Dynamics and biological relevance of DNA demethylation in *Arabidopsis* antibacterial defense

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DNA methylation is an epigenetic mark that silences transposable elements (TEs) and repeats. Whereas the establishment and maintenance of DNA methylation are relatively well understood, little is known about their dynamics and biological relevance in plant and animal innate immunity. Here, we show that some TEs are demethylated and transcriptionally reactivated during antibacterial defense in *Arabidopsis*. This effect is correlated with the down-regulation of key transcriptional gene silencing factors and is partly dependent on an active demethylation process. DNA demethylation restricts multiplication and vascular propagation of the bacterial pathogen *Pseudomonas syringae* in leaves and, accordingly, some immune-response genes, containing repeats in their promoter regions, are negatively regulated by DNA methylation. This study provides evidence that DNA demethylation is part of a plant-induced immune response, potentially acting to prime transcriptional activation of some defense genes linked to TEs/repeats.

epigenetics | RNA silencing | MAMP

The innate immune response is the first line of defense against pathogens and plays a critical role in antimicrobial defense. This response is initiated by host-encoded pattern-recognition receptors (PRRs) that recognize evolutionarily conserved pathogen-derived signatures, known as Microbe-Associated Molecular Patterns (MAMPs), and activate MAMP-Triggered Immunity (MTI) (1). In plants, the few well-characterized PRRs encode transmembrane receptor-like kinases with extracellular leucine-rich repeat (LRR) domains and intracellular kinase domains (1). Furthermore, plants have evolved another strategy to perceive microbial pathogens through disease resistance (R) proteins. Most R proteins belong to the nucleotide-binding site leucine-rich repeat (NB-LRR) class, which shares structural homologies with mammalian innate immune receptors, such as Nucleotide-binding oligomerization domain-containing protein 1 (NOD1) and NOD2, and are thus often referred to as NOD-like receptors (NLRs). They recognize, directly or indirectly, divergent pathogen virulence determinants known as effector proteins, and establish effector-triggered immunity (ETI) (2). In most cases, plant NLRs recognize indirectly pathogen effectors by sensing their effects on plant target proteins, as postulated by the "guard hypothesis" (3). Upon detection of MAMPs or pathogen effectors, plant cells rapidly induce a series of signaling events that involve, for instance, activation of mitogen-activated protein kinase (MAPK) cascades, production of reactive oxygen species (ROS), and differential expression of hundreds of immune-response genes, including short interfering RNAs (siRNAs) and microRNAs (miRNAs) (2, 4). Recently, several siRNAs and miRNAs were found to orchestrate MTI and ETI responses (4, 5), implying a key role of RNA silencing in the regulation of the plant immune system.

Small RNA-dependent DNA methylation is an RNA silencing phenomenon that prevents overexpression and proliferation of transposable elements (TEs) in different organisms. Despite their parasitic nature, TEs have been domesticated by host genomes, notably to modulate the expression of nearby genes during biotic and abiotic stress responses (6–8). In plants, this regulatory mechanism is referred to as RNA-directed DNA methylation (RdDM) and involves the biosynthesis of siRNAs that guide the DNA methylation of TEs and repeats (9). In the *Arabidopsis* RdDM pathway, RNA polymerase IV (PolIV) transcribes single-stranded RNAs, subsequently used as substrates by RNA-dependent RNA polymerase 2 (RDR2) to produce double-stranded RNAs (dsRNAs) (10). These dsRNAs are processed by DICER-Like 3 (DCL3) into ~23- to 24-nt siRNAs, which are loaded onto complexes composed in part of argonaute (AGO) proteins including AGO4 and AGO6 (10). AGO4 is recruited to target loci through base pairing between siRNAs and intergenic RNAs produced by RNA polymerase V (PolV) (11). Through a currently unidentified process, the protein Domains Rearranged Methyltransferase 2 (DRM2) is recruited onto the chromatin to direct both symmetric (CG, CHG) and asymmetric (CHH) methylation (in these methylation types H refers to any nucleotide base other than a G) (10). During DNA replication, CHH methylation is actively perpetuated by the combined action of siRNAs and DRM2, whereas CG and CHG methylation is maintained by Methyltransferase 1 (MET1) and Chromomethylase 3 (CMT3), respectively (10). Furthermore, *Arabidopsis* encodes DNA glycosylases/lyases that can actively erase DNA methylation, among which Repressor Of Silencing 1 (ROS1) negatively regulates RdDM (12).

Whereas DNA methylation has been mostly functionally characterized in plant and animal developmental processes (13), recent findings also indicate a role for this silencing pathway in repressing plant defense toward biotrophic pathogens (8, 14–16), which take up nutrients from living plant cells. Nevertheless, the mechanistic interplay between the dynamic regulation of the RNA silencing machinery and to the transcriptional activation of TEs and pathogen-responsive genes remains ill defined. In addition, little is known about the detailed physiological relevance of RNA-dependent DNA methylation in host–pathogen interactions. This study addresses those issues in the context of *Arabidopsis* antibacterial defense.

