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ORIGINAL ARTICLE

# Tobacco RNA-dependent RNA polymerase 1 affects the expression of defence-related genes in *Nicotiana benthamiana* upon *Tomato leaf curl Gujarat virus* infection

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## Abstract

***Main Conclusion*** **RNA-dependent RNA polymerase 1 of *Nicotiana tabacum* modulates ToLCGV pathogenesis by influencing a number of defence-related genes in *N. benthamiana* plants.**

**Abstract** Key means of plants protecting themselves from the invading viruses is through RNA silencing. RNA-dependent RNA polymerase-1 (RDR1) is one of the crucial proteins of the RNA silencing pathway, which is induced after infection by viruses. RDR1 functions in the generation of small interfering RNAs (siRNAs) against the viral genome, thus it is antiviral in nature. Here, we used the transgenic *Nicotiana benthamiana* plant expressing *N. tabacum NtRDR1* and observed reduced susceptibility towards *Tomato leaf curl Gujarat virus* (ToLCGV) infection compared to the wild-type *N. benthamiana* plants. To understand the reason for such reduced susceptibility, we prepared high-definition small RNA (sRNA) cDNA libraries from ToLCGV-infected wild-type *N. benthamiana* and *NtRDR1* expressing *N. benthamiana* lines and carried out next-generation sequencing (NGS). We found that upon ToLCGV infection the majority of siRNAs generated from the host genome were of the 24 nucleotide (nt) class, while viral siRNAs (vsiRNAs) were of the 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 estimated the changes in the expression of various defence-related genes, such as *Constitutively Photomorphogenic-9 (COP9) signalosome (CSN) complex subunit-7, Pentatricopeptide repeat containing protein (PPRP), Laccase-3, Glutathione peroxidase-1 (GPX-1), Universal stress protein (USP) A-like protein, Heat shock transcription factor B4 (HSTF-B4), Auxin response factor-18* (*ARF18*)*, WRKY-6* and *Short chain dehydrogenase reductase-3a.* The differential expression of these genes might be linked with the enhanced tolerance of *NtRDR1 N. benthamiana* transgenic plants to ToLCGV. Our study suggests that reduced expression of *subunit-7 of CSN complex* and *WRKY6,* and increased expression of *USPA-like protein* might be linked with the reduced susceptibility of *NtRDR1*-transgenic *N. benthamiana* plants to ToLCGV.

**Keywords** Defence-related genes · Geminivirus · High-definition adapters · Host siRNA · Next-generation sequencing · *Nicotiana tabacum* · *NtRDR1* · Plant virus · RNA-dependent RNA polymerase · Small RNA · *Tomato leaf curl gujarat virus*

## Abbreviations

| | |
|---|---|
| DCL | Dicer-like |
| GPX-1 | Glutathione peroxidase-1 |
| HSTF-B4 | Heat shock transcription factor B4 |
| Nb-RdR1 | NtRDR1-transgenic *N. benthamiana* |

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| | |
|---|---|
| Nb-RdR1-ToLCGV | ToLCGV-inoculated NtRDR1-transgenic *N. benthamiana* |
| Nb-ToLCGV | ToLCGV-inoculated *N. benthamiana* |
| PPRP | Pentatricopeptide repeat containing protein |
| ToLCGV | Tomato leaf curl Gujarat virus |
| USP | Universal stress protein |

## Introduction

RNA silencing, also known as RNA interference (RNAi), protects plants from invading viruses and viroids (Goldbach et al. 2003; Carbonell and Daros 2017; Prakash et al. 2017). The trigger for RNA silencing is the presence of double-stranded RNAs (dsRNAs), which are recognized by specific Dicer-like (DCL) proteins, and are cleaved into small RNAs (sRNAs) of 21–24 nucleotide (nt) in length (Xie et al. 2004; Carmell and Hannon 2004). These sRNAs can be either micro-RNA (miRNA) or small-interfering RNA (siRNA) (Xie et al. 2004). Various sized sRNAs generated by specific DCLs are further recruited by Argonaute (AGO) proteins, leading to silencing of RNA transcripts by post-transcriptional gene silencing (PTGS) or targeting the genomic DNA for transcriptional gene silencing (TGS) (Henderson et al. 2006; Ghildiyal and Zamore 2009). The selection of specific AGO protein to be associated with sRNA, is decided by the 5′ terminal nucleotide of sRNA (Mi et al. 2008).

RNA-dependent RNA polymerases (RDRs) are crucial enzymes which convert small- and single-stranded RNA (ssRNA) into long-dsRNA in either primer-dependent or primer-independent manner, leading to the amplification of the silencing signal (Devert et al. 2015). *Arabidopsis thaliana* genome encodes for six RDR proteins (*AtRDR1-6*) with varied functions (Wassenegger and 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). *RDR*α clade includes AtRDR1, -2 and -6 while RDR3, -4 and -5 belongs to *RDR*γ clade. Functional characterization of RDR3, -4 and -5 is yet elusive. However, the role of RDR1, 2 and 6 have been implicated in providing resistance against viruses through PTGS and TGS (Searle et al. 2010; Wang et al. 2010; Lewsey et al. 2016). Among RDRs, the expression of *RDR1* is induced upon infection with viruses and viroids, and also found to be increased after exogenous application of salicylic acid (SA) and jasmonic acid (JA) (Xie et al. 2001; Pandey and Baldwin 2007). Antiviral role of RDR1 and RDR6 has been implicated in several studies in *A. thaliana, N. benthamiana*, and *N. tabacum* (Dalmay et al. 2000; Xie et al. 2001; Qu et al. 2005; Schwach et al. 2005; Wang et al. 2010). During virus infection, RDR1 is involved in the production of 21-nt long virus-activated siRNA (vasiRNA) from the endogenous loci of *A. thaliana* genome, targeting transcripts of *A. thaliana* (Cao et al. 2014). *RDR1* down-regulated lines of tobacco showed 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, 2012). Our previous study suggested the presence of binding sites for Myb family of TFs on the promoter of *RDR1* of various plant species (Prakash and Chakraborty 2019). The extent of RNA silencing is affected when the accumulation of *RDR6* is inhibited in *N. benthamiana*, a plant species which lacks a functional RDR1 (Yang et al. 2004; Qu et al. 2005; Schwach et al. 2005). NtRDR1 suppresses RDR6-mediated antiviral RNA silencing in *N. benthamiana* (Ying et al 2010).

