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The virological model plant, *Nicotiana benthamiana* expresses a single functional RDR6 homeolog

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ABSTRACT

The RNA dependent RNA polymerase, RDR6 is involved in a variety of processes including the biogenesis of endogenous regulatory small RNAs, maintaining post-transcriptional gene silencing of transgenes and establishing efficient antiviral RNA silencing. In the virological model plant, *Nicotiana benthamiana*, functional studies of RDR6 has so far only been depended on RNAi based methodologies. These techniques however have inherent limitations, especially in the context of antiviral RNA silencing. To overcome this issue, we created *rdr6* mutant *N. benthamiana* by the CRISPR/Cas9 genome editing system. Using the mutant, most of the proposed functions of RDR6 was confirmed. Additionally, the *rdr6 N. benthamiana* plant recapitulated closely the phenotype of the equivalent *Arabidopsis* mutant. In summary, the *rdr6 N. benthamiana* described here may be employed as a model system not only for the better understanding of the role of RDR6 in pathogen elicited immune responses but in various developmental processes as well.

1. Introduction

RNA silencing is an evolutionarily conserved gene regulatory mechanism (Carthew and Sontheimer, 2009; Poulsen et al., 2013) and in plants, is also an important component of antiviral immunity (Burgyán and Havelda, 2011; Guo and Ding, 2019; Muhammad et al., 2019). Antiviral RNA silencing is triggered by viral double-stranded RNAs (dsRNA) of various origins (replicative intermediates, genomic intramolecular fold-back structures). These molecules are processed into primary viral small interfering RNAs (vsiRNAs) by RNase III-like enzymes (DCLs). The antiviral response is amplified by the actions of endogenous RNA-dependent RNA polymerases (RDRs), which synthesize dsRNAs using aberrant single-stranded (ss) viral RNAs as templates. These dsRNAs serve as substrates for the production of secondary vsiRNAs. Both classes of vsiRNAs are eventually incorporated into argonaute (AGO) protein containing RNA-induced silencing complexes (RISCs), which can limit virus replication via a variety of mechanisms. Viruses have evolved a number of ways to evade detection and neutralization by the above processes including the use of various viral proteins, which interfere with RNA silencing at different steps (viral suppressors of RNA silencing, VSRs).

Functional studies of plant RNA silencing have been greatly benefited from the availability of the extensive collections of *Arabidopsis thaliana* mutants, in which various components of the pathway are compromised. Use of this genetic resource for the understanding of host-virus interactions however, has been greatly restricted due to the fact that *A. thaliana* is a non-host for many viruses, including a number of important plant pathogens. Contrary, the native Australian tobacco plant, *Nicotiana benthamiana* exhibits unparalleled susceptibility to viruses. Additionally, it belongs to the *Solanaceae* family, along with the economically most valuable crop species (potato, pepper, tomato and tobacco) and as such, it is regarded as an appropriate model system to study their viral diseases (Goodin et al., 2008). Notwithstanding, up until now, *N. benthamiana*'s widespread use as a model plant has remained limited due to its allotetraploid nature and the still incomplete sequence of its 3.1 Gb genome (Bally et al., 2018). Functional gene studies in this species mostly rely on RNAi based methodologies (virus-induced gene silencing (VIGS), antisense RNA, short hairpin RNA (shRNA)). Since the 1990s these techniques have emerged as powerful tools, which allowed the assignment of functions to genes, even in plant species with limited genetic resources. Especially VIGS has gained widespread application (mostly due to its speed and robustness) not only in model plant species, but in agronomically important crops as well (Lange et al., 2013; Lacomme, 2015). Though widely used, sceptics often point out that RNAi suppresses gene expression indirectly and the knock-down is frequently only partial (which can also be beneficial for studying genes with vital functions). In addition, these techniques are also plagued by difficult to control off-target effects. Studying

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components of RNA silencing using RNAi presents a further paradox, *viz.*, one has to rely on the activity of those molecules which he wishes to disable. Further complicating the issue is that VIGS vectors (e. g. TRV, PVX) may also interfere with various endogenous cellular functions and cause unintended changes in the expression of non-target genes (Oláh et al., 2013). Viruses also often exhibit antagonistic or synergistic interactions with each other making the results even more complicated to interpret, especially in the context of host-virus interactions. Finally, the virus encoded VSRs and the immense amount of vsiRNA generally produced during viral infection can also incapacitate the silencing machinery, which may compromise the knock-down of the intended target genes. In summary, although RNAi based techniques are still valuable tools of functional genomics, solely they are not suitable to reliably assess the full spectrum of a gene's function. The advent of genome editing offers solutions for most of the problems detailed above. It enables one to generate *bona fide* null alleles of practically any gene with relative ease, even in genetically intractable species like *N. benthamiana*. More sophisticated versions of the technology are also emerging, which allows the creation of any desirable gene modifications at one's discretion (Puchta, 2017).