Results

Flagellin-Derived Peptide flg22 Derepresses RdDM Targets in *Arabidopsis* Leaves. Flagellin Sensing 2 (FLS2) is a well-characterized plant PRR, which senses the bacterial flagellin-derived peptide flg22, resulting in the differential regulation of hundreds of genes (1, 17, 18). We investigated the potential impact of flg22 on transcriptional gene silencing (TGS) by monitoring the transcriptional status of *AtGP1*, a gypsy long terminal repeat (LTR)-retrotransposon strongly targeted by siRNA-directed DNA methylation (Fig. S1),

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and synergistically controlled by CG methylation (Fig. S2). An *AtGP1* LTR region was fused with the β-glucuronidase *GUS* reporter gene and transformed into *Arabidopsis*. From these stable transformants we selected a reference line in which *GUS* expression was restored upon DNA methyltransferase inhibitor application (Fig. S3). When this line was treated with flg22, *GUS* expression was reactivated mostly within and around the leaf vasculature, notably at secondary veins and at the base of midveins (Fig. 1*A*). These effects correlated with a progressive increase in GUS and endogenous AtGP1 mRNA levels during flg22 elicitation (Fig. 1*B* and Fig. S4), and a higher expression of other well-characterized RdDM targets normally derepressed in DNA methylation-defective mutants (Fig. 1*C* and Fig. S2), suggesting that flg22 has an inhibitory effect on TGS. It is noteworthy that transcripts of *Onsen*, an LTR-retrotransposon strongly targeted by RdDM (Fig. S1), were induced upon flg22 treatment, but not in DNA methylation-defective mutants (Fig. 1*C* and Fig. S2). Thus, as recently reported (6), loss of DNA methylation is not sufficient to reactivate this TE.

Flg22-Triggered Derepression of RdDM Targets Is Associated with the Down-Regulation of a Subset of Coregulated TGS Factors. To get insights into the regulatory mechanisms by which flg22 triggers TGS release, we first monitored mRNA levels of TGS factors over a 9-h time course experiment. We found a significant down-regulation of the key components AGO4, AGO6, Nuclear RNA Polymerase D2 (NRPD2), Nuclear RNA Polymerase D7 (NRPD7), Nuclear RNA Polymerase E7 (NRPE7), Nuclear RNA Polymerase E5 (NRPE5), Involved in *De Novo* 2 (IDN2), KOW Domain-containing Transcription Factor 1 (KTF1), Defective in RNA-directed DNA methylation 1 (DRD1), and MET1 at 3 h and 6 h after flg22 treatment, which correlated with the up-regulation of the early defense-marker gene *Flg22-induced Receptor-Kinase 1* (*FRK1*) (Fig. 2*A*). The majority of these TGS factor mRNAs, which mostly encode components of RdDM activity, regained normal levels at 9 h posttreatment (Fig. S5), when induction of the late defense-marker gene *Pathogenesis-related gene 1* (*PR1*) typically sets in (Fig. 2*A*). A transgenic reporter of *AGO4* transcription was also transiently decreased during flg22 elicitation (Fig. 2*B*), suggesting that repression of *AGO4*, and perhaps other coregulated TGS factors, occurs at the transcriptional level, which may be linked to an overrepresentation of three motifs within their promoters, including the pathogen-responsive W-box element (Fig. S6) (19). Importantly, flg22-triggered down-regulation of TGS factor mRNAs was also associated with a decrease in AGO4 and NRPE5 protein levels (Fig. 2*C*) and a reduction in the expression of *ROS1* (Fig. 2*D*), a gene known to be robustly down-regulated in DNA methylation-defective mutants (20–22). Collectively, these results suggest that flg22 inhibits TGS, at least in part, by repressing RdDM activity.

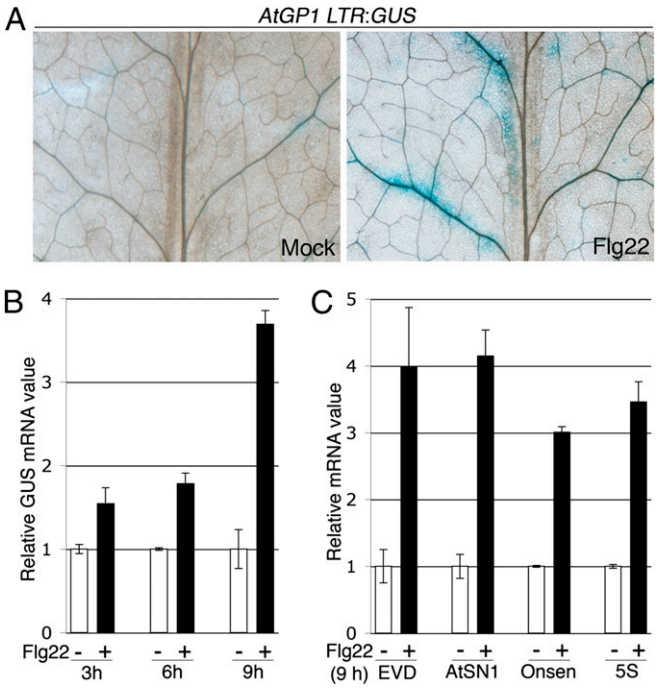

Fig. 1. (*A*) Five- to 6-wk-old *AtGP1 LTR:GUS* leaves were treated with water (mock) or 1 μM of flg22 for 24 h and stained with GUS. (*B*) As in *A* but over a 9-h time course and GUS mRNA levels were analyzed by RT-qPCR. (*C*) As in *B* at 9 h posttreatment and EVD (Evadé, AtCOPIA93), AtSN1 (*A. thaliana* short interspersed element 1), Onsen (AtCOPIA78), and 5S (5S rDNA) transcript levels were analyzed by RT-qPCR. Error bars: SD from three independent PCR results. Similar results were obtained in four independent experiments.