Geminiviruses are non-enveloped plant viruses with circular and single-stranded DNA genome, having genome size range of 2.5–3.2 kb (for monopartite viruses) and 4.8–5.6 kb (for bipartite viruses) (Navas-Castillo et al. 2011; Zerbini et al. 2017). Begomoviruses could associate with extra-viral components, such as betasatellites, for the successful establishment of diseases (Gnanasekaran and Chakraborty 2018; Gnanasekaran et al. 2019). *Tomato leaf curl Gujarat virus* (ToLCGV), belonging to *Begomovirus* genus of *Geminiviridae* family, is one of the predominant begomovirus species in India and therefore, is considered as a major constraint for the production of tomato in India (Chakraborty et al. 2008).

Next generation sequencing (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). 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 and as a result equal representation of various sRNAs in the library is achieved (Xu et al. 2015).

Previously, we have observed that *NtRDR1*-expressing lines of *N. benthamiana* showed reduced susceptibility against ToLCGV infection compared with the wild-type plants (Basu et al. 2018). Thus, to further understand the cause of such reduced susceptibility of *NtRDR1* transgenic plants against ToLCGV infection, we performed sRNA NGS analysis from ToLCGV-infected wild-type *N. benthamiana* and *NtRDR1*expressing transgenic lines of *N. benthamiana.* In the present study, we have reported that the expression of *NtRDR1* in *N. benthamiana* and ToLCGV infection causes differential accumulation of sRNAs. We also predicted target transcripts of differentially accumulated sRNAs and have observed that expression

of many defence-related genes are affected, which could be possibly linked with the reduced susceptibility of *NtRDR1*-expressing lines of *N. benthamiana*.

## Materials and methods

### Source of seeds, plant growth conditions, agro-infection and sample collection

Seeds of non-transgenic (referred in the manuscript as wild type) *N. benthamiana* plants were procured from Central Tobacco Research Institute (CTRI), Andhra Pradesh, India. Seeds of 35S-*NtRDR1*-transgenic *N. benthamiana* plant were a gift from Professor Hui Shan Guo, Chinese Academy of Sciences, Beijing, China. Seedlings were grown for 16 h of daylight in a growth room at 22 °C.

Infectious *Agrobacterium tumefaciens* strain EHA105 harbouring partial tandem repeat constructs of ToLCGV (DNA-A, GenBank accession no. AY190290; DNA-B, GenBank accession no. AY190291) was inoculated in wild-type *N. benthamiana* and *NtRDR1*-transgenic *N. benthamiana* plants as described previously (Kumari et al. 2010; Ranjan et al. 2014). *A. tumefaciens* strain EHA105 carrying the empty vector pCAMBIA2300 was used for mock-inoculation. Systemic leaves were collected from three biological replicates at 21 days post-inoculation (dpi), immediately kept in liquid-nitrogen and stored at − 80 °C until isolation of total RNA was carried out.

### Total RNA isolation for sRNA library preparation

Total RNA from the leaf samples were isolated by TRIzol reagent (Invitrogen) as per the manufacturer's instructions. mirVana miRNA isolation kit™ (Ambion) was used for total RNA purification as per the manufacturer's instructions. After elution of the RNA, ethanol precipitation was performed for concentrating total RNA by the addition of 3 volume of absolute ethanol, 0.1 volume of 3 M sodium acetate and 25 µg/mL glycogen (Ambion) and incubation at − 20 °C overnight. Sample was centrifuged at 15,000*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 min. 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 high definition (HD) adapters and next generation sequencing

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

### Bioinformatics analysis of small RNA sequences

Raw FASTQ files received by BaseClear were converted to FASTA format. Reads containing unassigned nucleotides were excluded and the 3′ HiSeq 2500 adapter sequence (TGGAATTC) was trimmed. The HD signatures (four assigned degenerate nucleotides at the ligating ends) of the reads were also trimmed. Reads were mapped (no mismatch was allowed) to the *N. benthamiana* genome and ToLCGV genome separately (DNA-A and DNA-B) using PatMaN (Prufer et al. 2008). The resulting read counts of small-RNA alignments were used to generate the principal component analysis (PCA) using the tools available within DESeq2 package (Love et al. 2014). For differential expression analysis, the read per million approach was used for the normalization of the reads (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 and to avoid false positive results. sRNA greater than two-fold change in expression were considered differentially expressed. To create Sequence logos for viral reads, reads were aligned to ToLCGV genome (Accession, DNA-A: AY190290; DNA-B: AY190291) and were separated based on size. Sequence logos were prepared using the 'ggseqlogo' package (Wagih 2017).

### Construction of heat map

To have a better understanding and visual representation of the differential expression of the sRNAs, a heat map was constructed. To generate the heat map, the Multi Experimental Viewer (MeV 4.9.0) software was used (Saeed et al. 2003). For clustering, Euclidean distance was used as the distance matrix, and the complete linkage clustering was used as the linkage method.

### Target prediction

Targets of the differentially expressed sRNAs were predicted employing the psRNA Target server (Dai et al. 2018). Targets of sRNAs were predicted against "*N. benthamiana*, transcript, Niben, 101" cDNA library. The 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: 2–13 nucleotide, number of

mismatches allowed in seed region: 2, HSP size: 19, bulge (gap) was allowed, penalty for extending gap: 0.5 and translation inhibition range: 10–11 nucleotide.

### Total RNA isolation for reverse transcription quantitative PCR (RT-qPCR)

Total RNA from the leaf samples (collected at 21 dpi) were isolated by TRIzol reagent (Invitrogen) as per the manufacturer's instructions. 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 oligo dT (1.0 µg/µl) were mixed with the required amount of nuclease-free water (considering the total volume of final mixture 20.0 µl) and incubated at 72 °C for 10 min followed by snap chilling (10 min) 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 Revert Aid 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 °C for 60 min, followed by heat inactivation of reverse transcriptase at 72 °C for 10 min. Relative expression of various transcripts was checked by qRT-PCR (Illumina EcoTM Real-Time PCR System). PowerUp™ SYBRTM Green Master Mix was used for relative quantification of the transcripts. Tubulin (NM_001325628.2) was used as an internal control (reference gene). Prism 8 (GraphPad) was used for plotting individual graphs.