Most plant species express several *RDR* genes (e. g. *A. thaliana* 6 of them) of which the antiviral roles of *RDR1* and *RDR6* have been extensively documented (Qu, 2010). Interestingly, the *RDR1* gene of the LAB strain of *N. benthamiana* is incapacitated by a 72 bp insertion, explaining the strain's hypersusceptibility to TMV infection (Yang et al., 2004). The *RDR6* gene of *N. benthamiana* has also been shown to be essential for intracellular PTGS (post-transcriptional gene silencing) and for the production of secondary vsiRNAs (Schwach et al., 2005; Qu et al., 2005; Vaistij and Jones, 2009). Although, these results are in general good agreement with the functions of RDR6 described in *A. thaliana* (using *rdr6* mutants), one should keep in mind that in *N. benthamiana* the above observations were made using plants in which RDR6 expression was knocked-down by shRNA. Recently, it has been shown that efficient shRNA mediated gene silencing requires RDR6 (Harmoko et al., 2013). Consistently, significant amount of residual RDR6 activity was found in *N. benthamiana* plants expressing RDR6-specific shRNA (Qin et al., 2012). These findings combined with the general problems of RNAi-mediated gene knock-down detailed above prompted us to attempt the mutagenesis of the *N. benthamiana RDR6* gene using CRIPSR/Cas9.

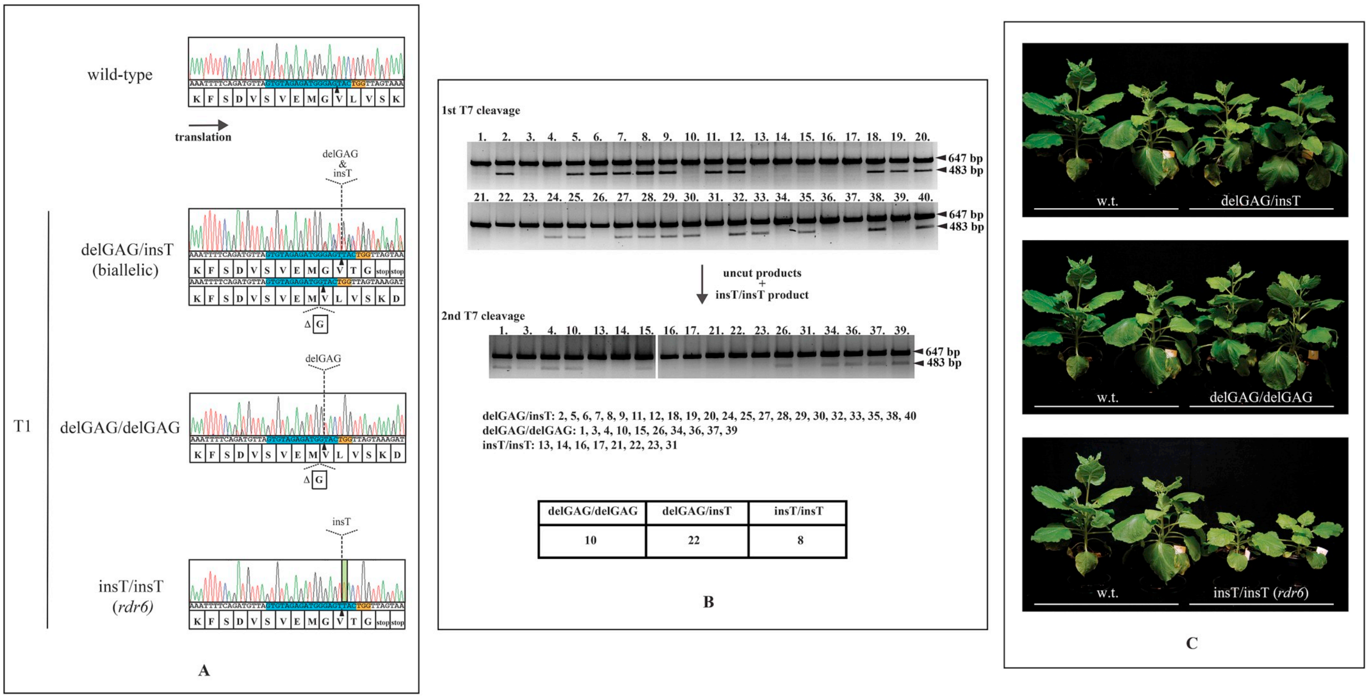
Here we report that the LAB strain of *N. benthamiana* expresses a single functional *RDR6* homeolog. Inactivation of this gene by CRISPR/Cas9 recapitulates the increased virus sensitivity of *rdr6* mutants of other plant species. The *rdr6* mutant *N. benthamiana* described in this report may represent a valuable resource not only for the better understanding of the role of RDR6 in plant antiviral defenses, but also unraveling the functions of various classes of endogenous siRNAs in different developmental processes.