Flg22 Triggers DNA Demethylation at Well-Characterized RdDM Loci. We next monitored DNA methylation levels at well-characterized RdDM targets during flg22 elicitation, using bisulfite sequencing (which identifies the positions of methylated and unmethylated cytosines). We found a progressive flg22-triggered demethylation at the retroelement *AtSN1*, which primarily occurred in the CHH context (Fig. 2*E*). Demethylation at *Onsen*'s *LTR* regions, which are almost exclusively composed of cytosines in the CHH context, was also detected, although a more transient effect occurred during flg22 elicitation (Fig. S7). Notably, DNA demethylation at both *AtSN1* and *Onsen* preceded the up-regulation of their cognate transcripts in multiple independent experiments (Fig. S7), suggesting that demethylation may contribute to the transcriptional activation of these TEs, although other chromatin modifications are also likely to be involved. Noteworthy, the mild effects observed on DNA demethylation levels also suggest that these epigenetic changes may occur in specific immune-response cells such as the ones that surround leaf vasculature and where *AtGP1* was transcriptionally reactivated (Fig. 1*A*).

ROS1 Facilitates Induction and Demethylation of *AtSN1* During flg22 Elicitation. The relatively rapid decrease in DNA methylation upon flg22 exposure suggested the possible implication of an active DNA demethylation process. Given that ROS1 is expressed in vegetative tissues and that it contributes to abiotic stress responses (12), we investigated its contribution in the above regulatory process. We first exposed a loss-of-function mutation in *ROS1* plants to flg22 and monitored the levels of some TEs that are controlled by RdDM. Flg22-mediated induction of *AtSN1* and *AtGP1*, which are known ROS1 targets (23), was altered in *ros1*-elicited plants (Fig. 2*F* and Fig. S8), whereas induction of the retrotransposon *Onsen* was unaffected in the same elicited mutants (Fig S8). These results indicate that transcriptional reactivation of a subset of TEs requires ROS1, which is consistent with a compromised CHH demethylation of *AtSN1* observed in flg22-treated *ros1* mutants (Fig. 2*G*). Therefore, ROS1 presumably contributes to the transcriptional activation of some TEs by constitutively pruning DNA methylation at these loci, thereby potentially accelerating the reduction in DNA methylation caused by the repression of TGS factors during the elicitation (Fig. 2 *A*–*C*). Nevertheless, it remains to be determined whether flg22-triggered repression of ROS1 mRNAs (Fig. 2*D*) also contributes to an eventual remethylation and resilencing of these TEs in a later phase of the elicitation.

DNA Demethylation Restricts Bacterial Multiplication in *Arabidopsis* Leaves and Is Associated with an Activation of the Salicylic Acid-Dependent Defense Response. To explore the potential link between DNA demethylation and antibacterial defense, we first tested the resistance of DNA methylation mutants to the bacterial pathogen *Pseudomonas syringae* pv. *tomato* strain DC3000 (*Pto* DC3000) by syringe inoculation assay. Using this inoculation method, a mild enhanced bacterial growth was observed in *ros1*-infected plants, but not in the *demeter-like 2* (*dml2*) and *dml3*-infected DNA glycosylase/lyase loss-of-function mutants (Fig. S9), supporting a role for ROS1-dependent

