Crosstalk between epigenetic silencing and infection by tobacco rattle virus in Arabidopsis

LAURA DIEZMA-NAVAS\textsuperscript{1,2}, ANA PÉREZ-GONZÁLEZ\textsuperscript{2}, HAYDEÉ ARTAZA\textsuperscript{4,†}, LOLA ALONSO\textsuperscript{4,‡}, ELENA CARO\textsuperscript{3}, CÉSAR LLAVE\textsuperscript{1,§} AND VIRGINIA RUIZ-FERRER\textsuperscript{1,}*\textsuperscript{,*}

\textsuperscript{1}Department of Microbial and Plant Biotechnology, Centro de Investigaciones Biológicas, CSIC, Ramiro de Maeztu 9, Madrid, Spain
\textsuperscript{2}Doctorado en Biotecnología y Recursos Genéticos de Plantas y Microorganismos Asociados, ETSI Agronómica, Alimentaria y de Biosistemas, Universidad Politécnica de Madrid, 28040, Madrid, Spain
\textsuperscript{3}Centro de Biotecnología y Genómica de Plantas, Universidad Politécnica de Madrid (UPM), Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), Campus de Montegancedo UPM, 28223, Pozuelo de Alarcón, Madrid, Spain
\textsuperscript{4}Bionformatic and Statistic Service, Centro de Investigaciones Biológicas, CSIC, Ramiro de Maeztu 9, 28040, Madrid, Spain

SUMMARY

DNA methylation is an important epigenetic mechanism for controlling innate immunity against microbial pathogens in plants. Little is known, however, about the manner in which viral infections interact with DNA methylation pathways. Here we investigate the crosstalk between epigenetic silencing and viral infections in Arabidopsis inflorescences. We found that tobacco rattle virus (TRV) causes changes in the expression of key transcriptional gene silencing factors with RNA-directed DNA methylation activities that coincide with changes in methylation at the whole genome level. Viral susceptibility/resistance was altered in DNA (de)methylation-deficient mutants, suggesting that DNA methylation is an important regulatory system controlling TRV proliferation. We further show that several transposable elements (TEs) underwent transcriptional activation during TRV infection, and that TE regulation likely involved both DNA methylation-dependent and -independent mechanisms. We identified a cluster of disease resistance genes regulated by DNA methylation in infected plants that were enriched for TEs in their promoters. Interestingly, TEs and nearby resistance genes were co-regulated in TRV-infected DNA (de)methylation mutants. Our study shows that DNA methylation contributes to modulate the outcome of viral infections in Arabidopsis, and opens up new possibilities for exploring the role of TE regulation in antiviral defence.

Keywords: Arabidopsis thaliana, disease resistance genes, DNA methylation, plant–virus interactions, plant viruses, RNA-dependent DNA methylation, Tobacco rattle virus, transposable elements.

INTRODUCTION

RNA-mediated epigenetic silencing includes de novo DNA cytosine methylation, maintenance of methylation and demethylation. In plants, DNA methylation targets mainly transposable elements (TEs) and repetitive DNA sequences, and plays essential roles in maintaining genome stability and regulating gene expression (Matzke and Mosher, 2014; Matzke et al., 2015). RNA-directed DNA methylation (RdDM) initiates and re-establishes silencing of TEs through de novo methylation of cytosines (Panda et al., 2016; Wendte and Pikaard, 2017; Wendte and Schmitz, 2018). The ‘non-canonical’ RdDM pathway entails Pol II transcription at DNA loci without epigenetic marks, synthesis of double-stranded RNA (dsRNA) by RNA-DEPENDENT RNA POLYMERASE 6 (RDR6), production of 21- to 24-nt siRNAs by RNase III-like DICER-LIKE 2 (DCL2), DCL3 or DCL4, and recruitment by ARGONAUTE 4 (AGO4) (Duan et al., 2015; Gao et al., 2010; Mari-Ordonez et al., 2013; McCue et al., 2015; Nuthikattu et al., 2013; Panda and Slotkin, 2013; Panda et al., 2016; Stroud et al., 2013). Alternatively, Pol II transcripts associate with AGO4 and undergo 3′–5′ exonuclease trimming by specific nucleases to produce DCL-independent siRNAs (sidRNAs) of 20 to 60 nts (Yang et al., 2016; Ye et al., 2016). The AGO4 loaded complexes base pair with nascent Pol II transcripts and guide DOMAIN REARRANGED METHYLTRANSFERASE 2 (DRM2) for low levels of DNA methylation of homologous sites (Cao and Jacobsen, 2002; Zhong et al., 2014). This initial methylation marks the target loci

*Correspondence: Emails: cesarllave@ciib.csic.es (César Llave); virginia.ruiz@uclm.es (Virginia Ruiz-Ferrer)
†Present address: Department of Clinical Science, University of Bergen, 5020, Bergen, Norway
‡Present address: Genetic and Molecular Epidemiology Group, Spanish National Cancer Research Center (CNIO), Madrid, Spain
§Present address: Department of Plant Physiology, Plant Biotechnology and Molecular Biology Group. Environmental Sciences and Biochemistry School, Castilla-La Mancha University, Toledo, Spain