2. Results and discussion

Since *N. benthamiana* is an allotetraploid species, one is expected to find two homeologs of most of the genes, one from each of the two parental subgenomes (Bally et al., 2018). Detailed analyses however, indicated that some genomic regions and even entire chromosomes were lost during the evolution of members of the Australian *Nicotiana* genus. As a consequence, only about half of the *N. benthamiana* genes involved in RNAi have a pair of identifiable and intact homeologs (Nakasugi et al., 2013). About a quarter of them have one intact and one inactive homeolog, while the rest of the genes have each lost their homeolog. For example, the main antiviral argonaute, AGO2 (Harvey et al., 2011; Scholthof et al., 2011) belongs to the last group, with only one identifiable homeolog left in the *N. benthamiana* genome. Consistently, CRISPR/Cas9 mediated inactivation of both alleles of the sole *AGO2* gene made the plant highly susceptible to virus infection (Ludman et al., 2017). The case of *RDR6* however is more complex. Currently, there are two assemblies of the *N. benthamiana* genome publicly available; the Sol Genomics assembly v1.01 and the Benthgenome assembly v0.5 (Bally et al., 2018). The two assemblies share greater than 99.9% identity, indicating that progenies of the same accession were used for the generation of both datasets (most likely the LAB strain of *N. benthamiana*). The Benthgenome assembly contains two *RDR6* cDNAs (Nbv6.1trP76280 and Nbv6.1trP44090). Compared to the *A. thaliana* RDR6, Nbv6.1trP76280 encodes a complete and predictably active RDR6 protein, while Nbv6.1trP44090 contains a premature stop codon and as a consequence the encoded protein is severely truncated and most likely inactive (it lacks the RNA dependent RNA polymerase, RdRp domain). BLAST search of the Sol Genomics database also retrieves two predicted *RDR6* cDNAs (Niben101Scf12609g01010.1 and Niben101Scf03832g02004.1). The lengths of the ORFs found in these cDNAs are close to the full-length *RDR6* gene (3756 bp and 3138 bp) and despite the presence of a few insertions and deletions, they both have the potential to encode intact RDR6 proteins with N-terminal RRM (RNA recognition motif) and C-terminal RdRp domains. Although, the Sol Genomics RDR6 cDNAs contain some assembly errors (Niben101Scf12609g01010.1 lacks the 3’ end of the ORF, some of the insertions in both Niben101Scf12609g01010.1 and Niben101Scf03832g02004.1 are simple sequence duplications due to erroneous assembly, Supplementary Fig. 1), they indicate the possibility that *N. benthamiana* may have two functional *RDR6* homeologs, similarly to other RNAi associated genes (e.g. *AGO1*, *AGO4*).

Earlier we cloned the nine argonaute genes of *N. benthamiana* (Fátyol et al., 2016). Their sequence analysis indicated that the *N. benthamiana* plant line we use in our laboratory was more closely related to the accession from which the Benthgenome assembly was derived. Accordingly, we were able to isolate a full-length RDR6 cDNA from our plant line, which was 99.9% identical to Nbv6.1trP76280 (with only 4 nucleotide mismatches out of 3594). To inactivate *N. benthamiana RDR6*, an sgRNA was designed targeting the 5’ end of the gene. The target region was selected from the conserved part of the ORF, which is also present in all of the predicted RDR6 cDNAs (Supplementary Fig. 2). The sgRNA and the plant codon optimized SpCas9 nuclease were expressed using the pK7WG2D binary plasmid. The vector was introduced into plant cells by the well-established *Agrobacterium*-mediated leaf disc transformation protocol (Clemente, 2006). Following selection on kanamycin containing media a T0 transformant was obtained, which exhibited mixed sequencing chromatogram in the targeted region of the *RDR6* gene (Supplementary Fig. 3). The observed chromatogram could be resolved as a mixture of two individual chromatograms: one containing a T insertion and the other a GAG trinucleotide deletion at the Cas9 cleavage site (3 nt upstream of the PAM sequence) (Fig. 1A). This was also confirmed by sequencing of individual cloned PCR products (data not shown). The single T insertion caused a frameshift in the *RDR6* reading frame and as a consequence led to a premature stop codon. The RDR6 molecule encoded by this allele is most likely dysfunctional since it lacks almost 90% of the protein, including the functionally essential RdRp domain. The GAG trinucleotide deletion resulted in the loss of a single Gly encoding GGA triplet at position 139. Given that the affected region lies outside of the functionally important domains of RDR6 (RRM or RdRp), the protein encoded by this allele is most likely functional. The CCTop plant CRISPR analysis online tool detected potential off target sites for the used sgRNA at the very ends of two highly related genes encoding leucine-rich repeat-containing proteins (gene IDs: Niben101Scf07027g04018.1 and Niben101Scf02203g04006.1). The sequences of these sites are identical and bear three mismatches relative to the *RDR6* target region. One of these mismatches is in the seed region (at the third position upstream of the PAM) and thus is likely to strongly reduce the probability of cleavage by Cas9. Accordingly, sequence analyses revealed no collateral mutations at these sites (Supplementary Fig. 4).