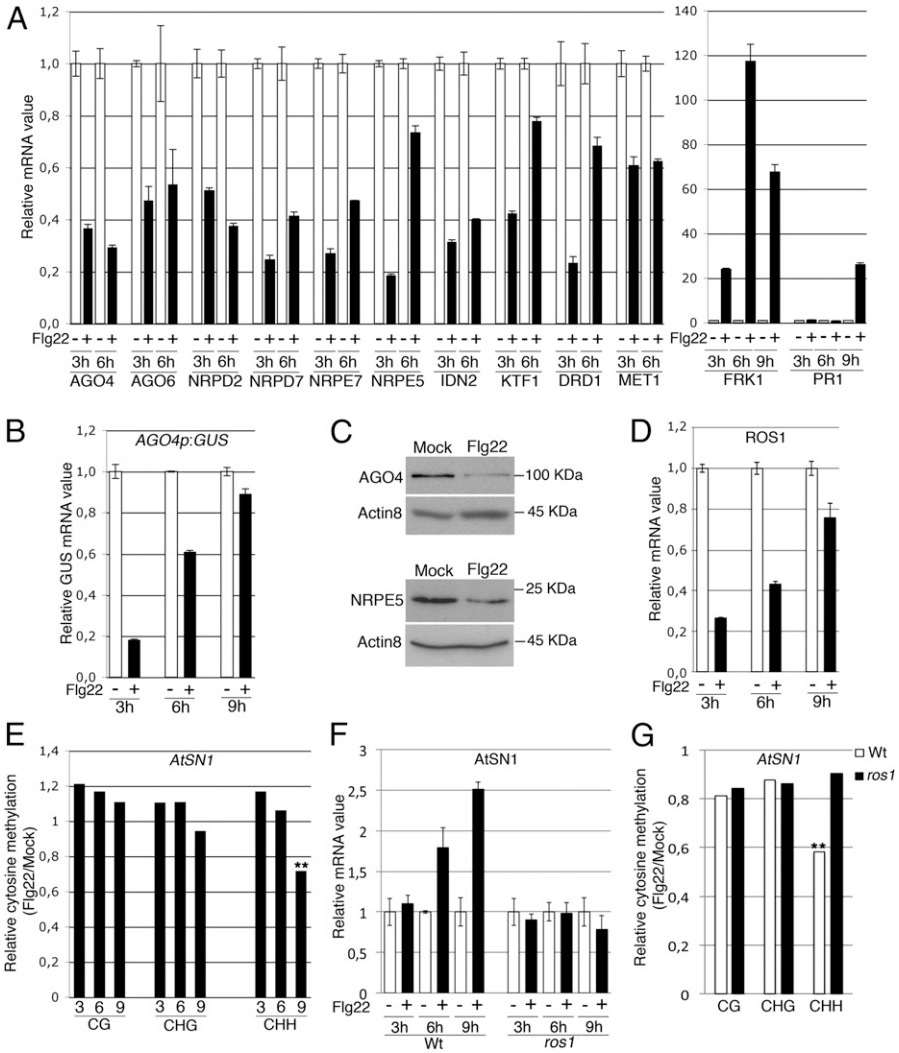

Fig. 2. (*A*) Five- to 6-wk-old WT leaves were syringe infiltrated with water (–) or flg22 (+) and mRNA levels of known TGS factors (*NRPD2*, *NRPD7*, *AGO4*, *AGO6*, *NRPE7*, *NRPE5*, *IDN2*, *KTF1*, *DRD1*, and *MET1*) were monitored at 3 and 6 h post-flg22 treatment with SD as in Fig. 1, *Left*. FRK1 and PR1 transcript levels were also analyzed at 3, 6, and 9 h posttreatment with SD as in Fig. 1, *Right*. Similar results were obtained in five independent experiments. (*B*) GUS mRNA levels were monitored as in *A* from *AGO4*p:*GUS*-elicited plants with SD as in Fig. 1. Similar results were obtained in two independent experiments. (*C*) WT plants were treated as in *A* for 9 h and AGO4, NRPE5, and ACTIN8 protein levels monitored by Western blot analyses. Similar results were obtained in two independent experiments. (*D*) WT plants were treated as in *A* and ROS1 transcript levels were analyzed at 3, 6, and 9 h posttreatment with SD as in Fig. 1. Similar results were obtained in three independent experiments. (*E*) Methylation levels at *AtSN1* analyzed by bisulfite sequencing in plants treated as in *A*. The region analyzed contains four CG, seven CHG, and 33 CHH. Asterisks represent significant differences (**$P < 0.01$). Similar results were obtained in five independent experiments, with kinetics of DNA demethylation starting at 6 or 9 h after flg22 treatment from one experiment to the other. (*F*) mRNA levels of *AtSN1* treated as in *A* in WT and *ros1–4* leaves with SD as in Fig. 1. Similar results were obtained in two independent experiments. Of note, the kinetics of DNA demethylation depicted in *E* are not directly comparable with the kinetics of AtSN1 induction presented in *F*. These experiments are independent. (*G*) Methylation levels at *AtSN1* in plants treated as in *A* for 6 h and analyzed as in *E*. Asterisks represent significant differences (**$P < 0.01$). Similar results were obtained in two independent experiments, with a kinetics of DNA demethylation occurring at 6 h in one experiment and 9 h in the other.

DNA demethylation in antibacterial resistance. This phenotype was also associated with an altered salicylic acid (SA)-dependent defense response as revealed by an attenuated flg22-triggered induction of PR1 in *ros1*- versus WT-elicited plants (Fig. 3*E*). On the contrary, bacterial titers were lower in mutants defective in RdDM activity (i.e., *nrpd2*, *drm1*/*drm2*, and Fig. S9) (8, 16), with stronger resistance achieved in mutants impaired in both the RdDM pathway and maintenance of CG or CHG methylation (i.e., *drm1*/*drm2*/*cmt3* referred to as the *ddc* mutant, *met1*/*nrpd2*, and Fig. S9). This is consistent with the constitutive expression of *PR1* observed in the *ddc* and *met1*/*nrpd2* mutants (Fig. 3*A*), and thus mimics, to some extent, the flg22-induced response observed in wild-type plants (Fig. 2*A*). Enhanced *PR1* expression in nontreated *met1*/*nrpd2* was also associated with constitutive cell death resembling the hypersensitive response (HR), a physiological response that often accompanies plant antimicrobial resistance and that is typically observed in mutants exhibiting autoimmune phenotypes (Fig. 3*B*) (24). Interestingly, this cell death phenotype was confined around secondary veins (Fig. 3*B*), in the same location as the tissues in which *AtGP1* was reactivated upon flg22 treatment (Fig. 1*A*). On the basis of these results we hypothesized that DNA demethylation may restrict bacterial propagation within and around leaf vasculature.