## Results

### *NtRDR1*-transgenic *N. benthamiana* plants showed reduced susceptibility against ToLCGV infection

Both wild-type and *NtRDR1*-transgenic *N. benthamiana* plants, infected with ToLCGV showed systemic symptoms such as mild leaf curling, vein chlorosis and stunted growth at 7–9 dpi. ToLCGV-infected *NtRDR1*-transgenic *N. benthamiana* plants showed symptom remission at 18–20 dpi as compared to wild-type plants. The transgenic *NtRDR1 N. benthamiana* mock plants did not show any phenotypic difference when compared to wild-type mock plants (Fig. 1).

### 24-nt long siRNAs are predominantly derived from the host genome while 21–22-nt long siRNAs are the major class of sRNAs generated from the ToLCGV genome

sRNA deep sequencing was performed by generating sRNA cDNA libraries using HD adapters to reduce the ligation bias. Deep-sequencing data suggested that most of the redundant as well as non-redundant host reads of mock-inoculated and ToLCGV-infected, wild-type and *NtRDR1*-transgenic lines, belong to the 24-nt siRNAs class (Fig. 2a–d). The 21-22-nt siRNAs were the most abundant class of sRNA derived from the ToLCGV genome (Fig. 2e). This finding was consistent with previous reports which suggested that 21–22-nt viral siRNAs (vsiRNAs) are the most abundant sRNAs among all size classes of sRNAs (Yang et al. 2011; Golyaev et al 2019). There was no significant difference in the siRNA population between wild-type and *NtRDR1*-transgenic lines either in mock-treated or in ToLCGV-infected plants, suggesting that presence or absence of RDR1 did not affect the generation of different size-classes of sRNAs.

Further, we analyzed the presence of specific nucleotide at the 5′ terminus of the vsiRNAs to determine the involvement of Argonaute (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, d, e). 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. 3c, f).

### 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 the offset-fold change method (Mohorianu et al. 2011). Differential expression analysis of sRNAs was performed between the following plants—wild-type *N. benthamiana* (Nb-WT) vs ToLCGV-inoculated *N. benthamiana* (Nb-ToLCGV), *NtRDR1*-transgenic *N. benthamiana* (Nb-RdR1) vs ToLCGV-inoculated *NtRDR1*-transgenic *N. benthamiana* (Nb-RdR1-ToLCGV), Nb-RdR1-ToLCGVvs Nb-ToLCGV (host aligned sRNAs) and Nb-RdR1-ToLCGVvs Nb-ToLCGV (ToLCGV aligned sRNAs).

Principal 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. 4a–d). The first two principle components describe 84% variation between Nb-ToLCGV and Nb-RdR1-ToLCGV (Fig. 4c) while approximately 95% variation was observed in rest of the three comparisons described above (Fig. 4a, b, d). PCA based on normalised small RNA expression profile suggested that the sRNA reads from biological replicates of the specific plants (same treatment) clustered together, while the different plants (different treatment) clustered separately. Several host- and virus-derived siRNAs (1129 host siRNAs in Nb-ToLCGV

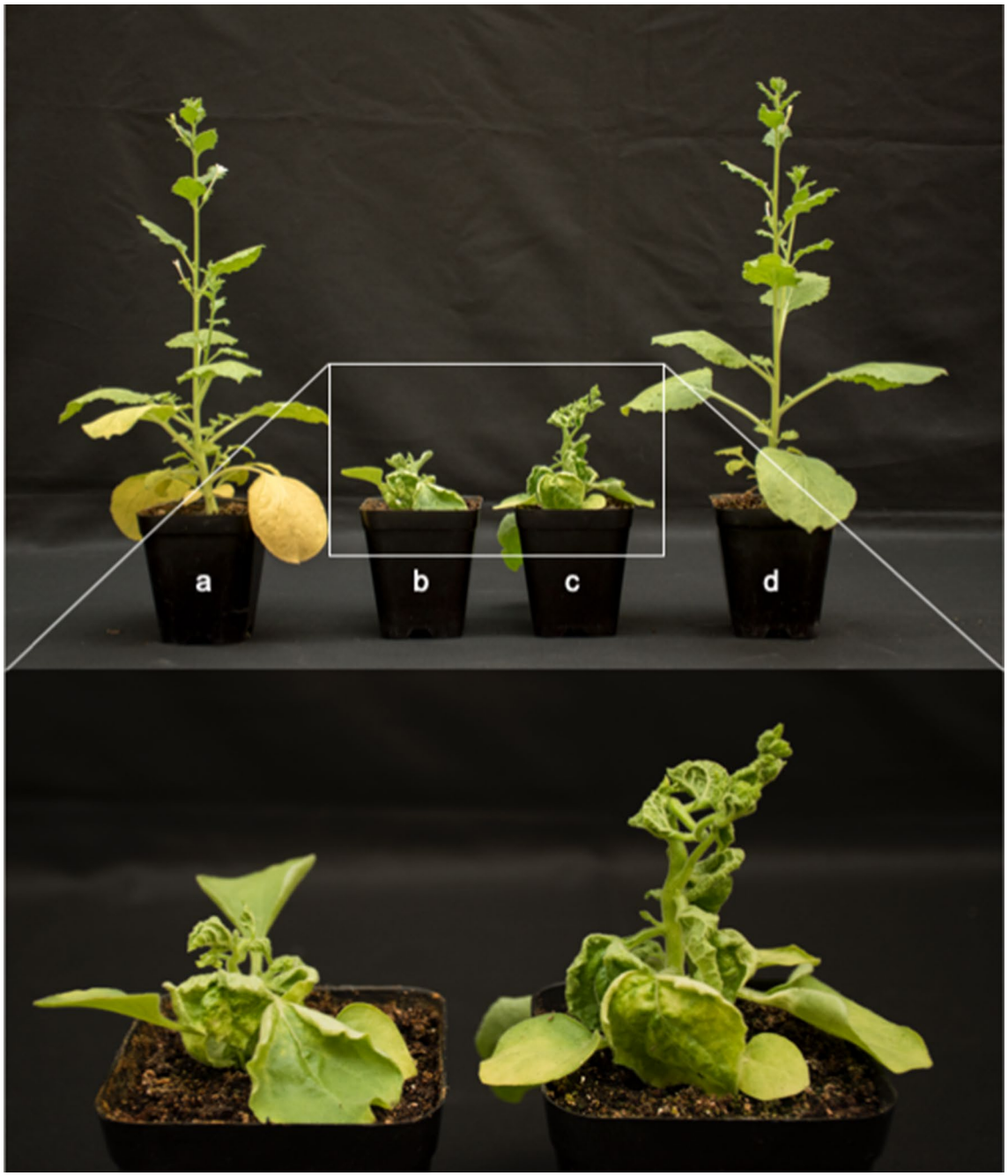


**Fig. 1** Photograph showing wild-type and transgenic *N. benthamiana* plants (expressing *NtRDR1*) infected with ToLCGV (30 dpi). Upper panel: **a** wild-type *N. benthamiana* (mock); **b** wild-type *N. benthamiana* infected with ToLCGV; **c** *NtRDR1 N. benthamiana* infected with ToLCGV; **d** *NtRDR1 N. benthamiana* (mock). Lower panel: enlarged view of **b** wild-type *N. benthamiana* infected with ToLCGV and **c** *NtRDR1 N. benthamiana* infected with ToLCGV