The above biallelic T0 plant was allowed two self-pollinate and T1



delGAG/insT: 2, 5, 6, 7, 8, 9, 11, 12, 18, 19, 20, 24, 25, 27, 28, 29, 30, 32, 33, 35, 38, 40
delGAG/delGAG: 1, 3, 4, 10, 15, 26, 34, 36, 37, 39
insT/insT: 13, 14, 16, 17, 21, 22, 23, 31

delGAG/delGAG	delGAG/insT	insT/insT
10	22	8

Fig. 1. Generation of *rdr6* mutant *N. benthamiana*. (**A**) Sequencing chromatograms of the targeted region of the *RDR6* gene are shown. The fragments were amplified by PCR from T1 plants and sequenced directly. The DNA sequence recognized by the sgRNA is highlighted with blue and the adjacent PAM motif with orange. The Cas9 cleavage site is indicated by an arrowhead. Translations of the target regions are also shown. (**B**) T7 endonuclease assay of the T1 progenies. A 647 bp fragment of the *RDR6* gene encompassing the target region was used as a substrate for the cleavage assay. The reactions were analyzed on 1.8% agarose gel. The 483 bp cleavage product is indicated by an arrowhead. The number of plants belonging to the different genotypes are given in the table. (**C**) Pictures of 2-month-old *N. benthamiana* plants.

plants were obtained. Inheritance of the mutations was analyzed by T7 endonuclease assay (Fig. 1B). Among the 40 randomly selected T1 plants, 22 delGAG/insT biallelic heterozygotes and 18 homozygotes (10 delGAG/delGAG and 8 insT/insT) were identified, which corresponded to the expected Mendelian ratios. The delGAG/insT biallelic heterozygotes and delGAG/delGAG homozygote plants looked phenotypically normal and were also fertile. Contrary, the insT/insT homozygotes (hereafter referred to as *rdr6* plants) were smaller than the age matched wild-type controls. Their leaves were also more elongated and serrated than that of the wild-type plants consistent with the precocious juvenile to adult transition observed in *Arabidopsis rdr6* mutants (Peragine et al., 2004) (Fig. 1C). The *rdr6* plants were not able to produce seeds. Detailed inspection of the flowers revealed reverse hercogamyc arrangement of the anthers and stigma, which favors outcrossing and insect-mediated pollination (in the parental LAB strain the homostylyc arrangement favors self-pollination, Supplementary Fig. 5) (Bally et al., 2018). Though not formally proven, this feature of the *rdr6* plant could be responsible for its sterility under laboratory conditions. Overall, the phenotype of *rdr6 N. benthamiana* (dwarfism, precocious vegetative phase change, decreased fertility) resembles that of the equivalent *Arabidopsis* mutant. Interestingly, in the RDR6 deficient *N. benthamiana* lines reported earlier no phenotypic alterations were noted compared to wild-type plants (Schwach et al., 2005; Qu et al., 2005; Vaistij and Jones, 2009). A plausible explanation for our disparate result, is that while our *rdr6* line is a *bona fide* null mutant the latter ones still possess easily detectable RDR6 activity, which is a consequence of the RNAi technique (shRNA-mediated knock-down) used for their production (Qin et al., 2012).