DNA Demethylation Restricts Bacterial Propagation Within Xylem Vessels. To assess the role of DNA demethylation in vascular propagation of *Pto* DC3000, we first determined whether this bacterium could propagate within *Arabidopsis* leaf vasculature. Using wound-inoculation assay of a GFP-expressing *Pto* DC3000 strain in wild-type leaf midveins, we observed bacterial propagation within xylem vessels and restricted to a few vascular bundles, as recently described in *Nicotiana benthamiana* leaves (Fig. 3*C*) (25). We next wound inoculated *Pto* DC3000–GFP in midveins and secondary veins of *ddc* and *met1*/*nrpd2* mutants and found that bacterial propagation was significantly impaired in these mutants with especially strong effects in *met1*/*nrpd2* secondary veins (Fig. 3*D*). Conversely, wound-inoculated *ros1* leaves displayed a significant increased bacterial spread within secondary veins, with chlorosis and necrosis developing in tissues adjacent to the inoculation sites (Fig. 3*F*), which is consistent with the intense transcriptional expression of *ROS1* in the vasculature (Fig. S10). Collectively, these results indicate that DNA demethylation restricts bacterial multiplication and propagation in *Arabidopsis* leaves, suggesting that some immune-response genes are likely to be directly controlled by siRNA-directed DNA methylation and ROS1-dependent DNA demethylation.

***RMG1* is a Disease Resistance Gene That Is Targeted by RdDM and ROS1-Dependent DNA Demethylation.** Plant *NLRs* encode key immune receptors whose overexpression was shown to trigger constitutive HR and/or *PR1* induction in some instances (26–28). The HR-like phenotype and enhanced *PR1* expression observed in *met1*/*nrpd2* (Fig. 3 *A* and *B*) therefore suggested that some *NLRs* might be more expressed in this mutant background, and perhaps, directly controlled by siRNA-directed DNA methylation. Sequencing of RNA extracted from flg22-elicited wild-type

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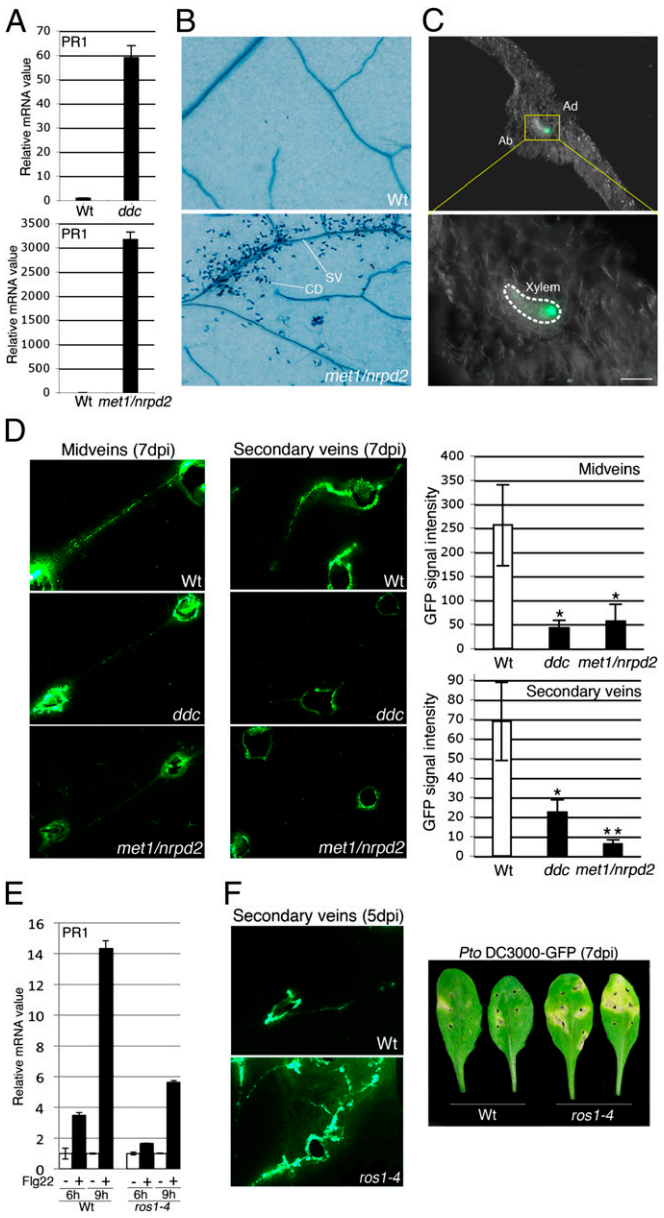

Fig. 3. (*A*) PR1 mRNA levels with SD as in Fig. 1. Similar results were obtained in two independent experiments. (*B*) Trypan blue-stained nontreated WT and *met1-3*($^{-/+}$)*/nrpd2-2* leaves. SV and CD stand for secondary vein and cell death, respectively. Cell death is observed around secondary veins but also at the base of some trichomes in the *met1/nrpd2* background. Similar results were obtained in two independent experiments. (*C*) Five- to 6-wk-old leaves of WT were wound inoculated with a toothpick in midveins with *Pto* DC3000–GFP at 5×10^7 cfu/mL and GFP signal monitored under UV light from transversal sections of leaf blades performed in between two inoculation sites. Pictures were taken at 7-d postinfection (dpi). Ad and Ab stand for adaxial part of the leaf and abaxial part of the leaf, respectively. (Scale bar, 0.05 mm.) Similar results were obtained in three independent experiments. (*D*) Five- to 6-wk-old leaves of WT, *ddc*, and *met1-3* ($^{-/+}$)*/nrpd2-2* were wound inoculated in midveins or secondary veins as in *C* and pictures were taken at 7 dpi (*Left*). GFP fluorescence intensity in midveins or in secondary veins (*Right*). Asterisks represent significant differences in GFP fluorescence intensity (*$P < 0.05$; **$P < 0.01$). Similar results were obtained in three independent experiments. (*E*) WT and *ros1–4* leaves were treated with water (mock) or 1 μM of flg22 for 6 and 9 h and PR1 transcript levels analyzed by RT-qPCR. Error bars: SD from three independent PCR results. Similar results were obtained in four independent experiments. (*F*) WT and *ros1–4* leaves were wound inoculated as in *C* and GFP signal monitored at 5 dpi as in *C* (*Left*). Pictures of bacterial disease symptoms on WT and *ros1–4* wound-inoculated leaves (*Right*). Similar results were obtained in four independent experiments.