compared to the Nb-WT, 278 host siRNAs in Nb-RdR1-ToLCGV compared to Nb-RdR1, 418 host siRNAs in Nb-RdR1-ToLCGV compared to Nb-ToLCGV and 2337 vsiRNAs in Nb-RdR1-ToLCGV compared to Nb-ToLCGV) were found to be differentially expressed in all the different combinations (Fig. 5, Fig. S1–S3, Table S1.1–S1.4). Targets of the differentially expressed siRNAs of 21–24-nt

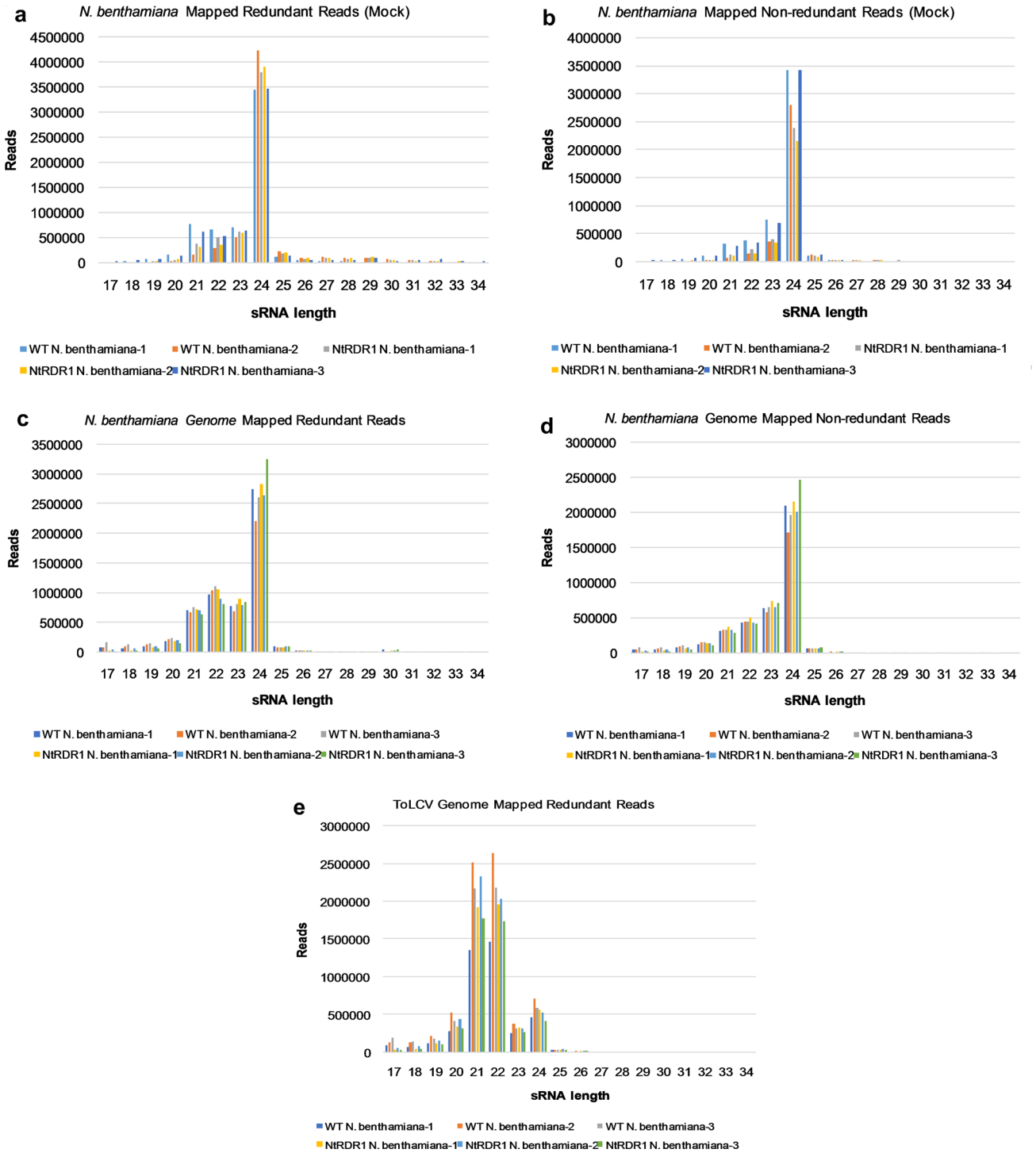


**Fig. 2** Size-class distribution of *N. benthamiana* and ToLCGV mapped sRNA reads. **a** Redundant and **b** non-redundant sRNAs reads form the sequenced cDNA sRNA libraries from mock-inoculated wild-type and *NtRDR1*-expressing lines of *N. benthamiana*. **c**, **d** *N. benthamiana* genome mapped redundant (**c**) and non-redundant (**d**) reads. **e** ToLCGV genome mapped redundant reads, from ToLCGV-infected wild-type and *NtRDR1 N. benthamiana* cDNA-sRNA libraries. Three biological replicates were used for this analysis (except wild-type *N. benthamiana* mock-treated samples, where only two biological replicates were used)

