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Article type : Original Article

Immune receptor genes and pericentromeric transposons as targets of common epigenetic regulatory elements

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Running Title:
sRNAs matching *PRR*/*NLR* genes and unlinked TEs

Keywords:
immune receptor genes, transposons, epigenetics, defense priming, RdDM, MOM1, Arabidopsis thaliana

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/tpj.14098

ABSTRACT

Pattern recognition receptors (PRR) and nucleotide-binding leucine-rich repeat proteins (NLR) are major components of the plant immune system responsible for pathogen detection. To date, the transcriptional regulation of *PRR*/*NLR* genes is poorly understood. Some *PRR*/*NLR* genes are affected by epigenetic changes of neighboring transposable elements (TEs) (*cis*-regulation). We analyzed whether these genes can also respond to changes in the epigenetic marks of distal pericentromeric TEs (*trans*-regulation). We found that Arabidopsis tissues infected with *Pseudomonas syringae* pv. tomato (*Pst*) initially induced the expression of pericentromeric TEs, and then repressed it by RNA-directed DNA methylation (RdDM). The latter response was accompanied by the accumulation of small RNAs (sRNAs) mapping to the TEs. Curiously these sRNAs also mapped to distal *PRR*/*NLR* genes, which were controlled by RdDM but remained induced in the infected tissues. Then, we used non-infected *mom1* (Morpheus' molecule 1) mutants that expressed pericentromeric TEs to test if they lose repression of *PRR*/*NLR* genes. *mom1* plants activated several *PRR*/*NLR* genes that were unlinked to MOM1-targeted TEs, and showed enhanced resistance to *Pst*. Remarkably, the increased defenses of *mom1* were abolished when MOM1/RdDM-mediated pericentromeric TEs silencing was re-established. Therefore, common sRNAs could control *PRR*/*NLR* genes and distal pericentromeric TEs and preferentially silence TEs when they are activated.

INTRODUCTION

The plant immune system relies on the ability of every cell to detect potential invaders and consequently trigger defenses. Two major types of immune receptors are responsible for such functions: the plasma-membrane embedded pattern recognition receptors (PRRs) that recognize microbe-associated molecular patterns, and the intracellular nucleotide-binding leucine-rich repeat (LRR) proteins (NLR) that detect pathogen-derived effectors. The activation of PRR/NLR receptors triggers massive gene reprogramming and synthesis of defense compounds. The structure and evolution of these proteins have been intensively studied (Jones *et al.*, 2016),

and the mechanisms regulating their activity have started to be elucidated (Halter and Navarro, 2015). Some PRR/NLR receptors are controlled at the transcriptional level, and others have post-transcriptional regulation by micro RNAs or phased secondary siRNA (Howell *et al.*, 2007; Chen *et al.*, 2010; Fei *et al.*, 2013; Boccara *et al.*, 2014; Fei *et al.*, 2016). The first group includes *RPS5, RPS2*, *RPS4*, *Laz5*, *SNC1*, *ADR1*, *ADR-L1* and *ADR-L2*, whose induction triggers defenses in the absence of ligands, often leading to dwarfism or spontaneous cell death (Tao *et al.*, 2000; Stokes *et al.*, 2002; Grant *et al.*, 2003; Yi and Richards, 2009; Palma *et al.*, 2010; Bonardi *et al.*, 2011; Collier *et al.*, 2011; Heidrich *et al.*, 2013; Boccara *et al.*, 2014).

The mechanisms that control the expression of *PRR*/*NLR* genes remain poorly understood. Some of these genes are affected by the epigenetic state of nearby transposable elements (TEs). This is observed in Arabidopsis tissues infected with *Pseudomonas syringae* pv. tomato (*Pst*), were induction of *NLR* genes is accompanied by hypomethylation of linked TEs (Dowen *et al.*, 2012). flg22 treatment also triggers activation of defense genes proximal to demethylated TEs (Yu *et al.*, 2013). In addition, the *NLR* genes are sensitive to histone marks of the proximal TEs. The *RPP7* gene contains a COPIA element in its first intron, whose histone methylation marks vary after infection, thus determining the generation of functional and non-functional *RPP7* transcripts (Tsuchiya and Eulgem, 2013). All these studies evaluate TEs and genes located in the chromosomal arms. So far, it is unknown if *PRR*/*NLR* genes are sensitive to epigenetic changes of pericentromeric TEs. These TEs are the most abundant in Arabidopsis, and are organized in clusters in gene-poor regions surrounding the centromeres (Sigman and Slotkin, 2016). Probably these elements modify their epigenetic state, or expression under biotic stress since pericentromeric chromatin undergoes structural changes under such condition. At the onset of *Pst* infection, this chromatin loses condensation and reduces the 5-methyl cytosine (5-mC) content (Pavet *et al.*, 2006). Later, pericentromeres show hypermethylation, suggesting that infected tissues restore 5-mC in these regions (Dowen *et al.*, 2012). However, the mechanisms underlying these alterations are unknown.