leaves uncovered 55 up-regulated NLR transcripts (induced more than twofold; Dataset S1). Among those, 15 carried repeats/siRNA clusters in their vicinity and six of these NLRs were expressed at higher levels in *met1/nrpd2* compared with wild-type plants (Fig. S11). One gene, *At4g11170*, referred to here as *Resistance Methylated Gene 1* (*RMG1*), was expressed at high levels in response to flg22 and in naïve *met1/nrpd2* relative to wild-type plants (Fig. S11) and displayed an earlier and sustained induction in flg22-treated RdDM-defective mutants compared with wild-type-elicited seedlings (Fig. S12). *RMG1* encodes a NB-LRR protein with a Toll/interleukin-1 receptor (TIR) domain at its N terminus. Interestingly, this disease resistance gene contains two helitron-related repeats in its promoter region referred to as *AtREP4* and *AtREP11* (Fig. 4*A*). *AtREP4*, which is the most distal repeat from *RMG1*'s transcription start, was strongly targeted by siRNAs and heavily methylated in all cytosine sequence contexts (Fig. 4*A* and Fig. S13), whereas DNA methylation at a region overlapping the 3′ end of *AtREP11* and the proximity of *RMG1*'s transcription start was weak in wild-type plants but drastically enhanced in all cytosine sequence contexts in the *ros1* mutant background (Fig. 4*A*). Furthermore, both basal expression and flg22-triggered induction of *RMG1* was compromised in *ros1* mutant plants (Fig. 4*B* and Fig. S14). Collectively, these results indicate that the disease resistance gene *RMG1* is a primary RdDM target and that both its basal expression and flg22-triggered transcriptional induction require ROS1 activity.

Discussion

On the basis of these data and on previous findings (8, 29), we conclude that induction of some TEs/defense genes involves a DNA demethylation process during antibacterial defense. In human cells, DNA demethylation of the *Interleukin-6* promoter facilitates the recruitment of specific transcriptional regulators during antiviral defense (30). Our data suggest that DNA demethylation in plants occurs in part through a combination of repression of a subset of coregulated TGS factors and constitutive ROS1-dependent active removal of DNA methylation at some TEs/defense genes. We hypothesize that such DNA demethylation may facilitate the recruitment of PolII and/or transactivators onto their promoters that contain pathogen-responsive elements (Figs. S13 and S15). If such transactivators are constitutively active, derepression will occur in unchallenged DNA methylation-defective mutants (e.g., *AtGP1* or *RMG1*). Alternatively, derepression may require induced and/or activated transactivators and would, therefore, occur solely during antibacterial defense, as observed for the RdDM targets *Onsen* and *WRKY22* (Fig. S2), a defense transcription factor whose flg22-triggered induction is enhanced in RdDM-defective mutants relative to wild-type seedlings (*SI Text* and Figs. S15–S19). We thus propose that DNA demethylation likely prime TE-, as well as defense gene-induction through the concomitant activation of their transactivators and/or the interference with other chromatin marks. Accordingly, higher levels of the active marks H3K4me3/H3K9ac, and lower levels of the repressive marks H3K9me2/H3K27me3, were detected at SA-responsive gene promoters in PolV-defective mutants (16). However, none of these promoters was directly targeted by RdDM, arguing for an indirect effect of those mutations on the chromatin-based status of SA-responsive genes. Transcriptional activation of primary RdDM targets may additionally require the constitutive presence of active chromatin marks at these loci as noticed in the body of *WRKY22* and at the 5′ part of the ORF of *RMG1* (http://epigara.biologie.ens.fr/cgi-bin/gbrowse/a2e/). If these epigenetic modifications are indeed present within the same cells, they would ensure a dual and antagonistic epigenetic control of these defense genes by maintaining, through DNA methylation in their promoters, a low basal expression level in normal growth conditions, while having a chromatin environment poised for a rapid and pervasive transcription under pathogen constraints when TGS is released. Such a chromatin-based regulatory mechanism would be well adapted to tightly control the basal- and pathogen-responsive- transcriptional status of immune-response genes such as *NLRs*, whose overex-