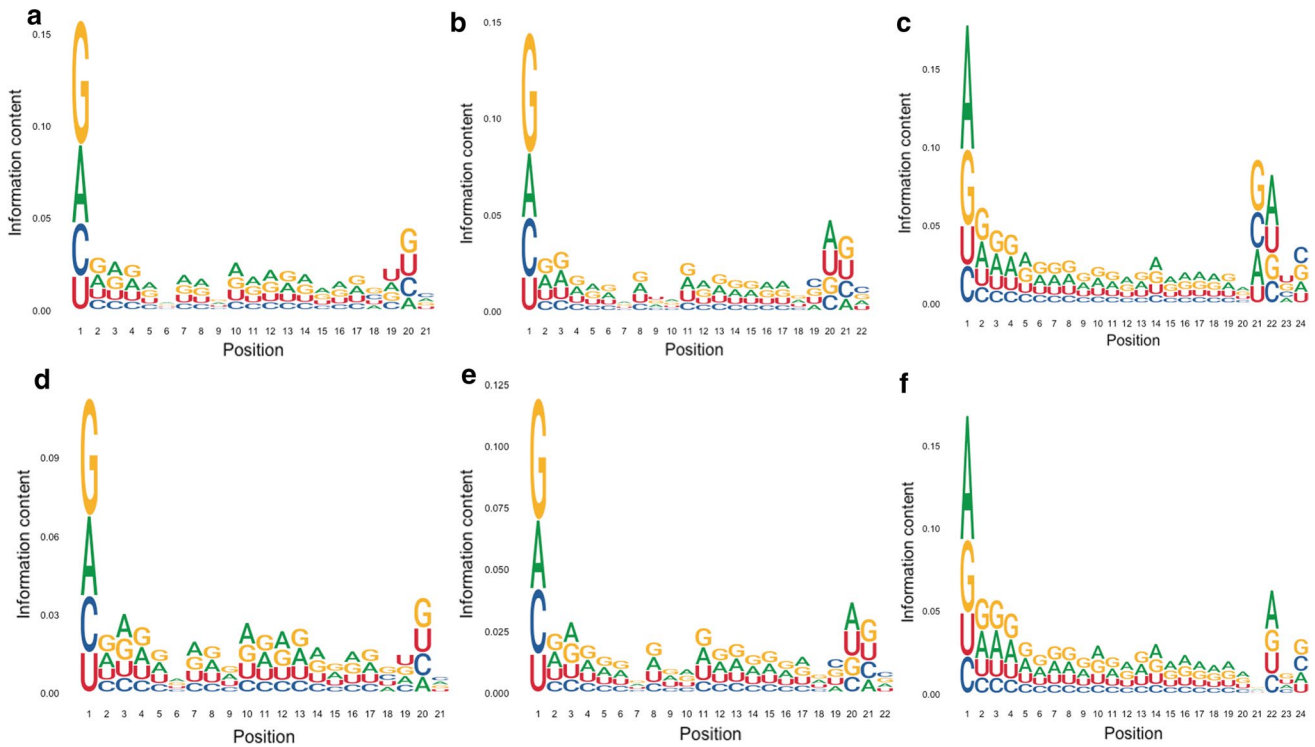


**Fig. 3** **a**–**c** Sequence logo analysis of ToLCGV siRNAs from ToLCGV-infected wild-type *N. benthamiana.* Profile of 21 nucleotide vsiRNAs (**a**), 22 nucleotide vsiRNAs (**b**), 24 nucleotide vsiRNAs (**c**). **d**–**f** Sequence logo analysis of ToLCGV siRNAs from ToLCGV-infected *NtRDR1* transgenic *N. benthamiana*. Profile of 21 nucleotide vsiRNAs (**d**), 22 nucleotide vsiRNAs (**e**), and 24 nucleotide vsiRNAs (**f**). 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

were predicted by psRNA Target server (Dai et al. 2018) (Table S2.1–S2-4).

### *NtRDR1* expression in *N. benthamiana* leads to increased accumulation of several defence-related genes

All the differentially expressed sRNAs of 21–24-nt range were checked for their putative target plant host transcripts (Table S2.1–S2-4). Several sRNAs were predicted to target mRNA transcript of various host genes. A summary of total number of differentially expressed sRNAs and their putative targets are given in Table S3. We checked the expression of nine host genes based on the target prediction data of differentially expressed sRNAs.

Gene expression analysis revealed that *Constitutively Photomorphogenic-9 (COP9) 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)* were enhanced in the *NtRDR1*-transgenic lines of *N. benthamiana* compared to the wild-type *N. benthamiana* plants (Fig. 6a–f). Interestingly, the expression of all of these genes was significantly reduced during ToLCGV infection in *NtRDR1*-transgenic lines.

In addition to these observations, during ToLCGV infection, the expression of *Laccase-3* was found to be significantly reduced in both, wild-type *N. benthamiana* as well as *NtRDR1*-transgenic lines compared with the mock plants (Fig. 6c). Moreover, the expression of *USP A-like protein* was increased in ToLCGV-infected *NtRDR1*-transgenic lines as well as in mock-inoculated *NtRDR1* lines as compared to the ToLCGV-infected wild-type *N. benthamiana* and mock-inoculated plants (Fig. 6e).

We also observed a significant reduction in the transcript accumulation of *Auxin response factor-18* (*ARF18*)*, WRKY-6* and *Short chain dehydrogenase reductase-3a* during ToLCGV infection in *NtRDR1*-transgenic lines compared to the mock plants as well as in ToLCGV-infected wild-type *N. benthamiana* plants (Fig. 6g–i).

## Discussion

*N. benthamiana* is widely used as a model organism for studying plant-pathogen interaction. Being a member of the *Solanaceae* family, *N. benthamiana* is closely related