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for subsequent Pol IV transcription to activate the canonical RdDM pathway, in which Pol IV transcripts are either converted into dsRNA by RDR2 and processed by DCL3 into 24-nt siRNAs or directly loaded onto AGO4 for 3′–5′ trimming into sidRNAs (Haag et al., 2012; Herr et al., 2005; Law et al., 2013; Panda et al., 2016; Wierzbicki et al., 2012; Ye et al., 2016). Pol V nascent transcripts provide scaffold RNAs that interact with AGO4 and AGO6, and guide DRM2 to reinforce DNA methylation of both DNA strands (Duan et al., 2015; Lahmy et al., 2016; Wierzbicki et al., 2009; Ye et al., 2016). AGO4 co-localizes with Pol II whereas AGO6 co-localizes with Pol V, suggesting that AGO4 and AGO6 function sequentially to silence RdDM targets (Duan et al., 2015). RdDM via DRM2 targets all cytosine contexts equally, whereas maintenance methylation occurs separately for each cytosine sequence context (Panda et al., 2016; Stroud et al., 2013; Wendte and Schmitz, 2018). METHYLTRANSFERASE 1 (MET1) and CHROMOMETHYLASE 3 (CMT3) catalyse maintenance of previously established DNA methylation at CG and CHG sites (where H = A, C or T), respectively, whereas maintenance of asymmetric CCH methylation is directed by DRM2 and CMT2 (Cao et al., 2003; Kankel et al., 2003; Stroud et al., 2014). Furthermore, plants encode several DNA glycoxylases/lyases that release transcriptional silencing by actively removing cystosine methylation (Ortega-Galisteo et al., 2008; Penterman et al., 2007). Among them, ROS1 is a negative regulator of RdDM that catalyses DNA demethylation to release homology-dependent transcriptional silencing of hypermethylated target DNA sequences (Gong et al., 2002; Zhu et al., 2007).

RdDM is a major positive regulator in plant disease resistance (Dowen et al., 2012; Yu et al., 2013). Arabidopsis mutants defective in both the RdDM pathway and maintenance of methylation show strong disease resistance towards a range of bacteria and against biotrophic oomycetes, but are more susceptible to necrotrophic fungi (Dowen et al., 2012; Lopez et al., 2011; Lopez Sanchez et al., 2016; Yu et al., 2013). In contrast, mutants affected in DNA demethylation display enhanced resistance to necrotrophic fungi, but increased susceptibility to hemibiotrophic fungal and bacterial pathogens (Le et al., 2014; Lopez et al., 2011; Lopez Sanchez et al., 2016; Yu et al., 2013). The regulatory effects of epigenetic silencing during infection with submicrobial agents are, however, poorly understood. RdDM targeting on plant DNA viruses leads to enhanced methylation of viral minichromosomes in geminivirus-infected plants or changes in histone modification and chromatin compacting in infections with caulimoviruses (Al-Kaff et al., 1998; Raja et al., 2008). Furthermore, plant viruses use viral suppressors of RNA silencing (VSRs) to induce repression of DNA methylation in their hosts (Pumplin and Voinnet, 2013; Ruiz-Ferrer and Voinnet, 2009). In the present study, we investigate the influence of DNA methylation in the control of viral proliferation and antiviral defence against tobacco rattle virus (TRV) in Arabidopsis. We further analyse changes in the transcriptional status and cytosine DNA methylation of TEs and their impact on the expression of neighbouring disease resistance genes.

RESULTS

TRV compromises the expression of DNA methylation genes in Arabidopsis inflorescences

