3 Sequence logo analysis of ToLCV siRNAs from wild-type *N. benthamiana* showing profile of (A) 21 nt vsiRNAs, (B) 22 nt vsiRNAs, (C) 23 nt vsiRNAs and (D) 24 nt vsiRNAs. The overall height of the stack indicates the sequence conservation at the particular nucleotide position, while the height of characters within the graph indicates the relative frequency of nucleotides at that position.

Fig. 4 Sequence logo analysis of ToLCV siRNAs from *NtRDR1* transgenic *N. benthamiana* showing profile of (A) 21 nt vsiRNAs, (B) 22 nt vsiRNAs, (C) 23 nt vsiRNAs and (D) 24 nt vsiRNAs. The overall height of the stack indicates the sequence conservation at the particular nucleotide position, while the height of characters within the graph indicates the relative frequency of nucleotides at that position.

Fig. 5 Principle component analysis of the differentially expressed sRNAs between (A) wild-type *N. benthamiana* & ToLCV infected wild-type *N. benthamiana*; (B) *NtRDR1 N. benthamiana* & ToLCV infected *NtRDR1 N. benthamiana*; (C) ToLCV infected NtRDR1 *N. benthamiana* & ToLCV infected wild-type *N. benthamiana* (sRNAs aligned to *N. benthamiana* genome) and (D) ToLCV infected *NtRDR1 N. benthamiana* & ToLCV infected wild-type *N. benthamiana* & (sRNAs aligned to ToLCV genome).

Fig. 6 Heat map of differentially expressed sRNAs in between (A) wild-type *N. benthamiana* & ToLCV infected wild-type *N. benthamiana*; (B) *NtRDR1 N. benthamiana* & ToLCV infected *NtRDR1 N. benthamiana*; (C) ToLCV infected NtRDR1 *N. benthamiana* & ToLCV infected wild-type *N. benthamiana* (sRNAs aligned to *N. benthamiana* genome) and (D) ToLCV infected *NtRDR1 N. benthamiana* & ToLCV infected wild-type *N. benthamiana* & (sRNAs aligned to ToLCV genome).

Fig. 7 Effect of *NtRDR1* overexpression and ToLCV infection on the transcript accumulation of various genes in *N. benthamiana*. Transcripts of the following genes were affected A- *subunit-7 of Cop-9 complex*, B- *Pentatricopeptide repeat-containing protein*, C- *Laccase-3*, D- *Glutathione peroxidase-1*, E- *Universal stress protein A-like protein*, F- *Heat shock transcription factor B4*, G-*Auxin Response Factor-18*, H- *WRKY-6* and I- *Short chain dehydrogenase reductase-3a*. For each samples, three biological replicates were used. Tubulin was used as an internal control. Error bars represent standard

deviation calculated from the three biological replicates. p-values denoted by '*', '**', '***' and '****' corresponds to 0.01-0.09, 0.001-0.009, 0.0001-0.0009 and <0.0001, respectively.

Fig. 8 Relative expression of *RDR6* in wild-type and *NtRDR1* transgenic *N. benthamiana* mock-infection and ToLCV infection by qRT-PCR. For each samples, three biological replicates were used. Tubulin was used as an internal control. Error bars represent standard deviation calculated from the three biological replicates. p-values denoted by '*' and '****' corresponds to 0.01-0.09 and <0.0001, respectively.

Supplementary
Tables S1.1. Lists of differentially expressed sRNAs in between wild-type *N. benthamiana* & ToLCV infected wild-type *N. benthamiana*.
Tables S1.2. Lists of differentially expressed sRNAs in between *NtRDR1 N. benthamiana* & ToLCV infected *NtRDR1 N. benthamiana.*
Table S1.3. Lists of differentially expressed sRNAs between ToLCV infected wild-type *N. benthamiana* & ToLCV infected *NtRDR1 N. benthamiana* (sRNAs aligned to *N. benthamiana* draft genome, Niben.genome.v1.0.1.scaffolds.nrcontigs).
Table S1.4. Lists of differentially expressed sRNAs between ToLCV infected wild-type *N. benthamiana* & ToLCV infected *NtRDR1 N. benthamiana* (sRNAs aligned to ToLCV genome DNA-A: AY190290; DNA-B: AY190291).

Table S2.1 Lists of targets of differentially expressed sRNAs between wild-type *N. benthamiana* & ToLCV infected wild-type *N. benthamiana*.
Table S2.2. Lists of targets of differentially expressed sRNAs between *NtRDR1 N. benthamiana* and ToLCV infected *NtRDR1 N. benthamiana.*
Table S2.3. Lists of targets of differentially expressed sRNAs between ToLCV infected *NtRDR1 N. benthamiana* and ToLCV infected wild-type *N. benthamiana* (targets of host aligned sRNAs).
Table S2.4. Lists of targets of differentially expressed sRNAs between ToLCV infected *NtRDR1 N. benthamiana* and ToLCV infected wild-type *N. benthamiana* (targets of ToLCV aligned sRNAs)
Table S3. Summary of total number of differentially expressed siRNAs and their targets.