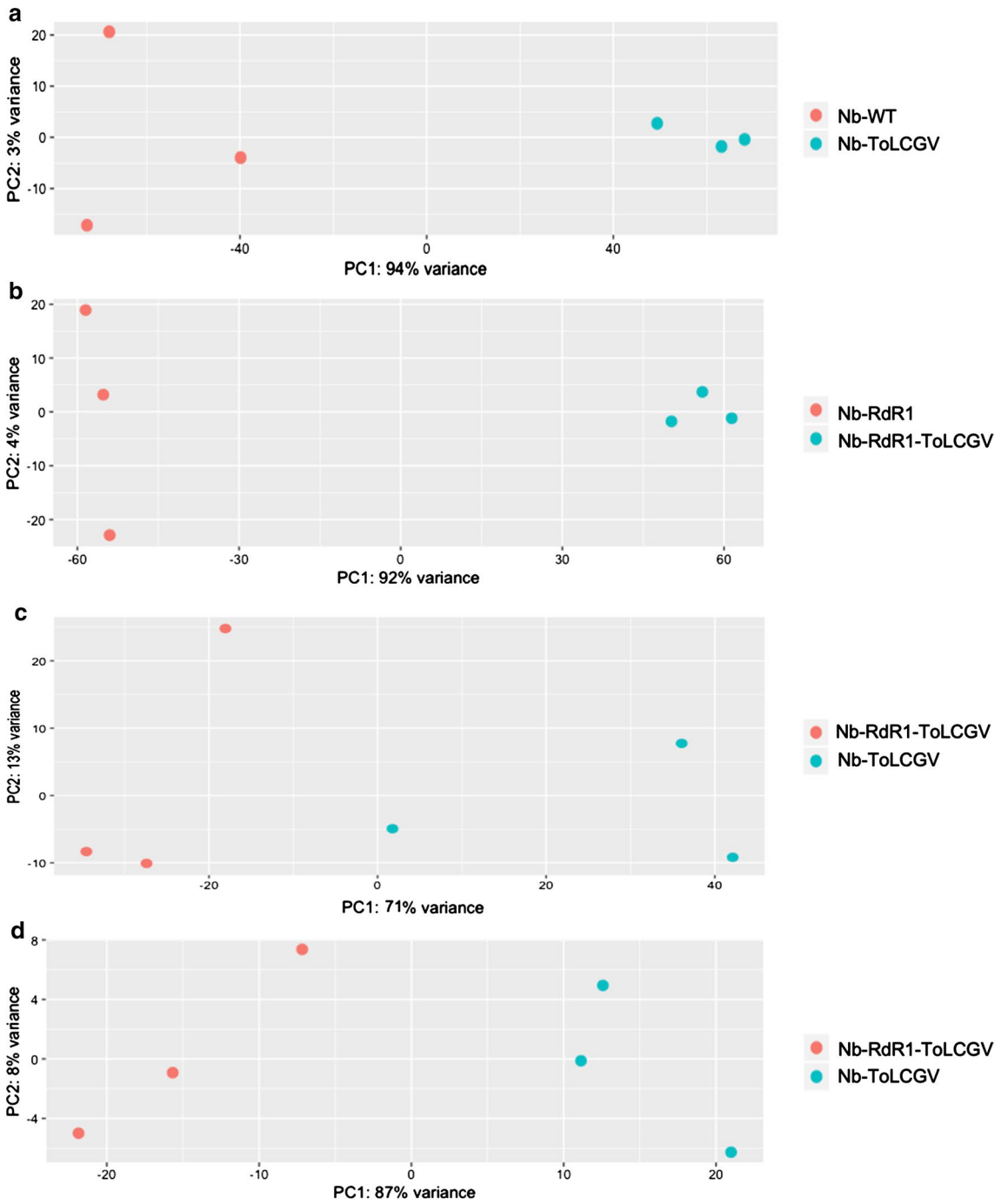


**Fig. 4** Principle component analysis of the differentially expressed sRNAs between **a** wild-type *N. benthamiana* and ToLCGV-infected wild-type *N. benthamiana*, **b** NtRDR1 *N. benthamiana* and ToLCGV-infected *NtRDR1 N. benthamiana*, **c** ToLCGV-infected *NtRDR1 N. benthamiana* and ToLCGV-infected wild-type *N. benthamiana* (sRNAs aligned to *N. benthamiana* genome) and **d** ToLCGV-infected *NtRDR1 N. benthamiana* and ToLCGV-infected wild-type *N. benthamiana* (sRNAs aligned to ToLCGV genome). Reads obtained from *N. benthamiana* mock- and ToLCGV-inoculated plants are indicated as Nb-WT and Nb-ToLCGV, respectively, whereas reads obtained from *NtRDR1*-expressing *N. benthamiana* mock-and ToLCGV-inoculated plants are mentioned as Nb-RdR1 and Nb-RdR1-ToLCGV, respectively

to *S. lycopersicum* (tomato) and *S. tuberosum* (potato), important crops for human nutrition. Thus, components and mechanisms of various biochemical pathways in such plants are believed to be conserved. *N. benthamiana* genome possesses $n = 19$ chromosome (more than 3.5 GB in size) and is sequenced (draft genome availableat https://solgenomics.net/organism/Nicotiana_benthamiana/genome).

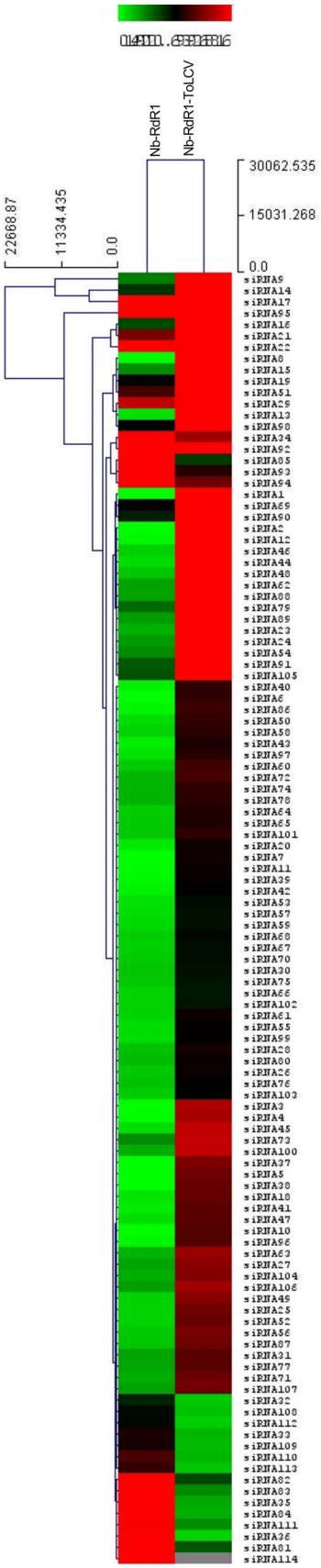


**Fig. 5** Heat map of differentially expressed sRNAs in between mock-inoculated *NtRDR1 N. benthamiana* (labelled as Nb-RdR1) and ToLCGV-infected *NtRDR1*-expressing *N. benthamiana* (labelled as Nb-RdR1-ToLCGV)

Although, *N. benthamiana* possesses the gene encoding for *RDR1,* one of the antiviral factors in RNA silencing pathway, it is a natural 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). It was suggested that in *N. tabacum* RDR1, but not RDR6, functions in the generation of vsiRNAs leading to antiviral RNAi (Xie et al. 2001; Rakhshandehroo et al. 2009) and therefore, is considered as the primary antiviral responder in tobacco. In addition, RDR1 also contributes to SA-mediated antiviral resistance (Ying et al 2010). RDR1 also acts as a defence protein against geminivirus infection and attenuates symptoms by enhancing the methylation of the viral genome (Basu et al. 2018).