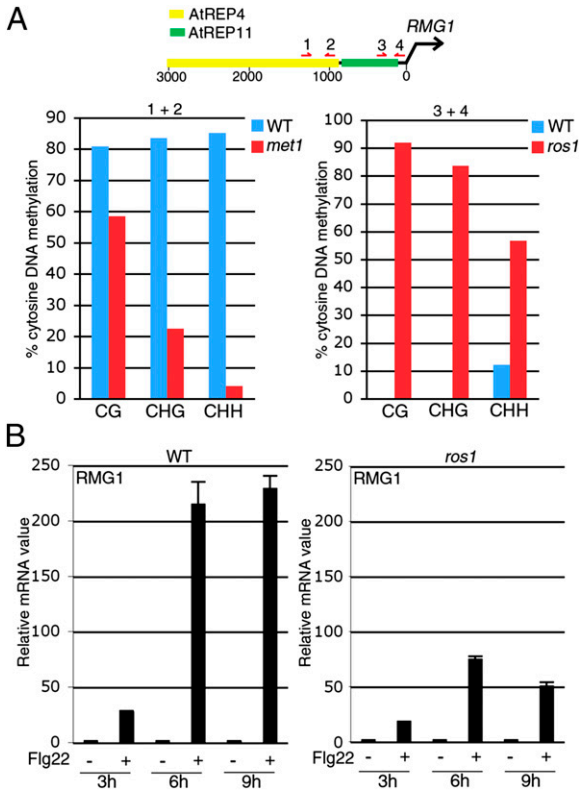

Fig. 4. (*A*) DNA methylation at *RMG1* promoter, a schematic representation of the *RMG1* promoter (*Upper*) is presented where red arrows represent the position of primers used for bisulfite sequencing on WT and *met1*$^{(-/-)}$ leaves (*Left* graph) or WT and *ros1–4* leaves (*Right*). The regions analyzed with primers (1+2) and (3+4) contain 12 CG, six CHG, 56 CHH, and two CG, three CHG, and 27 CHH, respectively. Similar results were obtained in three independent experiments. (*B*) RMG1 mRNA levels in 5- to 6-wk-old WT or *ros1–4* leaves treated with either water (–) or flg22 (+) for 3, 6, and 9 h with SD as in Fig. 1. Similar results were obtained in four independent experiments.

pression is often associated with severe fitness cost (26–28). Consistent with this idea, the disease resistance gene *Lazarus 5* (*LAZ5*), whose transcriptional expression requires the active chromatin mark H3K36me3 (31), is also targeted by siRNA/DNA methylation at its 5′ and 3′ ends (http://epigenomics.mcdb.ucla.edu).

We have shown that the basal- and/or flg22-induced expression of several MAMP-responsive *NLRs* was enhanced in DNA methylation defective mutants (Figs. S11 and S12). Although flg22-triggered induction of many *Arabidopsis NLRs* was previously reported (17, 18), very little is known on the underlying mechanisms involved in this regulatory process. We propose that flg22-triggered inactivation of TGS represents one of those mechanisms because it presumably contributes to the transcriptional activation of *RMG1* and perhaps other *NLRs*. This part of our study therefore sheds light on an as-yet unsuspected molecular link between MTI and *NLR*-dependent defense responses. This link may also have a posttranslational component, because flg22 triggered a decrease in the accumulation of protein levels of TGS factors (Fig. 2*C*) that might be sensed by NLR proteins, thereby activating an SA-dependent defense response (Fig. 2*A*). This scenario, although still speculative, would thus extend the classical "guard hypothesis" to the indirect detection of MAMPs by plant NLRs that would monitor the differential protein levels of "guarded" TGS factors during MAMP-triggered signaling events. Whether these hypothesized regulatory mechanisms contribute to the autoimmune phenotypes observed in the *met1*/*nrpd2* double mutant remains to be determined.

We have also demonstrated that DNA demethylation restricts leaf vascular propagation of *Pto* DC3000, which possibly gains access to the vasculature from wound inoculation sites or hydathodes (Fig. S20). This plant-induced vascular defense appears to be particularly effective at the base of midveins and proximal regions of secondary veins (Fig. 1*A*), which represent the only tissues where cells are still dividing at late stages of leaf development (32) and where maintenance of DNA methylation is likely needed to silence RdDM targets such as *AtGP1* retrotransposons. MAMP-triggered inactivation of TGS in these actively dividing cells may thus favor a potent derepression of a subset of immune-response genes *in cis*, including the *WRKY22* and *RMG1* described in this study. In a similar way, it might contribute to the strong antimicrobial defense response that is often observed in plant meristematic tissues (33). MAMP-triggered release of TGS may also lead to the production of additional TE-based substrates for DCL proteins and therefore favors the biosynthesis of *trans*-active siRNAs that would have the potential to silence modulators of plant defense in cells that surround sites of TE reactivation. Such a scenario would be consistent with the enhanced accumulation of TE-derived 21-nt siRNAs recently described upon SA treatment (8), and might contribute to the formation of an immune cell layer around the vasculature that would prevent bacterial propagation from xylem vessels to mesophyll cells and vice versa. Such a noncell autonomous regulatory mechanism has been initially described in the context of pollen development, where the derepression of some Athila retrotransposons in pollen vegetative cells was shown to trigger the production of 21-nt mobile TE-derived siRNAs that were *trans*-active in sperm cells (34). Intriguingly, the reactivation of TEs in pollen vegetative cells was associated with the down-regulation of a subset of TGS factors (34), therefore mimicking the flg22-triggered effects described in the present study. It is thus tempting to speculate that an endogenous regulatory mechanism might ensure a constitutive antimicrobial immune response in pollen vegetative cells that would protect them from pathogen invasion, thereby preserving the integrity of male gametes and limiting pollen transmission of pathogens, a common plant-to-plant spreading strategy used by many viruses (35).