As a first approach to infer the potential role of epigenetic silencing during viral infections, we used quantitative real-time RT-PCR (qRT-PCR) to assess changes in the relative transcript accumulation of a subset of genes involved in DNA methylation in plants exposed to TRV infection. Gene expression analyses were done using inflorescences as TRV is a seed-transmitted virus known to accumulate consistently in reproductive tissues (Donaire et al., 2008). Samples were collected from mock-inoculated plants (negative controls) or systemically infected plants at 7 and 14 days post-inoculation (dpi) because they represented the time points of maximal and minimal TRV accumulation in infected inflorescences, respectively (Fig. 1a). Among the methyltransferases involved in DNA methylation, MET1 exhibited a significant reduction of transcripts in TRV-infected plants compared to mock-inoculated controls at 14 dpi, whereas DRM2 or CMT3 were unaffected (Fig. 1b). We found that genes encoding KOW-DOMAIN CONTAINING TRANSCRIPTION FACTOR 1 (KTF1, also known as SPT5L), NRPE1 (largest subunit of Pol V), RDR2 and, to a lesser extent, AGO6 were significantly up-regulated at 7 dpi (Fig. 1c). Genes encoding KTF1 and RDR2 remained induced at 14 dpi, whereas AGO4, AGO9 and DCL3 transcripts were significantly reduced at this time point (Fig. 1c). During TRV infection, qRT-PCR showed increasing levels of ROS1 transcripts at 7 dpi (Fig. 1d). The remaining genes tested in our study were apparently unaltered by TRV infection. In conclusion, our data suggest a bimodal response to TRV infection. An early first stage (7 dpi) was characterized by (i) activation of genes encoding RNA polymerases RDR2 and Pol V, which generate scaffold transcripts for siRNA production and AGO4/6/9-bound siRNAs binding, respectively, (ii) induction of KTF1, an adaptor protein that binds Pol V-derived transcripts, and (iii) enhanced expression of the repressor of RNA silencing ROS1 (He et al., 2009). At later stages of infection, RdDM genes encoding DCL3, and the executors AGO4 and AGO9 as well as the downstream effector MET1 were down-regulated. These results confirmed changes in the expression of DNA methylation genes in response to TRV infection.

TRV triggers changes in DNA methylation in infected plants

To test if changes in DNA methylation gene expression led to global changes in methylation, we conducted genome-wide methylation profiling (MethylC-seq) of inflorescence tissue from mock-inoculated and TRV-infected plants at 14 dpi. The
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Fig. 1  TRV alters the expression of DNA methylation genes involved in transcriptional gene silencing. (a) TRV genomic RNA1 in Col-0 Arabidopsis inflorescences. Mock-inoculated controls (baseline) were included to discriminate background amplification. (b)–(d) Transcript accumulation of DNA methylation genes. Samples were collected from inflorescence tissues at 7 and 14 days post-inoculation (dpi). Relative expression levels were quantified by qRT-PCR using CBP20 as internal control. Two [DOMAIN REARRANGED METHYLTRANSFERASE 2 (DRM2), INVOLVED IN DE NOVO 2 (IDN2), KOW DOMAIN-CONTAINING TRANSCRIPTION FACTOR 1 (KTF1), DICER-LIKE 3 (DCL3), RNA-DEPENDENT RNA POLYMERASE 2 (RDR2)], three [TRV, CHROMOMETHYLASE 3 (CMT3), ARGONAUTE (AGO), INCREASE IN BONSAI METHYLATION 1 (IBM1), REPRESSOR OF SILENCING 1 (ROS1), NUCLEAR RNA POLYMERASE D1 (NRPD1-POL IV), NUCLEAR RNA POLYMERASE E1 (NRPE1-POL V)] or four [METHYLTRANSFERASE 1 (MET1)] independent biological replicates were analysed. 10× means that values are 10 times bigger than those represented in the graph. Values are expressed using the ΔΔCt method to derive relative fold change ($2^{-\Delta\Delta C}$) ± standard errors. Two-tailed t-test was used to compare transcript accumulation (* P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001).
Bisulphite conversion rate was high (99.6%) in both samples and similar coverage of methylated cytosines (mC) (98.2%) was found in mock-inoculated and TRV-infected plants (Table S1). DNA methylation levels in CG, CHG and CHH showed a rise at the transcription start sites, while the methylation levels of CG also displayed a tendency to increase at the gene bodies. The abundance of genome-wide methylated cytosines in the three possible contexts was comparable between TRV-infected and mock-inoculated plants (Table S1). A deeper inspection of the distribution of global methylation revealed that canonical coding regions (including 5’ UTR, CDS, intron and 3’ UTR regions) were slightly hypomethylated in all the CG, CHG and CHH contexts in TRV-infected plants compared to mock-inoculated controls (Table S1). We applied a linear mixed-effect model to identify differentially methylated regions (DMR) based on the static window method under TRV infection and mock inoculation (Liang...
accumulation was enhanced in the ago4 and ago6 genotypes by not significant due to inter-sample variability. Conversely, TRV at 7 dpi compared to wild-type plants, although differences were presented within the protein-coding gene bodies. Furthermore, we observed hypo- and hypermethylation of cytosines within DMRs at either promoter or protein-coding regions. We found a substantial enrichment of differentially methylated CGs or CHGs within 1.5 kb upstream of the transcriptional start site of protein-coding genes (Table S2). In contrast, DMRs were poorly represented within the protein-coding gene bodies. Furthermore, we observed hypo- and hypermethylation of cytosines within DMRs at both sequence contexts in TRV-infected plants compared to the mock-inoculated ones. Nevertheless, 62% of the TRV-responsive DMRs identified at CG and CHG contexts were hypomethylated.