Pericentromeric and non-pericentromeric TEs differ in many structural and functional traits, including size, high-order chromatin organization and epigenetic control (Sigman and Slotkin, 2016). In general, the Arabidopsis TEs placed on chromosome arms near genes are repressed by transcriptional gene silencing (TGS) through RNA-directed DNA methylation (RdDM). In the canonical pathway, this mechanism is initiated by recruitment of RNA polymerase IV (Pol IV) at *loci* with histone 3 lysine 9 dimethylation (H3K9me2). There, Pol IV transcribes a single-stranded RNA that is converted into double-stranded RNA by RNA-dependent RNA polymerase 2 (RDR2), and processed into 24 nt small RNAs (sRNAs) by DICER-like 3 (DCL3). These sRNAs are incorporated into Argonaute 4 (AGO4) and guided to the target *loci*, where RNA polymerase V (Pol V) transcribes scaffold RNAs complementary to AGO4-associated sRNA, and domains rearranged methyltransferase 2 (DRM2) directs *de novo* DNA methylation. Subsequently, after cell division, H3K9me2 directs deposition of DNA methylation, reinforcing TE repression by RdDM. In addition, this mechanism maintains the heterochromatic state of flanking regions between TEs and neighbor genes (Zemach *et al.*, 2013; Matzke and Mosher, 2014; Stroud *et al.*, 2014; Sigman and Slotkin, 2016). Pericentromeric TEs are also repressed by 5-mC and H3K9me2, and basal DNA methylation is primarily mediated by chromatin remodeler DDM1 (Decreased DNA Methylation 1). Different enzymes maintain non-CG methylation at pericentromeric (chromomethylases CMT2/CMT3) or non-pericentromeric TEs (DRM2) (Zemach *et al.*, 2013; Matzke and Mosher, 2014; Stroud *et al.*, 2014; Sigman and Slotkin, 2016), showing that repression of both kinds of elements involves specialized mechanisms. The targets of these chromatin-remodeling factors are not completely defined, particularly under stress. RdDM silences a subset of pericentromeric TEs that are also repressed by the chromatin remodeling factor MOM1 (Morpheus' molecule 1) (Amedeo *et al.*, 2000; Steimer *et al.*, 2000; Yokthongwattana *et al.*, 2010; Nishimura *et al.*, 2012). Among them, the LTR/Gypsy TEs *TSI* is a major target, and its activation in *mom1* mutants occurs without changes in 5-mC marks (Habu *et al.*, 2006; Vaillant *et al.*, 2006; Numa *et al.*, 2010; Yokthongwattana *et al.*, 2010). Interestingly, the small "conserved MOM1 motif 2" (CMM2) restores repression of common MOM1/TGS targets in the *mom1* background (Caikovski *et al.*, 2008; Mlotshwa *et al.*, 2010; Nishimura *et al.*, 2012). On the other hand, MOM1 shows epistatic, synergic or antagonist relationships with Pol IV or Pol V over common

targets (Numa *et al.*, 2010; Yokthongwattana *et al.*, 2010), indicating that different sets of proteins regulate silencing of pericentromeric TEs. Interestingly, some RdDM components were found necessary to maintain basal repression of plant immune cascades, but this has not been evaluated for MOM1, even though this protein controls pericentromeric TEs whose expression could be affected by stress conditions (Pavet *et al.*, 2006; Dowen *et al.*, 2012; Probst and Mittelsten Scheid, 2015).

This work analyzes the expression of pericentromeric TEs (*TSI* and *Athyla6a*) in *Pst*-infected tissues, and the effect of their over-expression on *mom1* defense responses. We show that *Pst* triggers the late repression of *TSI* and *Athyla6a* by RdDM, and the accumulation of sRNAs that target *PRR/NLR* genes and multiple distal TEs. In addition, we found that non-infected *mom1* plants express several *PRR*/*NLR* genes, whose basal repression is recovered upon pericentromeric TE re-silencing. Our results suggest that common sRNAs could affect the expression of *PRR*/*NLR* genes and pericentromeric TEs.

RESULTS

Pst infection alters the expression of different TEs

We selected two sets of TEs to study their expression during *Pst* infection. This included *Athila6a* and *TSI* (LTR/Gypsy) as typical pericentromeric elements embedded in constitutive heterochromatin (Amedeo *et al.*, 2000; Caikovski *et al.*, 2008), and *Atlantys2A* (LTR/Gypsy) and *Ta11* (LINE) as TEs from chromosome arms with heterochromatic or euchromatic structure, respectively (Pecinka *et al.*, 2010). We quantified TE transcripts in wild-type plants using non-treated (T0) and mock- (10 mM $MgCl_2$) or pathogen- (10^7 cfu/ml) infiltrated samples taken at 24 h post-treatment (hpt). All TEs were slightly activated by mock-inoculation, suggesting their sensitivity to mechanical stress (2 to 5-fold transcript increase mock *vs* T0, Fig 1a). In contrast, both TE sets responded differently to pathogen infection. *Athila6a* and *TSI* had lower expression in *Pst*- than in mock-treated samples, and *Atlantys2A* and *Ta11* were activated by infection (>7-fold difference for *Pst vs* mock*;* Fig 1a). This indicated that pericentromeric and non-pericentromeric TEs have different

regulations, and *TSI* and *Athila6a* lose their induction by mock-inoculation in the infected tissues.

To assess whether *TSI* and *Athila6a* were repressed by *Pst* treatment, we analyzed public sRNA-seq data ((Zhang *et al.*, 2011); reads at NCBI/GEO GSE19694) and detected a 8.3- and a 4.1-fold increase of sRNAs homologous to *TSI* and *Athila6a* in *Pst*- compared to mock-treated samples, respectively (Fig 1b; Table S1). These sRNAs mostly had 24 nt in length (Fig 1c). Then, we quantified TE transcripts in the RdDM mutants *nrpd1a-4* (Pol IV major subunit), *dcl2/3/4* (DCL) and *nrpd1b-11* (Pol V major subunit). After infection, none of the mutants reduced *TSI* expression (*Pst* vs mock) (Fig 1d; Fig S1a), and neither *nrpd1a-4* nor *dcl2/3/4* plants repressed *Athila6a* (Fig S1b). In contrast, *Atlanthys2A* and *TA11* remained activated in infected *nrpd1a-4* and *nrpd1b-11* tissues (Fig S1c, Fig S1d). As *TSI* is a major target of TGS mediated by MOM1 (Steimer *et al.*, 2000), we monitored its expression in *mom1-5* mutants. *Athila6a* was also included in this study. This TE is repressed by MOM1 (Steimer *et al.*, 2000), but uses a different set of proteins than *TSI* for its silencing (Vaillant *et al.*, 2006, Slotkin, 2010). Both TEs were expressed in *mom1-5* as expected (Fig 1d; Fig S1e) and *TSI* -unlike *Athila6a*- (Fig S1e) lost repression in infected tissues.