Systemic acquired resistance (SAR) is an inducible broad-spectrum immune response in plants that restricts the spread of pathogens and prevents infection in distal tissues (36). The SAR signal SA, whose production is increased in response to pathogens or flg22 (37, 38), is known to trigger massive changes in gene expression and to induce DNA demethylation at SA-induced TEs (8, 39). Furthermore, bacterial-induced SAR was recently shown to confer transgenerational resistance toward unrelated pathogens including *Pto* DC3000 (14, 15). Based on these findings and on the present work, we speculate that pathogen- or MAMP-induced production of SA might trigger DNA demethylation of TEs/defense genes both locally but also in systemic unchallenged tissues including reproductive organs, thereby orchestrating transgenerational immune priming. Additionally, siRNA pools that are produced from pathogen- or MAMP-challenged tissues, including TE-derived siRNAs mentioned above, might trigger long-distance mobile silencing that could modulate the transcriptional response to pathogens in the offspring. Investigating the contribution of DNA demethylation and pathogen-responsive siRNAs in transgenerational immune priming will therefore be essential to unravel the mechanisms by which pathogens drive the selection of new phenotypes through epigenetic and epigenetic-directed genetic changes.

Materials and Methods

Plant Growth Conditions and Treatments. Most of the plants used in this study were grown at 23 °C with an 8-h photoperiod. Five- to 6-wk-old leaves from different genotypes were syringe infiltrated with either water or flg22 synthetic peptide (Genscript), at 1 μM concentration. The treatments of *Arabidopsis* seedlings with flg22 or DNA methyltransferase inhibitor are described in *SI Materials and Methods*.

Transgenic Plant Materials and DNA Contructs. *AtGP1 LTR:GUS* and *ROS1*p:*ROS1*-GUS constructs were generated as described in *SI Materials and Methods*. These constructs were transformed in the Col-0 accession. The *AtGP1 LTR*:

GUS #16 reference line was selected based on its sensitivity to a DNA methyltransferase inhibitor (*SI Materials and Methods*).

Histochemical GUS Staining. Five- to 6-wk-old leaves of *AtGP1 LTR*:*GUS* #16 were syringe-infiltrated with either water or flg22 peptide at 1 μM concentration and collected at 24 h posttreatment. They were GUS stained as described in *SI Materials and Methods*. Five- to 6-wk-old unchallenged *ROS1*p:*ROS1-GUS* leaves were GUS stained similarly.

Bacterial Infections. Bacterial infections were performed by syringe infiltration or wound inoculation on 5- to 6-wk-old *Arabidopsis* leaves from different genotypes. *Pto* DC3000 and a GFP-tagged *Pto* DC3000 were used for this study. For syringe-inoculation assay, *Pto* DC3000 was used at a concentration of 10^5 colony-forming units per milliliter (cfu/mL) and bacterial titers were monitored by serial dilution assays. For wound inoculation, *Pto* DC3000–GFP was used at a concentration of 5×10^7 cfu/mL and inoculated in either midveins or secondary veins with a toothpick. Bacterial propagation was then analyzed as described in *SI Materials and Methods*. To determine the presence of *Pto* DC3000–GFP in xylem vessels, transversal sections of leaves were conducted by cutting polystyrene rod containing leaf transversally with a razor blade.

Real-Time RT-PCR Analyses. Total RNA was extracted using RNeasy Plant Mini kit (Qiagen). RNA samples were reverse transcribed into cDNA using SuperScript III Reverse Transcriptase (Invitrogen) with a mix of random hexamers and oligodT. The cDNA was quantified using a SYBR Green qPCR kit (Roche LightCycler 480 SYBR Green I Master) and gene specific primers. PCR was performed in 384-well plates heated at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 10 s and annealing at 60 °C for 40 s. A melting curve was performed at the end of the amplification. Transcript levels were normalized to that of Actin2. All primer sequences are listed in Dataset S2.

Bisulfite Conversion and Sequencing. Total genomic DNA was extracted using DNeasy Plant Mini kit (Qiagen) and bisulfite treated using EpiTect Bisulfite kit (Qiagen). The PCR products were purified and cloned as described in *SI Materials and Methods*. Height to 23 clones were sequenced from naïve leaf samples, whereas 15–25 clones were sequences from mock-treated and flg22-treated leaf samples. The bisulfite conversion efficiency was tested by confirming the absence of DNA methylation at a nonmethylated region (see *SI Materials and Methods* for details). All primer sequences are listed in Dataset S2.

Cell Death Observations. Five- to 6-wk-old leaves from different genotypes were stained with Trypan Blue as described in *SI Materials and Methods*.

Western Blot Analyses. Total protein extracts from 5- to 6-wk-old *Arabidopsis* leaves, treated with either water or flg22 synthetic peptide at 1 μM concentration, were obtained using the Tanaka method and resolved on SDS/PAGE. Protein blot analysis was performed using antibodies raised against an AGO4 and NRPE5 peptides (gifts from T. Lagrange, Laboratoire Génome et Développement des Plantes, Perpignan, France), ACTIN8 (monoclonal antiactin plant; Sigma).

Small RNA Library/Sequencing and Data Mining. A small RNA library was made from 5-wk-old Col-0 leaf samples and deep sequenced by Fasteris (Geneva) on the Illumina HiSEq. 2000. Details of data processing are described in *SI Materials and Methods*.

RNA Library/Sequencing and Data Mining. RNA libraries were made from 5- to 6-wk-old Col-0 leaf samples (treated with either water or flg22 at 1 μM concentration for 6 h) and deep sequenced by Fasteris (Geneva) on the Illumina HiSEq. 2000. Details of data processing are described in *SI Materials and Methods*.

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