A key step of sense transgene induced PTGS (S-PTGS) is the conversion of aberrant RNAs into dsRNAs. This step is catalyzed by RDR6. The dsRNAs are then processed by DCL2 and -4 into 22 and 21 nt siRNAs, respectively. *RDR6* deficiency is expected to interfere with the S-PTGS coupled siRNA production. We wanted to test whether the *RDR6* homeolog, which we had inactivated was indeed responsible for the above activity. Leaves of *rdr6* mutant and control wild-type plants were infiltrated with a CaMV promoter driven GFP expression vector either alone or combined with CaMV driven GFP-IR (GFP inverted repeat) and RDR6 expression constructs. Three days later the GFP transgene expression and GFP derived siRNA production was monitored by northern blot analyses (Fig. 2). GFP mRNA expression was significantly higher in the mutant plants than in the wild-type controls indicating that S-PTGS was compromised in the absence of the RDR activity supplied by the mutagenized *RDR6* homeolog. Co-expression of GFP-IR restored silencing demonstrating that the RDR6-independent IR-PTGS (inverted repeat induced PTGS) was still operational in the mutant plants. GFP siRNA levels were negatively correlated with that of GFP mRNA. Over-expression of the RDR6 protein increased GFP siRNA production even further. Consistent with the role of RDR6 supplying mostly DCL2 and -4 with dsRNA substrates, the increase was most substantial in the 21–22 nt size range.

Involvement of RDR6 in antiviral RNA silencing was demonstrated earlier (Qu, 2010). The production of secondary vsiRNAs mainly depends on RDR6. If the *RDR6* homeolog, which we had inactivated is the main source of the above RDR activity, one would expect elevated sensitivity of our *rdr6* plant line to virus infection. To test this, plants with altered RDR6 alleles (insT/insT, delGAG/delGAG, delGAG/insT) and wild-type controls were challenged with different viruses. The symptoms exhibited by PVX (*Alphaflexiviridae*) infected wild-type, delGAG/delGAG homozygote and the delGAG/insT biallelic heterozygote plants were indistinguishable (recovery by 21 dpi, emerging new leaves with diminished symptoms and no GFP signal, Fig. 3A). This observation proves that the delGAG allele is indeed functional and even a single copy of it can provide wild-type level of antiviral protection. In contrast, PVX infected *rdr6* mutants (insT/insT) exhibited dwarfism with strongly symptomatic, GFP positive apical leaves. Sometimes even apical necrosis was evident in these plants. Recovery of the *rdr6* plants

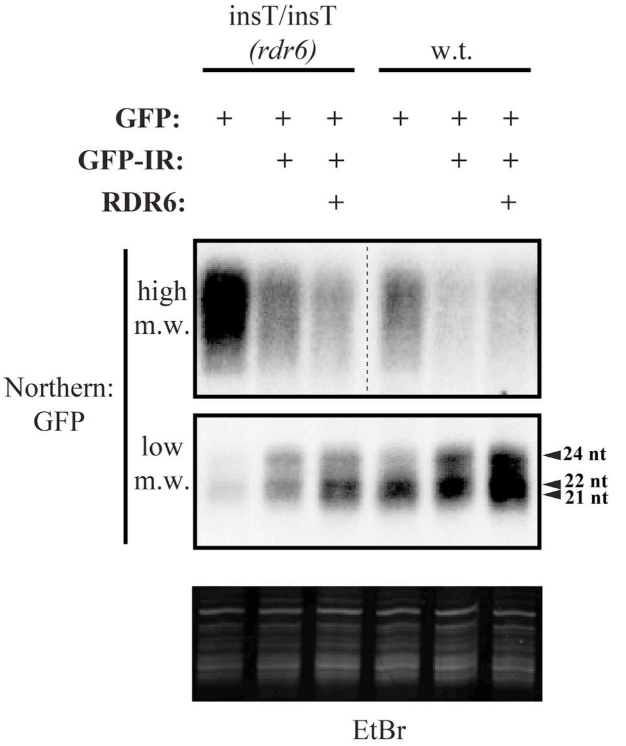


Fig. 2. Sense-PTGS is compromised in *rdr6* mutant *N. benthamiana*. Leaves of wild-type and insT/insT (*rdr6*) homozygote plants were infiltrated with the indicated *Agrobacteria* strains. Three days later RNA was prepared from the infiltrated leaves. GFP mRNA (top) and siRNA (bottom) levels were monitored by northern blots. Small RNAs of different sizes are indicated by arrowheads. The EtBr stained gel is shown as loading control.

was never observed and compared to RDR6 proficient plants they also accumulated more viral RNA and protein (Fig. 3B). The levels of vsiRNAs were also monitored in the PVX infected plants by northern blot analysis (Fig. 3C). Of the tested plant lines, wild-type and *ago2* N. *benthamiana* plants produced the highest amounts of vsiRNAs. Inactivation of both alleles of *RDR6* (insT/insT homozygote) led to a drastic decline in vsiRNA levels, consistent with the overriding contribution of the RDR6-dependent amplification cycle to vsiRNA production. The delGAG/delGAG homozygote and the delGAG/insT biallelic heterozygote plants produced intermediary levels of vsiRNAs. Apparently however, these vsiRNA levels were still sufficient to provide protection from virus infection comparable to that observed in wild-type plants (see above).