Finally, we analyzed possible causes of *TSI* repression by *Pst* treatment in wild type tissues, by evaluating its behavior at 3, 5, 7 and 24 hpi. Interestingly, *TSI* transcripts initially increased (3-7 hpi) and then decreased (24 hpi) in these tissues (Fig S1f). Thus, *TSI* is activated by stress and then re-silenced by RdDM like other TEs (Schoft *et al.*, 2009; Slotkin *et al.*, 2009; Pecinka *et al.*, 2010; Nuthikattu *et al.*, 2013).

sRNAs matching *PRR/NLR* genes and TEs accumulate in infected tissues

As RdDM simultaneously controls the pericentromeric TEs *TSI* (Fig 1d) and *Athila6a* (Fig S1b), and *PRR*/*LRR* genes (Lopez *et al.*, 2011; Dowen *et al.*, 2012; Yu *et al.*, 2013; Zheng *et al.*, 2013), we explored whether common sRNAs could match both kinds of *loci*. For this purpose, we accessed public sRNA-seq data ((Zhang *et al.*, 2011); GSE19694) and selected all sRNAs that increased in *Pst- vs* mock-

treated samples and mapped with zero mismatch to *PRR/NLR* genes (ORF and 2 Kb promoter region, genes listed in Table S6; see Materials and Methods). These sRNAs, hereafter called "R-sRNAs", matched 27 *PRR/NLR* genes, sometimes targeting TEs fragments inserted within the genes (“proximal TEs”) (Table S2). Subsequently, we mapped these R-sRNAs with the whole Arabidopsis TE set (TAIR10) and found that some of them had 100% homology to “distal TEs” (TEs placed at more than 2 kb from ORF + promoter regions of *PRR/NLR* genes). Then, *Pst*-infected tissues accumulated R-sRNAs targeting either (*i*) *PRR/NLR loci* or (*ii*) *PRR/NLR loci* and distal TEs (Fig 2a). R-sRNAs from the first group accounted for 30% of reads and mapped to 15 *PRR/NLR* genes, while those of the second group (70% of reads) matched 12 *PRR/NLR* genes (*ADR1-L1*, *RLK7*, *CRK37*, *Laz5*, *ADR1*, others) and 206 distal TEs (Fig 2a; Table S3). Interestingly, 43% of the 206 distal TEs were located in the proximity of pericentromeres (chromosome 1 is shown as representative in Fig 2b).

In silico analyses indicated that the R-sRNAs from group *ii* (33) mapped to 1443 regions in 206 distal TEs, suggesting that many of them have multi alignment to these TEs. The R-sRNAs matching *ADR1-L1* and *RLK7* (12 and 8 unique sequences, respectively) had the largest number of distal targets (730 positions from 37 annotated distal TEs for *ADR1-L1*; 624 positions from 94 annotated distal TEs for *RLK7*) (Fig 2c, Fig 2d), whereas R-sRNAs homologous to *ADR1* had fewer targets (13 positions from 13 annotated distal TEs). We found that *ADR1-L1, RLK7* and *ADR1* had constitutive expression in *nrpd1b-11* and *nrpd1a-4* mutants, indicating that these genes are controlled by RdDM (Fig 2e). Then, we selected *RLK7* to further examine its regulation. *RLK7* sRNAs were present in wild type plants but not in *nrpd1a-4* or *dcl2/3/4* mutants impaired in sRNA biogenesis, indicating that they derive from the canonical RdDM pathway (Fig 2f). The *RLK7* sRNAs increased after infection and accumulated at 24 hpi, (Fig 2g) corroborating the analysis of sRNA-seq data (Table S2; (Zhang *et al.*, 2011)). Despite this, *RLK7* was induced in the infected tissues, and this also applied for *ADR1-L1* and *ADR1* (Fig 2h). Therefore, infected tissues accumulated R-sRNAs mapping to *RLK7*, *ADR1-L1* and *ADR1*, but did not silence these genes. As discussed below, under this condition these R-sRNAs could be recruited to their second type of targets, the distal TEs that may have been activated.