DNA (de)methylation and TRV proliferation

We next asked how regulation of DNA methylation during the infection affected TRV accumulation in Arabidopsis inflorescences. To answer this question, we inoculated a set of loss-of-function single cmt3, double drm1 drm2 (referred to as drm1/2 hereafter) and triple drm1 drm2 cmt3 (referred to as ddc hereafter) mutants with impaired methyltransferase activities as well as single ago4, ago6, and ago9 mutants. Loss-of-function mutants of MET1 were not tested in this study since met1 knockout homozygous lines were unable to produce inflorescences (Bartee and Bender, 2001). qRT-PCR assays revealed that the kinetic of TRV accumulation in Arabidopsis inflorescences was drastically altered in some DNA methylation mutants. TRV RNA was significantly decreased in ddc mutants at 7 dpi with respect to the wild-type background (Fig. 2a). Mean values of TRV accumulation indicated a ~1.5-fold reduction of TRV levels in drm1/2 and ago6 mutants at 7 dpi compared to wild-type plants, although differences were not significant due to inter-sample variability. Conversely, TRV accumulation was enhanced in the ago4 and ago6 genotypes by 14 dpi (Fig. 2a). In addition, TRV levels in the hypermethylated mutant ros1 were strongly decreased at 7 dpi compared to control plants, whereas ros1 was hypersusceptible to TRV at 14 dpi (Fig. 2a). Collectively, our observations suggested that DNA (de) methylation is an important regulatory system controlling TRV proliferation as the infection progresses.

DNA methylation affects expression of SA-dependent PR1 gene in TRV-infected plants

Several studies showed that salicylic acid (SA)-dependent defence responses are enhanced in DNA methylation defective mutants following inoculation with biotrophic bacterial or necrotrophic fungal pathogens (Lopez et al., 2011; Yu et al., 2013). Hence, we first examined the potential effect of DNA methylation in plant defence during viral infections by measuring PATHOGENESIS-RELATED 1 (PR1), a marker of systemic acquired resistance used to monitor SA-dependent defence, in Arabidopsis inflorescences (Koornneef and Pieterse, 2008). Although PR1 is not directly targeted by DNA methylation (Lopez et al., 2011), qRT-PCR revealed that PR1 transcripts were strongly elevated in ddc infected-mutants compared to wild-type plants at 7 and 14 dpi, whereas loss of CMT3 was associated with reduced expression of PR1 at 7 dpi (Fig. 2b). This observation suggests that DRM and CMT3 methyltransferases could have antagonistic effects on PR1 regulation during the infectious process. Similarly, AGO mutations affected PR1 expression in different ways at 7 or 14 dpi (Fig. 2b). In ago4 mutants, PR1 transcripts were up-regulated at 7 dpi, but repressed at 14 dpi. Loss of AGO6 in TRV-infected plants correlated with reduced PR1 levels at 7 dpi, whereas ago9 mutations caused increasing amount of PR1 transcripts at 14 dpi (Fig. 2b). Finally, ros1 mutants accumulated PR1 transcripts after TRV inoculation to significantly lower levels than wild-type plants at 14 dpi (Fig. 2b). Our observations suggested that expression of SA-dependent PR1 was affected by mutations involving components of the DNA methylation machinery following TRV infection in Arabidopsis plants.

TRV infection promotes transcriptional activation of several TEs

We wondered if down-regulation of DNA methylation effector genes during TRV infection contributes to release TE silencing. To test this idea, we first analysed the transcriptional status of several TEs that are regulated by the RdDM pathway using TRV-infected inflorescences. The class I long terminal repeat (LTR)/Copia-retroelement ATCOPIA4, the LTR/GYPSY-like retroelements ATGP1 and ATHILA2, the non-LTR/short interspersed nuclear element ATSN1, and the class II DNA transposon TAG2 were selected for analysis. qRT-PCR indicated that ATHILA2, ATSN1 and TAG2 transcripts were significantly reactivated at 7 dpi, whereas ATHILA2 and ATGP1 displayed elevated transcript levels at 14 dpi compared to mock-inoculated plants (Fig. 3a). These findings suggested a response to TRV infection that promoted the transcriptional activity of several TEs.

To determine if variations in the transcriptional status of TEs in TRV-infected plants correlated with changes in their methylation profile, we conducted in-house bisulphite sequencing using retroelements of class I (ATCOPIA4, ATSN1) and DNA transposons of class II (TAG2). DNA extracted from inflorescences of TRV-infected or mock-inoculated Arabidopsis at 14 dpi was treated with bisulphite reagent to convert unmethylated cytosines into uracil, followed by PCR to amplify a specific DNA sequence of ~200 bp. When methylation was differentially analysed for both
symmetric (CG and CHG) and asymmetric (CHH) sequences, we found that TAG2 exhibited a modest but significant reduction in CG methylation, whereas CG methylation at the ATSN1 locus was increased in TRV-infected inflorescences (Fig. 3b). These results were reproduced when methylation was assessed using MethylC-seq data and the Bismark computational tool (Fig. S1) (Krueger and Andrews, 2011). These findings indicate that TE activation is not necessarily linked to reduced cytosine methylation as expected if TE expression was solely governed by DNA methylation during TRV infection, and suggest a transcriptional regulation of TEs independent of DNA methylation (Zervudacki et al., 2018). Methylation levels remained unaltered for non-responsive ATCOPIA4 in mock-inoculated and TRV-infected plants (Fig. 3b).