The sensitivity of the *rdr6* plant line to infections by viruses belonging to different viral *genera* was also tested. Not unlike PVX, the outcome of TuMV (*Potyviridae*) infection was strongly influenced by the RDR6 status of the plants (Fig. 4A). Compared to wild-type, the *rdr6* plants accumulated more viral RNA and by 21 dpi they almost always exhibited apical necrosis. In contrast, Fny-CMV (*Bromoviridae*) infection was not affected by RDR6 (Fig. 4B). The *rdr6* mutants were slightly more stunted than the age matched controls regardless of the infection. Apart from that, the symptoms caused by Fny-CMV were not significantly different between the two plant cohorts. This observation was in agreement with a previous report demonstrating that shRNA-mediated down-regulation of RDR6 had not aggravated CMV infection (Schwach et al., 2005). Finally, we also infected *rdr6* and control plants with CymRSV (*Tombusviridae*), which resulted in rapid plant death (by 7–10 dpi) regardless of the plants' genotype (Fig. 4C). Tombusviral P19 is a strong suppressor of RNA silencing and may be able to mask the antiviral activity of RDR6. To avoid the interference by P19, the above infections were repeated with a VSR deficient form of CymRSV (CymRSV-ΔP19) (Fig. 4D). The mutant virus produced much milder symptoms than the wild-type one and signs of recovery became obvious in plants of all genotypes by 14 dpi. Importantly however, even after 21 dpi significant delay in recovery was noted in the *rdr6* plants compared to the controls, which was also reflected in the higher viral RNA levels they retained. This observation indicated that RDR6-dependent secondary vsiRNAs contributed to symptom recovery in virus infected plants. In summary, the observed virus sensitivity of the *rdr6* plants was in general good agreement with previous findings made in RDR6i plant lines (Schwach et al., 2005; Qu et al., 2005; Vaistij and Jones, 2009).

3. Conclusions

- To avoid inherent problems associated with RNAi-mediated gene knock-down, we used the CRISPR/Cas9 genome editing tool to create *rdr6* mutant *N. benthamiana.*
- Using the mutant, the essential role of RDR6 in both PTGS and antiviral RNA silencing was unambiguously confirmed in this plant species. Our results also prove that despite its allotetraploid genome