Defenses against *Pst* are primed in *mom1* plants

Normally, *PRR*/*NLR* genes and pericentromeric TEs are repressed under basal conditions. We reasoned that if both types of *loci* have some kind of co-regulation, then activation of TEs could disrupt basal repression of *PRR*/*NLR* genes. To evaluate this, we monitored defense responses in non-infected *mom1* plants that over-express pericentromeric TEs (Fig S2a) (Habu *et al.*, 2006; Vaillant *et al.*, 2006; Numa *et al.*, 2010; Yokthongwattana *et al.*, 2010). Individual *mom1-5* plants grown in soil started to express *PR1* at the age of 8 weeks, and this was more evident at 9 weeks (Fig 3a). Similarly, groups of *mom1-5* plants grown either in soil or in a sterile synthetic medium induced *PR1* at the stage of 4-8 leaves (Fig S2b). However, *PR1* was not activated in young mutant plants (Fig S2b). We used samples with different *PR1* transcript levels ("-", "+", "++", "+++") to analyze possible causes of *PR1* expression in the mutant. Plants expressing *PR1* also activated *RMG1,* and sometimes *ADR1*, *RPS4* or *RLK7* (Fig S3). Therefore, several *PRR/NLR* genes lose negative regulation in *mom1-5* and this may cause *PR1* induction. Transcriptome analysis had not reported over-expression of *PRR/NLR* genes in young *mom1* mutants (Yokthongwattana *et al.*, 2010; Stroud *et al.*, 2012; Moissiard *et al.*, 2014) although some defense genes were up-regulated in these plants (Habu *et al.*, 2006). Then, we re-evaluated published data looking for minor but significant differences between *mom1* and wild type plants (>1.3-fold change) and thus detected 25 *PRR/NLR* genes with mild induction in the mutant (Table S5). *RLK7* was included among these genes and curiously, *RLK7* activation correlated with *RLK7* R-sRNAs accumulation in *mom1-5* plants (Fig 2f). Here again, the *PRR*/*NLR* gene was not repressed by the homologous sRNAs in the presence of activated pericentromeric TEs.

The transcriptional changes described for *mom1-5* affected its immunity since *Pst* growth was lower in mutant than in control plants (Fig 3b). Similarly, *mom1-2* and *mom1-1* mutants had enhanced pathogen resistance. Curiously, the young *mom1-5* plants that had not yet expressed *PR1* also restricted pathogen proliferation (Fig S2c), suggesting that they were prone to activate defenses. In agreement with this, the defense gene markers *PR1*, *ICS1* (*ISOCHORISMATE SYNTHASE 1*, responsible for SA generation) and *RMG1* were induced by mock-inoculation in

mom1-5, and after infection these genes showed earlier or stronger activation in the mutant (Fig 3c). Then, in the absence of MOM1 plants are prone to activate defense genes.

MOM1-silenced TEs are distal to *PRR/NLR* genes induced in *mom1*

The causes of *PRR*/*NLR* genes induction in *mom1* are unknown. One possibility is that these genes are close to the TEs that lose negative regulation in the mutant ("MOM1-TEs"). These elements (77 TEs) mostly belong to the LTR/Gypsy subfamily (Numa *et al.*, 2010; Yokthongwattana *et al.*, 2010). We selected 10 kb windows containing MOM1-TEs (5 kb toward 5' and 3' ends) to analyze if they included defense genes (1380 genes including 396 *PRR/NLR* and 984 biotic stress genes; see Materials and Methods). These regions contained 1 pre-tRNA, 49 TEs, 3 pseudogenes and 35 genes (Fig 4a; Table S4). The last group included *At2g11000* from the biotic stress class, which encodes a non-functional homolog of yeast MAK10 (Pesaresi *et al.*, 2003), whose role in plant immunity has not been demonstrated. Subsequently, we examined whether LTR/Gypsy TEs were enriched in regions surrounding the *PRR/NLR* genes induced in *mom1* (25 genes defined as "MOM1-*PRR/NLR*", Fig S4, Table S5). As control, 10 sets of 30 randomly selected *PRR/NLR* genes were evaluated. TEs located inside or near *PRR/NLR* genes (into the ORF or 5 kb toward 5' and 3' ends) were listed and classified in superfamilies (TAIR10). Although ten classes of TEs were detected in the proximity of MOM1-*PRR/NLR* genes, none of them was enriched in MOM1-*PRR/NLR* relative to randomly-selected *PRR/NLR* genes (Fig 4b; Fig S4). Importantly, LTR/Gypsy elements were poorly represented in both gene sets, whereas RC/Helitron and DNA/MuDR TEs were the most abundant. Therefore, MOM1-*PRR/NLR* genes are not proximal (< 10 kb) to MOM1-TEs and are not enriched in other TE superfamilies.

Only three LTR/Gypsy elements are placed near or within the MOM1-*PRR/NLR* genes, *AtGP8* (*AT1TE39495*), *Athila6a* (*AT2TE61100*) and *Athila7* (*AT4TE29285*), inserted in *ADR1*, *RLP23* and *RMG1*, respectively (Fig 4c; Fig S5a). None of them are MOM1 recognized targets (Numa *et al.*, 2010; Yokthongwattana *et al.*, 2010), suggesting that they do not mediate *PRR/NLR* gene activation in *mom1-5*.

AtGP8 is inserted into the *ADR1* gene promoter, and is the only one of these elements with H3K9me2 marks that could be eventually associated with MOM1 activity (Numa *et al.*, 2010) (Fig 4c; Fig S5a). We evaluated the *AtGP8* H3K9me2 levels by ChiP-qPCR in samples that express or not the *ADR1* gene (Fig 4d). The former were adult *Pst*-infected Col-0 and mature non-infected *mom1-5* plants, and the latter Col-0 and *mom1* seedlings. At seedling stage, both genotypes contained similar H3K9me2 levels, indicating that MOM1 is dispensable for deposition of this mark. In adult plants (*Pst*-infected wild type tissues and non-infected *mom1* plants), the H3K9me2 reduction accompanied *ADR1* expression (Fig 4d). Therefore, MOM1 is required to maintain the H3K9me2 mark during development, and its depletion correlates with gene induction. However, among the 25 *PRR/NLR* genes that were induced in *mom1* (Table S5), *ADR1* was the only one containing H3K9me2 in a Gypsy element whose release may determine gene expression. In contrast, the H3K9me2 marks at *RMG1* promoter did not change in *mom1* or in *Pst*-infected wild type tissues (Fig S5b), suggesting that they do not control gene expression. Thus, our results suggest that activation of *PRR/NLR* genes in *mom1-5* does not result from MOM1-mediated epigenetic changes affecting proximal TEs.