DMRs identified in our analysis contained 29 out of 31 000 TEs in Arabidopsis, of which five were found on methylated CHG and the remaining 24 on methylated CG (Table S3), suggesting that TRV infection had a modest impact on TE methylation in a genome-wide scale.

**DNA (de)methylation controls the expression of several TE-containing disease resistance genes in TRV-infected plants**

A detailed inspection of the Arabidopsis Information Portal (Version Araport11, https://www.araport.org) revealed that 29.25% \( (n = 548) \) of the disease resistance genes in the Plant Resistance Genes database (PRGdb; http://prgdb.org) contained at least one TE upstream of the transcription initiation site (up to ~1500 bp) (Table S4). To test if DNA methylation plays a role in controlling the expression of disease resistance genes with nearby TEs, a set of representative genes containing leucine-rich repeat (LRR) and/or nucleotide-binding (NB) domains was analysed by qRT-PCR in systemically infected DNA methylation...
mutants. We found that transcripts of the At2g15042 LRR family gene as well as transcripts of RECOGNITION OF PERONOSPORA PARASITICA 4 (RPP4, At4g16860) containing a N-terminal Toll and interleukin-1 (TIR)-like domain displayed a significant down-regulation in inflorescences of ddc, ago4 and ros1 mutants compared to wild-type Col-0 plants (Fig. 4). DANGEROUS MIX2H (DM2H, At3g44670), which encodes a resistance protein of the TIR-NBS-LRR class, was also repressed in ddc and ago4 mutants, but induced in ros1 (Fig. 4). Conversely, the At1g59218 locus encoding a disease-resistant protein with a coiled-coil N-terminal domain (CC-NBS-LRR) was up-regulated in ddc and ago4, but repressed in ros1 (Fig. 4). Transcripts of the LRR receptor-like kinase RECEPTOR-LIKE KINASE IN FLOWERS 1 (RKF1, At1g29750) were also increased in ddc mutants, but remained unaltered in ago4 and ros1 mutants (Fig. 4). Collectively, our data indicated that the expression of the disease resistance genes tested in this study was largely influenced by the methylation status of the plant.

Fig. 4 Expression of disease resistance genes in DNA methylation mutants in Arabidopsis. Transcript accumulation of transposable element-containing disease resistance genes was quantified by qRT-PCR using CBP20 as internal control. Samples were collected from inflorescence tissues of DNA methylation Arabidopsis mutants [drm1 drm2 cmt3 (ddc), ago4, and ros1] at 14 days post-TRV inoculation. Values from two (At2g15042, RPP4) or three (At1g59218, DM2H, RKF1) independent biological replicates are represented as fold change ($2^{-\Delta\Delta C_t}$) ± standard errors. Two-tailed t-test was used to compare transcript accumulation (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001).
We tested whether virus infection altered the expression of the above RdDM-targeted disease resistance genes. qRT-PCR revealed differential accumulation of At2g15042 and At1g59218 transcripts in systemically infected inflorescences relative to mock-inoculated controls at 14 dpi (Fig. 5). In contrast, RPP4, RKF1 and DM2H transcripts exhibited a high degree of inter-sample variability in independent biological experiments and no significant difference between infected and mock-treated plants was found (Fig. 5). Although TRV infection interfered with the DNA methylation machinery by down-regulating the expression of several key DNA methylation factors, our observations suggested that it was not sufficient to globally affect the expression of resistance genes that were regulated by DNA methylation-dependent mechanisms.

Fig. 5 Effect of TRV infection on the expression of disease resistance genes in Arabidopsis. Transcript accumulation of transposable element-containing disease resistance genes was quantified by qRT-PCR using CBP20 as internal control. Samples were collected from wild-type inflorescence tissues at 7 and 14 days post-inoculation (dpi). Values from two (At2g15042, RPP4) or three (At1g59218, RKF1, DM2H) independent biological replicates are represented as fold change ($2^{-\Delta\Delta Ct}$ ± standard errors. Two-tailed t-test was used to compare transcript accumulation (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001).