In the present study, it was found that ToLCGV infection induces symptoms in both wild-type as well as *NtRDR1*-transgenic *N. benthamiana* at 7–9 dpi. However, at around 20 dpi, *NtRDR1*-transgenic lines were found to show reduced symptoms, suggesting that the antiviral NtRDR1 was functioning in *NtRDR1*-transgenic *N. benthamiana*. At 20 dpi, ToLCGV-infected wild-type *N. benthamiana* plants exhibited more stunted growth as compared to ToLCGV-infected *NtRDR1 N. benthamiana* plants (Fig. 1). However, there was no difference in the phenotype of mock-inoculated wild-type and *NtRDR1*-transgenic plants.

NGS analysis revealed that the sRNAs generated from the host *N. benthamiana* genome were most abundant in 24-nt class. However, the sRNAs generated from the ToLCGV genome were predominantly of 21–22-nt in size. This suggests that DCL2 and DCL4 function as most predominant dicer proteins in the antiviral silencing pathway in *N. benthamiana* against ToLCGV while DCL3 is involved in the production of siRNAs from the host genome. DCL2 and DCL4 are known to function mostly in the PTGS while DCL3 functions in the TGS molecular pathway (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 onto specific Argonaute (AGO) is determined by the 5′ terminal nucleotide characterization of vsiRNA (Takeda et al. 2008). A bias for 'A' at 5′ terminus of the vsiRNA leads to the sorting of vsiRNAs with AGO2 and AGO4 while 'U' and 'C' at 5′ terminus of the vsiRNA are responsible for loading onto AGO1 and AGO5, respectively. So far, the presence of 'G' as the 5′ terminal nucleotide of vsiRNAs has not been linked with the sorting

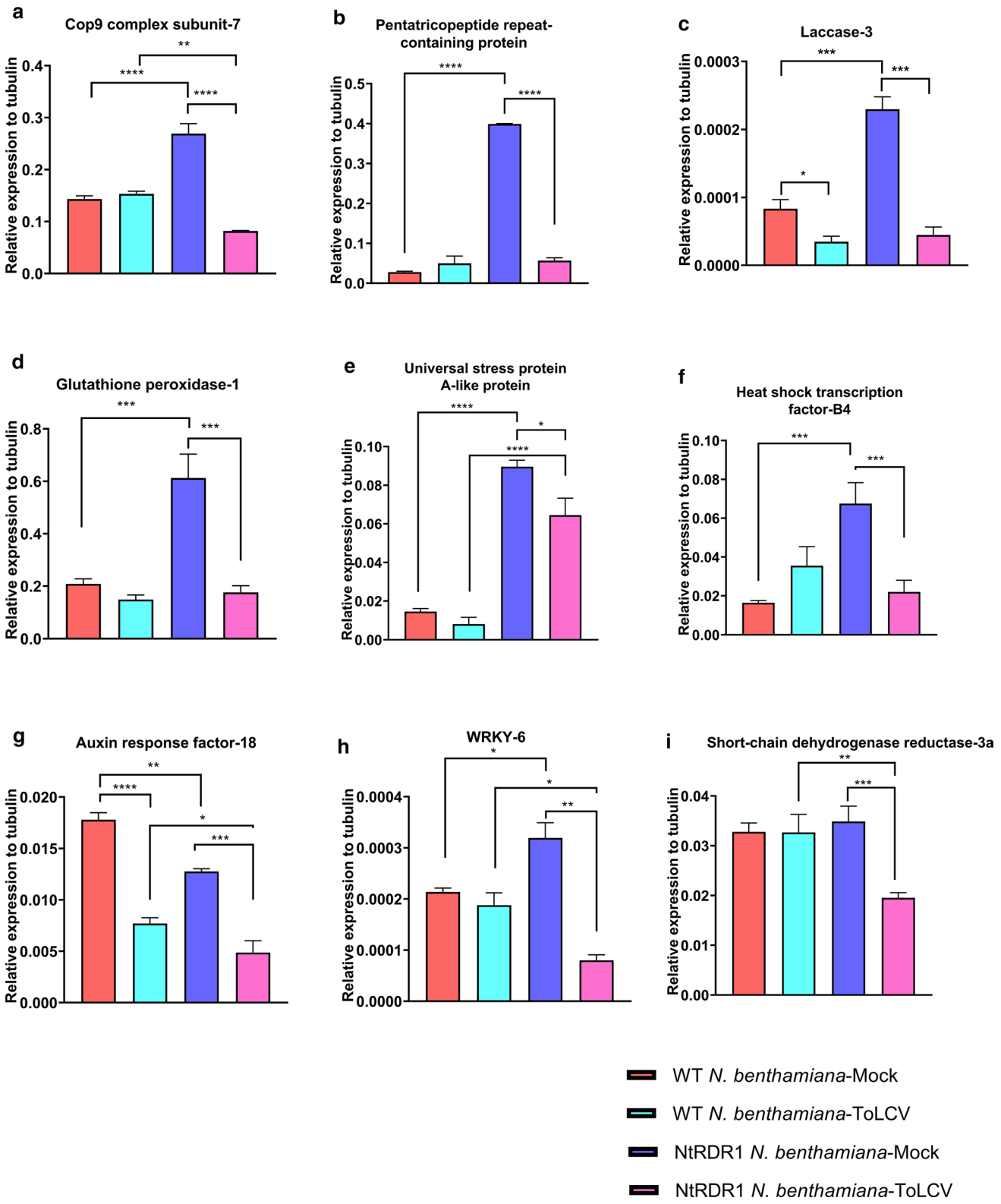


**Fig. 6** Effect of *NtRDR1* expression and ToLCGV infection on the transcript accumulation of various genes in *N. benthamiana.* Transcripts of the following genes were evaluated **a** *Subunit-7 of Cop9 complex*. **b** *Pentatricopeptide repeat-containing protein*. **c** *Laccase-3*. **d** *Glutathione peroxidase-1*. **e** *Universal stress protein A-like protein*. **f** *Heat shock transcription factor B*. **g** *Auxin Response Factor-18*. **h** *WRKY-6*. **i** *Short chain dehydrogenase reductase-3a*. For each sample, 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

with any AGO. Results from our study show that 24-nt long vsiRNAs from both wild-type as well as *NtRDR1*-transgenic lines are predominated with 'A' at the 5′ terminus suggesting that AGO2/AGO4 are involved in the sorting of 24-nt long vsiRNAs.