Involvement of RdDM in *mom1* defense regulation

Finally, we assessed whether the activation of *PRR/NLR* genes in *mom1* was strictly associated with over-expression of RdDM-targeted TEs. We used *mini-MOM1* plants for this purpose, since they rescue the capacity to silence TEs co-regulated by MOM1/RdDM, but not TEs regulated by MOM1 independently of RdDM (Caikovski *et al.*, 2008; Nishimura *et al.*, 2012). After quantifying *PR1* transcripts in soil-grown plants of different ages (3, 5 or 9 weeks or 4, 8 and 20-30 leaves) we found that none of the *mini-MOM1* samples expressed this gene (Fig 5a). In addition, *mini-MOM1* had reduced *RMG1*, *ADR1, RPS4* and *RLK7* expression compared to *mom1-1* plants. This was particularly evident for *RMG1,* with more than 400-fold differences in transcript levels in both genotypes (Fig 5b). As expected, *miniMOM1* was more susceptible to *Pst* than *mom1-1* plants and, notably, they responded similarly to wild type plants (Fig 5c). Therefore, the CMM2 domain that mediates RdDM-dependent TE silencing, is sufficient to maintain basal repression of *RMG1*, *ADR1*, *RPS4*, *RLK7* and *PR1*, and its absence determines pathogen defense priming.

DISCUSSION

We found that *Ta11* (At1TE89775), *Atlantys2A* (At3TE91745), *TSI* and *Athila6a* alter their expression in *Pst*-infected tissues. These changes are consistent with the general sensitivity of plant TEs to stress (Bucher *et al.*, 2012), and with the response of these particular elements to mechanical damage, heat treatment, or other injuries (Fig 1a; (Pecinka *et al.*, 2010; Wang *et al.*, 2013)). The factors that determine either induction (*Ta11*/*Atlantys2A*) or repression (*TSI*/ *Athila6a*) of these TEs by *Pst* are unknown. Neither the TE type nor the presence of nearby genes would explain such a difference. *Atlantys2A, TSI* and *Athila6a* belong to the same subfamily (LTR/Gypsy) and have a different regulation. *Ta11* and *Atlantys2A* respond in a similar way but only *Ta11* is inserted within a gene (AT1G72920, from the Toll-Interleukin-Resistance (TIR) domain family; TAIR10). Local epigenetic signatures do not correlate with TE expression either. Among the nine chromatin profiles defined for Arabidopsis (from state 1: active euchromatin, to state 9: silenced heterochromatin; (Sequeira-Mendes *et al.*, 2014)), *TSI*, *Athila6a* and *Atlantys2A* have analogous heterochromatin states (8/9), and *TA11* has a euchromatin state (2) (ARAPORT11; (Cheng *et al.*, 2017)). In contrast, chromosomal location may account for the differential regulation observed here, since this factor is key for controlling TE expression (Sigman and Slotkin, 2016).

We found that *TSI* was initially activated and subsequently repressed by *Pst*-treatment (Fig S1c). The genomic regions containing *TSI* lose 5-mC marks after infection (Pavet *et al.*, 2006) and it has been suggested that this could trigger the re-methylation of these domains (Dowen *et al.*, 2012). RdDM could help replenish 5-mC in these regions since it mediates repression of *TSI* and *Athila6a* by *Pst* (Fig 1d). Therefore, although pericentromeric TEs are basally controlled by the DDM1-CMT2/3 pathway (Zemach *et al.*, 2013; Stroud *et al.*, 2014; Sigman and Slotkin, 2016), they could be re-silenced by RdDM in *Pst*-infected tissues that lose condensation and DNA methylation of pericentromeres (Pavet *et al.*, 2006), which would help prevent a massive TE burst. Alternatively, the effect of RdDM on *TSI* repression described here may represent the response of a small subset of pericentromeric TEs. In turn, the non-pericentromeric TEs, *Ta11* and *Atlantys2A* that

are activated at 24 hpi (Fig S1d) do not remain induced at 5 dpi (Dowen *et al.*, 2012), suggesting that they would also be re-silenced at late stages of infection.

We observed that *ADR1-L1*, *RLK7* and *ADR1* are controlled by RdDM (Fig 2e). However, these genes are not silenced by the homologous 24 nt sRNAs present in infected tissues (Fig 2h). Such sRNAs also match distal TEs, and some of them have a large number of targets (> 600 sites for sRNAs homologous to *ADR1-L1* and *RLK7*) (Fig 2c). Interestingly, the *RLK7* induction coexisted with *RLK7* sRNAs accumulation in both *Pst*-infected wild type tissues (24 hpi; Fig 2g, Fig 2h) and non-treated *mom1* plants (Fig 2g, Fig S3). Therefore, sRNAs with perfect match to *PRR*/*NLR* genes and distal TEs could preferentially silence the TEs upon their transcriptional activation (Fig 6). This would be consistent with previous studies showing that under basal conditions, sRNAs dependent on POL IV are generated from pericentromeres and do not repress these regions, but they presumably silence distal homologous TEs (Li *et al.*, 2015; Sigman and Slotkin, 2016). Interestingly, sRNAs derived from *Athila* regulate the stress-related gene *UBP1b* in *trans* (McCue *et al.*, 2013). On the other hand, only a small proportion of the defense genes that alter their expression in Pol V or ROS1 mutants are associated with proximal TEs and Pol V/ROS1-dependent DNA methylation, suggesting that they are regulated by DNA methylation in *trans* (Lopez Sanchez *et al.*, 2016). The origin of the common sRNAs described here is unknown. Probably, they are transcribed from the TEs when these elements are expressed (in wild type infected plants or naive *mom1* mutants). Even so, it will be important to determine why common sRNAs do not silence *PRR*/*NLR* genes when pericentromeric TEs are being expressed. Possibly, Pol V or other RdDM components are preferentially recruited to pericentromeric *loci* in infected tissues. In this sense, some particular viral, endogenous or transfected non-coding RNAs act as decoy to repress some targets and activate others, probably by deviating PTGS components (Franco-Zorrilla *et al.*, 2007; Blevins *et al.*, 2011; Miller *et al.*, 2016).