TEs and nearby disease resistance genes are co-regulated in DNA methylation mutants

Given that TEs indirectly modulate the expression of their neighbouring genes (Dowen et al., 2012; Ito et al., 2011; Piya et al., 2017; Yu et al., 2013; Zervudacki et al., 2018), we wondered if the expression of disease resistance genes during the infection could be connected to the epigenetic regulation of associated TEs. We first determined if TEs in the resistance genes tested in this study were targets of DNA methylation by monitoring TE transcript accumulation in DNA (de)methylation mutants at 14 dpi. The LTR/Copia retroelement (At4TE42895) of the ATCOPIA93 family located at the promoter region of RPP4 was down-regulated in ddc, ago4 and ros1 mutants (Fig. 6a). Transcripts of LTR/GYPSY retroelement (At2TE26610) of the ATGP2N family
that overlapped with both the promoter and 5' region of the TRV-induced At2g15042 gene were significantly reduced in ago4 and ros1 (Fig. 6a). A rolling-circle (RC)/HELITRON DNA transposon (At3TE65675) of the ATREP5 family located upstream of the transcription initiation site of DM2H was distinctively repressed in ddc, but induced in ago4 (Fig. 6a). Finally, transcripts of a non-LTR/long interspersed nuclear retroelement (At1TE33670) of the AtLINE1-2 family located upstream of RKF1 were more abundant in the ddc mutant than in the wild-type control (Fig. 6a). These results indicate that genetic inactivation of DNA (de)methylation...
strongly affected the expression of TEs associated with disease resistance genes. Interestingly, our results showed that misregulation of TEs and disease resistance genes (with the exception of DM2H) varied in the same direction in the DNA methylation mutants tested (Figs 5 and 6a). This finding suggests that TEs and their neighbouring genes were co-regulated in these mutants.

Next, we used qRT-PCR to examine the transcriptional status of the above TEs in response to TRV infection at 7 and 14 dpi. qRT-PCR showed that ATCOPIA93 and ATGP2N transcripts were markedly activated in TRV-infected inflorescences at 7 and 14 dpi, respectively (Fig. 6b). ATREPS transcripts accumulated lower transcript levels in infected plants at 7 dpi, whereas ATLINE1-2 transcripts were not significantly different in TRV-infected and mock-inoculated plants (Fig. 6b). Altogether, our data confirmed the previous observation that TRV infection influences the expression of TEs within disease resistance gene clusters regulated by (de)DNA methylation.

**DISCUSSION**

Previous studies have shown epigenetic modifications in response to viral infections in plants (Buchmann et al., 2009; Duan et al., 2012; Yang et al., 2011, 2016; Ye et al., 2016; Zhang et al., 2011; Zhao et al., 2016). Here we take a leap forward by providing a novel view of the crosstalk between viral performance. We prove that TRV-infection in Arabidopis leads to misregulation of several key components of the transcriptional gene silencing machinery. Our results revealed an inducible activation of RDR2, Pol V and AGO6 coding genes in the RdDM pathway in early colonized inflorescences, concomitantly with the peak of TRV accumulation. This was followed by a repression of genes encoding DCL3 and the RdDM effectors AGO9 and AGO4 shortly afterwards, when TRV levels were strongly decreased. This response was further accompanied by down- and up-regulation of the ROSI demethylase and the MET1 methyltransferase at 7 and 14 dpi, respectively. This scenario of dual gene regulation of transcriptional silencing factors seems to differ to that seen during antibacterial defence in Arabidopsis in which a significant down-regulation of DNA methylation genes was observed (Yu et al., 2013). Our MethylC-seq analysis served to identify DMRs in the methylome of plants infected with TRV, supporting the idea that methylation and demethylation events occur in response to viral infections. Our analysis identified a preponderance of hypomethylated DMRs that was consistent with the induction of the demethylase ROS1. In addition, repression of MET1 and DRM2-associated AGO4 or AGO6 may contribute to hypomethylation by negatively impacting maintenance of CG and CHH methylation, respectively, in infected inflorescences (Panda et al., 2016; Wendte and Schmitz, 2018).