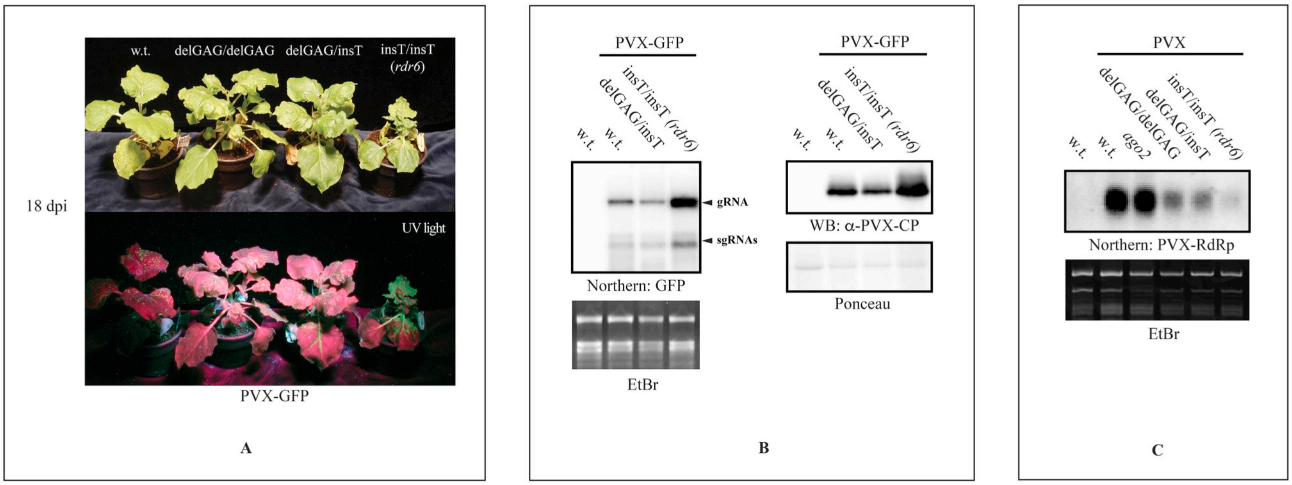


Fig. 3. The *rdr6* mutant *N. benthamiana* plants are hyper-susceptible to PVX infection. (**A**) Photographs of PVX-GFP infected plants of different genotypes. Picture of the same plants was also taken under UV light (bottom). (**B**) Accumulations of viral RNA and protein (CP) in different genotypes of plants were analyzed by northern and western blots. (**C**) Analyses of vsiRNA levels in *N. benthamiana* of the indicated genotypes. PVX-specific vsiRNAs were detected using a fragment of the PVX RdRp gene as a probe. In the first lanes of the northern and western blots samples prepared from uninfected w. t. plants were loaded as negative controls. EtBr stained RNA gels (northern) and Ponceau stained protein filter (western) are shown as loading controls.