The *nrpd1a-4* and *dcl2/3/4* mutants are impaired in sRNAs biogenesis. In contrast, *mom1* keeps such capacity but fails in silencing some pericentromeric TEs acting downstream of RdDM. Then, the activation of *PRR*/*NLR* genes may have different origins in *mom1* and RdDM mutants. Several *PRR*/*NLR* genes remain

repressed by RdDM in non-infected tissues ((Lopez *et al.*, 2011; Dowen *et al.*, 2012; Yu *et al.*, 2013; Zheng *et al.*, 2013); this study Fig 2h), so that plants impaired in sRNA generation (*nrpd1a-4*, *dcl2/3/4*) would lose such capacity, leading to de-repression of these genes (*cis* regulation). In contrast, MOM1 does not seem to modulate *PRR*/*NLR* gene expression in *cis.* The role of MOM1 in RdDM is particularly associated with pericentromeric *loci*, whereas *PRR*/*NLR* genes are not recognized MOM1 targets (Habu *et al.*, 2006; Numa *et al.*, 2010; Yokthongwattana *et al.*, 2010). The *PRR*/*NLR* genes that are induced in *mom1* are unlinked to MOM1-silenced TEs (Fig S4; Fig 4b), and at least two of these genes, *RLK7* and *ADR1*, have homology with sRNAs matching pericentromeric TEs (Fig 2c). Then, the induction of *PRR*/*NLR* genes in *mom1* may respond to *trans* regulation. Interestingly, the CMM2 domain that rescues *TSI* silencing (Fig 5b) also re-establishes basal *PRR*/*NLR* genes repression and pathogen susceptibility (Fig 5c) in the *mom1* background, suggesting that the defense phenotypes result from failures in MOM1/RdDM-mediated TE silencing. The RdDM pathway could be exacerbated and the recruitment of sRNAs could be favored towards pericentromeric TEs in *mom1* plants. Alternatively, *mom1* could accumulate lncRNAs derived from pericentromeric TEs that function as inducers of *PRR*/*NLR* genes in *trans*. However, the latter mechanism would not work on young *mom1* plants (2 weeks old), since they express pericentromeric TE (Steimer *et al.*, 2000) but do not activate *PRR*/*NLR* genes (Fig 3a, Fig S2b).

Finally, it is interesting to note that *mom1* plants do not express *PR1* in early stages of development, but do so when they are older (Fig 3a; Fig S2b). The size of the mutant was similar to that of the control plant at all analyzed conditions. After mock or *Pst* infiltration, *PR1*, *ICS1* and *RMG1* show an earlier and stronger activation in the mutant. Then, *mom1* is primed to induce defenses and it manifests this trait with aging, unlike other chromatin mutants that show constitutive defense expression, such as *rdr2*, *rdr6*, *dcl2*, *dcl3*, *dcl4*, *sni1*, *acd11*, *pie*, *sef*, *hta9/hta11* and *bal* plants (Mosher *et al.*, 2006; Yi and Richards, 2007; March-Diaz *et al.*, 2008; Palma *et al.*, 2010; Boccara *et al.*, 2014). Moreover, MOM1 is required to maintain basal repression of several *PRR*/*NLR* genes and this is also manifested at adult stage. Then, MOM1 could contribute to control age-dependent defense priming

through regulation of immune receptor genes. However, the role of MOM1 in the control of these genes during development requires further investigation.

EXPERIMENTAL PROCEDURES

Plant material

Arabidopsis thaliana mom1-2, *mom1-5*, *mom1-1* mutants and *miniMOM1* transgenic plants were kindly provided by Dr. Jerzy Paszkowski (The Sainsbury Laboratory) and Dr. Ortrun Mittelsten Scheid (Gregor Mendel Institute). *nrpd1a-4* (SALK_083051) and *nrpd1b-11* (SALK_029919) seeds were provided by Dr. Meyers (Department of Plant & Soil Sciences and Delaware Biotechnology Institute), and *dcl2/3/4 (dcl2-1/3-1/4-2* CS16391) seeds were obtained from ABRC. Plants were germinated on Murashige and Skoog media (Sigma-Aldrich) for ten days, transferred to soil and then grown under 8 h light/16 h dark cycles at 23 ºC.

Plant infection and pathogen growth

Pseudomonas syringae pv. *tomato* DC3000 grown on King's B medium supplemented with kanamycin and rifampicin was used to inoculate leaves at concentrations of 10^5 cfu/mL (quantification of bacterial content) or 10^7 cfu/mL (gene expression analysis), as previously described (Pavet *et al.*, 2005). Mock treatments included inoculation of 10mM MgCl2 solution (vehicle of bacterial suspension).