Viruses hijack components of ubiquitin 26S (Ub-26S) proteasome proteolytic pathways for supporting its own replication by diverting Ub-26S proteasome pathways to new targets such as the modification of AGO protein to suppress the gene silencing (Alcaide-Loridan and Jupin 2012; Byun et al. 2014). CSN regulates E3 ligases by de-conjugating RUB1 (related to Ub) from CRL (Cullin–RING ligases), a multi-subunit enzyme (Schwechheimer and Isono 2010). Through NGS analysis and target prediction of differentially expressed sRNAs, 4.7-fold higher expression of siRNAs that target the *subunit 7 of CSN*, was found in *NtRDR1*-transgenic *N. benthamiana* compared with the wild-type *N. benthamiana*. This was further validated by RT-qPCR analysis. This study revealed that the expression of *subunit 7 of Cop 9 signalosome complex* is decreased in the ToLCGV-infected *NtRDR1*-transgenic *N. benthamiana* but not in theToLCGV-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 ToLCGV infection. Therefore, it could be hypothesized that host has increased level of siRNAs for silencing the components of Ub-26S proteasome pathway so that ToLCGV fails to usurp the Ub-26S proteasome pathway for its benefit (Alcaide-Loridan and Jupin 2012; Randow and Lehner 2009). However, detailed experimental evidence needs to be carried out to verify such notion in the future.

To our knowledge, very few scientific studies have been conducted to understand role of lignin in plant-virus interaction. Reports suggested that increased expression of genes involved in the lignin and SA biosynthesis pathways is linked with enhanced defence against plant viruses (Malinovsky et al. 2014; Anjanappa et al. 2017). Several researchers have suggested the crucial role of lignin in providing defence 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, 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 ToLCGV infection in wild-type as well as in *NtRDR1*-transgenic *N. benthamiana* (Fig. 6c). ToLCGV-infected plants produced enhanced level of siRNAs targeting *Laccase-3,* so that, probably, the rate of lignin degradation would be limited, which would provide strength to the plant during ToLCGV infection. Such a hypothesis requires further experimental evidence in order to be proven correct.

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

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

WRKY6 functions as a positive regulator of the immune response in plants. In *N. attenuata*, WRKY6 is required for resistance against herbivory attack (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* in transgenic *NtRDR1*-transgenic *N. benthamiana* 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, reduced accumulation of *WRKY6* transcripts in ToLCGV-infected *NtRDR1*-transgenic *N. benthamiana* plants was observed. Since, WRKY6 functions in providing resistance to necrotrophic pathogens (probably by increased accumulation of JA), and since JA signaling is antagonistic to SA signaling (Thaler et al. 2012), the reduced expression of *WRKY6* transcripts in ToLCGV-infected *NtRDR1*-transgenic *N. benthamiana* plants might be due to the activation of genes involved in SA and systemic acquired resistance (SAR) pathway. SA and SAR have been implicated in providing defence to the host against viruses (Carr et al. 2010).

In plants, trans-acting siRNAs (tasiRNAs) are produced from the RNA polymerase II-dependent *TAS1-4* transcripts. miRNA-mediated target cleavage of *TAS1-4* transcripts acts as a source for the generation of tasiRNAs. Following the cleavage of *TAS* transcripts, RDR6 converts the remaining transcript into dsRNA. Finally, DCL4 cleaves such dsRNAs into 21-nt long tasiRNAs in a phased manner (Chen et al. 2010). PPRP might be targeted by the tasiRNAs generated from the miRNA173-targeted *TAS1* and *TAS* 2

transcripts, while *ARFs* 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, the present study suggests a possible role of PPRP in plant virus pathogenesis. ToLCGV infection in wild-type *N. benthamiana* triggers an increase in the transcript levels of *HSTF-B4* and *PPRP,* suggesting response of host towards ToLCGV infection. In contrast, the opposite trend was observed in *NtRDR1*-transgenic *N. benthamiana* lines, where, the expression of *HSTF-B4* and *PPRP* was high in mock-inoculated plants and reduced upon ToLCGV infection. Elucidation of such changes in the *HSTF-B4* and *PPRP* transcripts in transgenic lines needs further experiments. Probably, induction of symptom appearance in the host plants infected with ToLCGV 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 short-chain dehydrogenases/reductases (SDRs) in the plant-virus interaction, it is difficult to explain the reduced expression of *SDR3a* in ToLCGV-infected *NtRDR1*-transgenic *N. benthamiana* and therefore, it necessitates further detailed investigation.

## Conclusions

Our study revealed that during ToLCGV infection in *N. benthamiana*, the size of the majority of host siRNAs is 24 nt, while vsiRNAs are of 21–22-nt, suggesting that host genes are silenced by TGS while the viral genes are silenced by the PTGS pathway. In addition, we tried to understand the reason for the reduced susceptibility of *NtRDR1*-transgenic *N. benthamiana* lines. 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 ToLCGV infection (compared with the ToLCGV-infected wild-type *N. benthamiana*) is linked with the reduced susceptibility of *NtRDR1*-transgenic *N. benthamiana* plants.

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*Author contribution statement* SC and TD did the conceptualization. Agro-infection and RNA isolation were performed byVP and AKS. VP generated sRNA library. AS performed read normalization and differential expression analysis. Differentially expressed sRNAs and their predicted targets were analysed by VP and SC. Relative expression analysis was done by VP. VP prepared the original draft of the manuscript. SC, TD, VP, AS and AKS edited the manuscript. SC and TD arranged the funds.

## Compliance with ethical standards

**Conflict of interest** The authors have no competing financial interests.

**Data deposition** The data discussed in this manuscript have been deposited in NCBI's Gene Expression Omnibus (Edgar et al. 2002) and are accessible through GEO Series accession number GSE144074 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE144074).

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