In wild-type plants, TRV levels increased significantly in early colonized inflorescence tissues and then sharply decreased likely due to activation of antiviral defences (Donaire et al., 2008). We found that the triple ddc methylase mutant displays reduced viral titres at 7 dpi. This effect was likely attributed to defects in DRM-dependent methylation as the single cmt3 mutant exhibits a wild-type phenotype. Conversely, we reported a phenotype of hypersusceptibility to TRV in inflorescences of ago4 and ago6 mutants at 14 dpi, indicating that AGO4 and AGO6 may have important antiviral functions. These findings support the idea that DNA methylation plays a role in controlling viral proliferation as the infection progresses in Arabidopsis inflorescences. Similarly, DNA demethylation was also relevant in directing the fate of TRV infection as mutant plants with a dysfunctional ROS1 displayed enhanced resistance at 7 dpi, but increased susceptibility at 14 dpi. The question is how perturbations in DNA methylation could shape the outcome of viral infections in Arabidopsis. Arabidopsis mutants impeded in DNA methylation respond to (hemibiotrophic pathogens by activating a defence arsenal controlled by SA that results in increased basal resistance (Lopez et al., 2011; Lopez Sanchez et al., 2016; Luna and Ton, 2012). Accordingly, we observed that increasing levels of PR1 in ddc mutants correlate with higher resistance to TRV, whereas low PR1 transcripts in ros1 or ago4 mutants coincide with higher susceptibility. Although the contribution of PR1 to antiviral defence is unclear (Carr et al., 2010; Fu and Dong, 2013), our data suggest a link between an altered SA-dependent defence response in DNA methylation mutants and TRV proliferation. It is further tempting to propose that effects caused by DNA methylation on TRV proliferation could be coupled to the control of TEs (McCue et al., 2015). In our study, we proved that infection with TRV leads to a significant activation/derepression of ATGP1, ATSN1, TAG2 and ATHILA2. Although we found modest changes in CG methylation in ATSN1 and TAG2, enhanced activation of these TEs during TRV infection was not necessarily connected with partial losses of DNA methylation. This finding suggests that activation/derepression of TEs in TRV-infected plants could take place independently of DNA methylation, perhaps due to transcription factor availability, as reported recently (Zervudacki et al., 2018). Nevertheless, we observed that transcription of at least ATCOPIA93, ATGP2N and ATREPS was altered in TRV-infected methylated mutants, suggesting that TEs may be regulated through DNA methylation-dependent and -independent mechanisms. TEs are mostly dormant in plants, but they can be derepressed in response to multiple stresses, providing promoter enhancer activities for adjacent plant genes (Huettel et al., 2006; Kashkush et al., 2003; Lisch, 2009; Zervudacki et al., 2018). During TRV infection, several TEs (ATCOPIA93, ATGP2N, ATREPS) located in disease resistance gene clusters were activated/derepressed. This finding brings out the possibility that misregulation of promoter-associated TEs may influence expression of nearby
disease resistance genes during TRV infection. It has been reported that transcriptional activation of TEs due to active demethylation contributes to prime the expression of neighbouring defence genes as part of the antimicrobial defence in Arabidopsis (Dowen et al., 2012; Yu et al., 2013; Zervudacki et al., 2018). Also, changes in DNA methylation in promoter TEs are responsible for DNA demethylase-mediated positive regulation of defence genes involved in fungal resistance in Arabidopsis (Le et al., 2014). In a recent study, a few defence genes whose expression was altered in response to biotrophic oomycetes were cis-regulated by DNA methylation/demethylation of TEs in their promoters (Lopez Sanchez et al., 2016). These findings fit with a general model in which DNA methylation exerts transcriptional control over some defence genes during non-stressful conditions, whereas DNA demethylation activates defence responses in plants exposed to microbial pathogens. In our study, we found that the expression of several disease resistance genes and their promoter-associated TEs was transcriptionally co-regulated in DNA methylation mutants infected with TRV. However, the antiviral role of TE-containing resistance gene clusters and how changes in TE expression mediate on TRV resistance remain unclear.

In conclusion, we have shown that TRV infection leads to significant changes in the expression of essential effectors of DNA (de)methylation in Arabidopsis reproductive tissues. We provide evidence suggesting that DNA (de)methylation contributes to viral infection by influencing TRV accumulation throughout the infection process. We have shown that TRV infection triggers activation/derepression of TEs, and that TE regulation likely entails both DNA methylation-dependent and -independent mechanisms. Finally, our results suggest a co-regulation of TEs and nearby disease resistance genes in TRV-infected DNA (de)methylation mutants. Our findings provide new insights into the regulatory roles of epigenetic silencing in compatible viral infections, and alert from potential findings provide new insights into the regulatory roles of epigenetic control of viral infections in plants.

**EXPERIMENTAL PROCEDURES**

**Plant and virus models**

Arabidopsis thaliana plants were grown in controlled environment chambers under 16 h/8 h of light/dark at 22 °C. Wild-type and mutant homozygous lines used in the study were in the ecotype Col-0 background and were PCR genotyped using the primers listed in Table S5. Arabidopsis mutant homozygous lines for cmt3-11t, drm1-2 drm2-2 and drm1 drm2 cmt3 were kindly supplied by Steve Jacobsen (University of California, Los Angeles, CA, USA). The homozygous ago4-2, ago6-3 and ago9-3 mutant lines were donated by James Carrington (Donald Danforth Plant Science Center, St. Louis, MO, USA). The Arabidopsis T-DNA insertion line for ros1 (SALK_045303C) was obtained from the Salk Institute Genomic Analysis Laboratory (La Jolla, CA, USA) (SiGnAL, http://signal.salk.edu/cgi-bin/tdnaexpress). TRV was derived from an infectious clone of which pTRV1 was described previously (Liu et al., 2002). pTRV2-GFP contains the soluble modified yellowfish green fluorescence protein (smGFP) coding gene adjacent to the promoter region of the pea early browning virus (PEBV) (Fernandez-Calvino et al., 2014).

**Viral inoculation**

Plants were rub-inoculated on rosette leaves using fresh inoculum prepared from Nicotiana benthamiana leaves systemically infected with TRV-GFP as described (Fernandez-Calvino et al., 2014). Arabidopsis control plants were mock-inoculated with extracts from healthy leaves and processed as the infected plants. Viral infection was corroborated by RT-PCR using TRV sequence-specific primers (Table S5).