Gene expression

Gene expression was analyzed by RT-qPCR, except for *TA11* in Fig S1d, where we used RT-sqPCR. Reverse transcription was performed by using 2 µg of total RNA treated with RQ1 DNAsa (Promega), random hexamer primers and M-MLV reverse transcriptase (Promega) to synthesize cDNA. qPCR was performed with Master Mix (Biodyanimics), as follows: 10 min at 95 °C; 45 cycles of 15 sec at 95 °C, 30 sec at 60 °C, and 30 sec at 72 °C, using primers described in Table S7. *UBQ5* (*Ubiquitin 5*; *At3g62250*) was used as a reference gene. Reaction efficiency was in the range of 90-100% for all analyzed genes. Relative expression of target genes was calculated by the $2^{-\Delta Ct}$ or $2^{-\Delta\Delta Ct}$ methods.

sRNA blots

Blots were performed as Tomassi et al (Tomassi *et al.*, 2017), with minor modifications. Total RNA (30 µg) was loaded in 17% polyacrylamide gels and then transferred to HyBond-N+ membranes (GE Healthcare) for their hybridization with a digoxigenin-labelled oligonucleotide probe complementary to *RLK7* sRNA. The U6 sRNA probe was used as RNA-loading control. Oligonucleotides are detailed in Table S7.

Bioinformatic analysis

TEs considered as MOM1 targets are elements over-expressed in *mom1-2* plants (Table S2; (Yokthongwattana *et al.*, 2010)). In-house perl scripts and Galaxy software (galaxy.org) were used to select defense genes located in the proximity of TEs (± 5 kb), and to identify TEs inserted close to immune receptor genes. This last analysis was applied to all TE superfamilies described in TAIR10 (LTR/Copia, DNA/En-Spm, DNA/Mariner, DNA/MuDR, DNA/Pogo, LINE/L1, DNA/Tc1, DNA, SINE, DNA/HAT, DNA/Harbinger, LTR/Gypsy, RC/Helitron, RathE2_cons, RathE3_cons, RathE1_cons, LINE). The abundance of TEs in the ORF and proximal 5 kb toward 5 ' and 3' ends were determined for the 25 immune receptor genes induced in *mom1* or 10 sets of 30 randomly selected genes of this type. Statistical differences between both gene groups were determined by using Poission distribution ($p<0.05$) as previously described (Numa *et al.*, 2010).

Public sRNA-seq data from leaf samples treated with 10 mM $MgCl_2$ (mock) or *Pst* DC3000 (2.10^7 cfu/mL) (Gene Expression Omnibus, accession GSE19694; (Zhang *et al.*, 2011)) was used to analyze accumulation of sRNAs in infection. sRNAs with at least 3 raw sequence reads were mapped to the Arabidopsis nuclear, chloroplast and mitochondrial genomes (TAIR10) with zero mismatch by using Bowtie software (Langmead B). The *PRR*, *NLR*, *RLK* (receptor-like kinases) and *RLP* (receptor-like proteins) genes analyzed here (Table S6) are those from the GO term "Biological Process response to biotic or abiotic stimulus" that are classified as sensitive to biotic stress (TAIR10). We refer to them as "*PRR*/*NLR*" genes because these are the most abundant classes in the list. Alternatively, we call them "immune receptor genes" based on sequence data since many of them have not yet been evaluated at the functional level". Among them, we selected genes whose sRNAs

increase at least two-fold in *Pst vs* mock condition (Table S2). Data was processed as previously described (Zhang *et al.*, 2011; Zavallo *et al.*, 2015).

Public data from GEO was used to analyze *mom1* transcriptomes (accessions GSE17940, GSE38286 and GSE54677 for *mom1-2*, (Yokthongwattana *et al.*, 2010; Stroud *et al.*, 2012; Moissiard *et al.*, 2014); accession GSE5771 for *mom1-1*, (Habu *et al.*, 2006)). The new analysis uses Fisher's exact test (FDR, 0.05) with Infostat Software to select defense genes activated in the mutants, focusing in genes from the GO term "Biological Process response to biotic or abiotic stimulus" (TAIR10).

Chromatin immune-precipitation

ChIP-qPCR experiments used a classical protocol (Gendrel *et al.*, 2005) with few modifications. Anti-H3 (Abcam ab12079), Anti-H3K9me2 (Abcam ab1220) and Dynabids Protein G (Invitrogen) were used to treat and precipitate DNA. Specific *ADR1, RMG1* and *Ta2* regions were amplified by qPCR using primers listed in Table S7. *Ta2* was used as control since its H3K9me2 content does not vary in *mom1-2* and *mom1-1* plants (Habu *et al.*, 2006; Vaillant *et al.*, 2006; Numa *et al.*, 2010). The $2^{-\Delta\Delta Ct}$ method (H3K9me2/Ta2/H3) was used to determine the H3K9me2 content in the indicated *PRR/NLR* gene regions.

ACKNOWLEDGMENTS

This work was supported by grants from Agencia Nacional de Promoción Científica y Tecnológica (PICTs 2014-3255 and 2016-2986 to MEA; PICT 2014-1532 to SA; PICT 2015-157 to PC), and Secretaría de Ciencia y Tecnología, Universidad Nacional de Córdoba to MEA. DC and FN are CONICET fellows; SR, PC, SA and MEA are career investigators of CONICET. The manuscript was revised by Gabriela Diaz Cortez.

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

D.A.C., F.N., D.Z. and S.R. conducted the experiments. P.C, S.A. and M.E.A. designed the experiments and wrote the paper.

SUPPORTING INFORMATION – SHORT LEGENDS

Figure S1 - Modulation of *TSI* expression by components of the RdDM machinery.