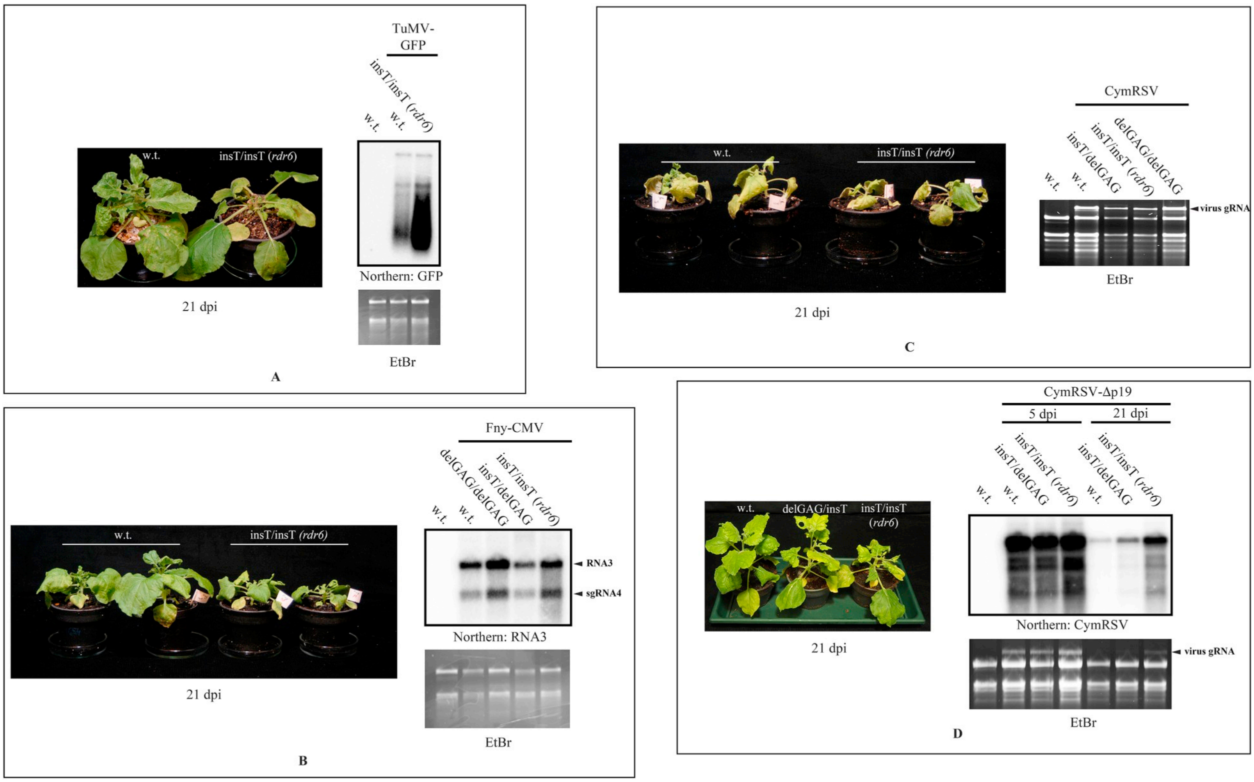


Fig. 4. Differential effects of RDR6 on *N. benthamiana*'s sensitivity towards virus infections. (**A**) The *rdr6* mutant *N. benthamiana* is hypersensitive to TuMV infection. The mutant plants exhibited apical necrosis by 21 dpi and also accumulated more viral RNA than the wild-type ones. The *RDR6* status of the plants did not influence the outcome of infection by either Fny-CMV (**B**) or CymRSV (**C**). Accumulations of virus RNAs were also comparable in the plants regardless of their *RDR6* status. Recovery of CymRSV-ΔP19 infected plants was delayed by RDR6 deficiency (D). By 21 dpi the apical leaves of *rdr6* plants exhibited stronger symptoms and retained higher amounts of viral RNA than that of RDR6 proficient control plants. TuMV, Fny-CMV and CymRSV-ΔP19 accumulations were judged by northern blots. CymRSV levels were monitored on EtBr stained agarose gel. In the first lanes of the northern blots RNA samples prepared from uninfected w. t. plants were loaded as negative controls.

N. benthamiana expresses a single functional RDR6 homeolog.

- The *rdr6 N. benthamiana* mutant line described in this report may represent an important resource to reliably assess the involvement of RDR6 in pathogen elicited defense responses.

4. Materials and methods

4.1. Plasmid construction

Plasmid construction was performed using conventional techniques (Sambrook and Russel, 2001). The bi-functional Cas9-sgRNA targeting plasmid was created as described before (Ludman et al., 2017). The *RDR6* target region was selected using the CCTop-CRISPR/Cas9 target online predictor tool (http://crispr.cos.uni-heidelberg.de/) (Stemmer et al., 2015). The full-length *N. benthamiana RDR6* ORF was assembled in pGEM-T easy plasmid vector from two sub-fragments, which were PCR amplified from leaf cDNA. The full-length ORF was subsequently cloned into pBIN61 binary vector. FLAG and HA epitope tags were added to the N-terminus of the protein using oligonucleotides. Sequences of all plasmids were verified by sequencing.

4.2. Plant transformation

Agrobacterium-mediated leaf disc transformation of *N. benthamiana* was performed essentially as described (Clemente, 2006). Briefly, *N. benthamiana* plants were cultured under sterile conditions on MS medium for 2–3 months. Leaf discs were cut from the plants and infected with a C58C1 *A. tumefaciens* strain carrying the binary Cas9-sgRNA targeting construct. The infected discs were incubated on MS104 agar plates for two days. Next, the discs were transferred onto MS104 agar plates supplemented with 300 μg/ml kanamycin and 500 μg/ml cefotaxime. Every month the discs were passaged onto fresh selective plates. Approximately after three month, the appearing shoots were transferred onto rooting medium. Finally, plantlets with fully developed root system were transplanted into appropriate soil mix and reared under standard greenhouse conditions.

4.3. T7 endonuclease assay

The 648 bp segment of the *RDR6* gene encompassing the target region was PCR amplified from T1 progenies using Phire Plant Direct PCR Kit (ThermoFisher). The first round of T7 endonuclease (New England Biolabs) digestion was carried out using 200 ng of PCR product as substrate according to the manufacturers' instructions. The cut PCR products corresponded to delGAG/insT biallelic heterozygotes. In the next round of cleavage, 100 ng of the uncut products were annealed with 100 ng of the equivalent *RDR6* fragment amplified from an insT/insT homozygote plant (verified by sequencing) and subsequently subjected to T7 cleavage as above. The cleavage products were analyzed on a 1.8% TBE-agarose gel. The cleaved PCR products were derived from delGAG/delGAG homozygotes while the uncut fragments were representing insT/insT homozygotes (*rdr6* mutants).

4.4. RNA analyses

RNA preparations, conventional and small RNA analyses were performed as described before (Fátyol et al., 2016).

4.5. *Agroinfiltration, protein analysis*

Agroinfiltration of *N. benthamiana* leaves and Western blot analysis of protein lysates were carried out as described (Fátyol et al., 2016).

4.6. *Virus inoculations*

Virus inoculations were performed as described before (Ludman et al., 2017). The experiments were repeated at least three times. The results were highly reproducible and representative data are shown. The protein and RNA samples used for northern and western blots were pooled and prepared from the plants, which constituted the given treatment group.

Note added in proof

While this manuscript was under preparation a paper by Matsuo and Atsumi was published in the journal Planta, which also described the generation of *rdr6* mutant *N. benthamiana* by CRIPSR/Cas9 (Matsuo and Atsumi, 2019). The mutant plant line exhibited the same phenotype as ours. In the Matsuo and Atsumi paper the role of RDR6 in PTGS was also confirmed however, the antiviral role of RDR6 was not assessed.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virol.2019.08.017.

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