**Real-Time RT-PCR**

Total RNA was extracted using the TRizol reagent (ThermoFisher Scientist, Carlsbad, CA, USA) according to the manufacturer’s instructions and genomic DNA was digested using DNase I (Sigma-Aldrich, St. Louis, MO, USA). One-step qRT-PCR was done as described previously (Fernandez-Calvino et al., 2016). CBP20 (At5g44200) transcripts were used for normalization because of their similar levels of expression in mock-inoculated and TRV-infected tissue in all genotypes tested. Relative gene expression was determined by the ΔΔCt method as 2−ΔΔCt (fold change) where ΔΔCt for each gene is the difference between the average ΔCt in a treated sample minus the average value of expression in control sample. Two to four independent biological replicates were used with two or three technical replicates each. Samples consisted of RNA from inflorescences pooled from 12–14 plants. Differences between experimental and control groups were tested using the two-tailed t-test (Student’s t test) with Welch’s correction. A list of sequence-specific primers used in this study is provided in Table S5.

**Whole genome bisulphite sequencing and data analysis**

Genomic DNA (gDNA) was extracted from Arabidopsis inflorescences using DNeasy Plant Mini kit (Qiagen, Hilden, Germany). Each sample consisted of gDNA from inflorescences pooled from 12–14 plants. Library construction and whole genome bisulphite sequencing (WGBS) were done by BGI-Tech on the Illumina HiSeq2000 using paired-end-reads for 50 cycles. Short Oligonucleotide Alignment Program was used to align the clean reads against the reference genome (TAIR10). Bisulphite sequencing data was analysed using the Web Service for Bisulfite Sequencing Data Analysis (WBSA) (Liang et al., 2014). The WGBS analysis package was downloaded and installed following the guidelines in http://wbsa.big.ac.cn/download/wgs_pipeline_custom.pdf. The input consists of NGS paired-end 100 bp reads.
from TRV-infected and mock-inoculated as well as the TAIR10 genome reference. The output was classified in two functional genic regions (promoter and gene body) with high and low methylation levels. Identification of methylation sites was based on a binomial distribution (N, P) using a 0.01 FDR corrected P value, where the probability P was configured with the 0.005 value as described (Liang et al., 2014). A linear mixed-effect model was applied to identify DMRs under TRV infection and mock inoculation based on the static window method. The prediction of DMRs was made using the WBSA independent module described in http://wbsa.big.ac.cn/download/dmr_pipeline_custom.pdf. The genes with DMRs within their body and flanking sequences were regarded as DMR-associated genes. Identification of DMRs was done in strings of CN, CG and CH, and the Wilcoxon test (P ≤ 0.01) used if both samples had sufficient coverage in these windows and the methylation level of one sample was greater, at least 0.2 (delta methylation level), than that of the other (Liang et al., 2014). The deep sequencing datasets generated in this study have been deposited in the GEO database under accession numbers GSE117419.

Bisulphite conversion and sequencing

DNA from inflorescences of 6-week-old Arabidopsis plants was extracted using a standard CTAB method. Bisulphite treatment was done using the EZ DNA Methylation Gold kit (Zymo Research, Irvine, CA, USA) following the manufacturer’s instructions. Modified DNA was amplified by PCR and cloned into pGEM-T Easy (Promega, Madison, WI, USA). PCR was performed with KAPA2G (Sigma-Aldrich, Kapa Biosystems, Cape Town, South Africa) with the following programme: denaturation (98 °C for 3 min), nine cycles of initial amplification (98 °C for 30 s, 50 °C for 15 s, 72 °C for 30 s), followed by 29 cycles of amplification (98 °C for 30 s, 60 °C for 15 s, 72 °C for 30 s). Oligonucleotides used for bisulphite analysis are detailed in Table S5. For sequencing, ~20 clonates were selected per each treatment. CyMate software was used to compare the converted clones to the original unconverted sequences, to count the converted/unconverted cytosines at each site and to calculate the percentage of methylation (Hetzl et al., 2007). Two independent samples consisting of a pool of approximately 80 plants each were collected and analysed.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher’s web site:

Fig. S1 Differential analysis of transposable element (ATSN1, TAG2 and ATCOPIA4) cytosine methylation using MethylC-seq data and the Bismark computational tool in CG, CHG and CHH contexts from mock-inoculated and TRV-infected inflorescences (14 days post-inoculation).

Table S1 Summary of MethylC-seq data.

Table S2 List of DMR-associated genes in both CG and CHG contexts.

Table S3 List of DMR-associated TEs in both CG and CHG contexts.

Table S4 List of potential disease resistance protein-coding genes as reported in the Plant Resistance Genes database (PRGdb; http://prgdb.org).

Table S5 List of primers used in this study.