Figure S2 - Defenses against *Pst* in *mom1* mutant plants.

Figure S3 - *mom1-5* mutants are prone to activate *PRR/NLR* genes.

Figure S4 - Abundance of TEs in the proximity of *PRR/NLR* genes activated in *mom1* (MOM1-*PRR/NLR* genes).

Figure S5 - Effects of MOM1 on the regulation of *RMG1* H3K9me2 content.

Table S1. *Loci* matching sRNAs quantified in mock- and *Pst*-infected tissues homologous to *TSI* and *Athila6A*.

Table S2. *PRR/NLR* genes with 100% homology to sRNAs accumulated in wild type tissues infected with *Pst.*

Table S3. Two sets of *PRR/NLR* genes with homology to R-sRNAs accumulated after *Pst* infection.

Table S4. List of genes located in proximity to MOM1-TEs.

Table S5. *PRR/NLR* genes activated in non-infected *mom1* plants.

Table S6. *PRR/NLR* genes used to analyze homology with R-sRNA.

Table S7. Primers and conditions used in RT-qPCR experiments.

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FIGURE LEGENDS

Figure 1 - Pericentromeric TEs are silenced by RdDM in *Pst*-infected tissues. (a) Expression of pericentromeric (*TSI*, *Athila6a*) and non-pericentromeric (*Atlantys2A*, *Ta11*) TEs evaluated in wild type plants under basal (T0), mock- or *Pst*-infiltration conditions (24 h post-treatment -hpt-). *UBQ5* was used as a reference gene. Values indicate changes in relation to T0 obtained by the $2^{-\Delta\Delta Ct}$ method and they represent the mean + SD of three technical replicates. Different letters indicate significant differences ($p< 0.05$; by ANOVA using Tukey's multiple comparison test). (b) Abundance of sRNAs matching *TSI* and *Athila6a* (see Table S1) in mock- or *Pst*-treated wild type samples analyzed at 14 hpt. (c) Percentage of 24 and 21 nt sRNAs matching *TSI* and *Athila6a* in *Pst*-infected wild type plants. (d) Relative *TSI* expression in *Pst*- *vs* mock- treated leaves (24 hpt) in wild type plants, and *nrpd1a-4*, *dcl2/3/4*, *nrpd1b-11* or *mom1-5* mutants, according to data shown in Fig S1. Values indicate *Pst*/mock ratio as $\log_2$ of $2^{-\Delta\Delta Ct}$. Three independent experiments showed similar results. *: significant differences among *Pst*- and mock-treated samples (*t* test $p< 0.05$).

Figure 2 - Control of *PRR/NLR* genes by sRNAs accumulating in infected tissues. (a) Some sRNAs that increase after *Pst* infection are identical to *PRR/NLR* genes (*i*) or to *PRR/NLR* genes and distal TEs (*ii*). Number of putative targeted *loci* and percentage of RPM of sRNAs are indicated for both groups [*i:* 15 *PRR/NLR* genes; 30% RPM; *ii*: 12 *PRR/NLR* genes, 206 TE; 70% RPM]. (b) Relative chromosome localization (x axis; centromere in white) and abundance (y axis) of the distal TEs from group *ii.* Chromosome 1 (44 TEs from group *ii*) is shown as representative. (c) Total positions matching R-sRNAs (homologous to the 206 distal TEs) for each *PRR/NLR* gene included in group *ii* (a). (d) Scheme of sRNAs mapping to the *ADR1-L1 loci* (inserted TE *AT4TE76870*), the pericentromeric TE *AT3TE45080* and other 36 distal-TEs. White boxes represent RC/Helitron TEs (ATREP10 family). (e,h) *ADR1-L1*, *RLK7* and *ADR1* expression in non-treated wild type, *nrpd1a-4* or *nrpd1b-11* plants (e); or (h) non-treated, mock- or *Pst*- treated (24 hpi) leaves of wild type plants. *UBQ5* was used as a reference gene. Values in (e) and (h) were obtained by the $2^{-\Delta\Delta Ct}$ method relative to Col-0 (e) or T0 (h) and they represent the mean + SD of three technical replicates. Different letters indicate

significant differences among samples (*t* test p<0.05 [e]; p< 0.05; by ANOVA using Tukey's multiple comparison test [h]). (f,g) sRNA blots hybridized with sRNAs homologous to *RLK7* and distal TEs. Blots include non-treated and mock- or *Pst*-treated (24 hpi) wild type (Col-0), *nrd1a-4* and *dcl2/3/4* samples (f); or non-treated (T0) and *Pst*-treated (7 and 24 hpi) Col-0 and *mom1* samples (g). Equal loading was monitored with *U6* sRNAs probes.

Figure 3 - *mom1* plants are primed to induce resistance against *Pst.* (a) *PR1* expression in wild type (Col-0) and *mom1-5* plants. Samples were taken at the stage of 8 or 9 weeks when they had 15-20 leaves. (b) *Pst* content in wild type (Col-0), *mom1-2*, *mom1-5* and *mom1-1* leaves at 1 and 3 days post-infection (dpi). cfu: colony-forming units. Values represent the mean + SD of two technical replicates containing 6 leaf discs each. *: significant differences between mutant and wild type (Col-0) samples (*t* test p<0.05). Similar results were obtained in three independent infection experiments. (c) *PR1*, *RMG1* and *ICS1* expression in wild type and *mom1-5* samples taken from non-treated (T0), mock-inoculated (10 mM $MgCl_2$) or *Pst*-infected leaves at different time points. Values indicate differences against Col-0 (a) and T0 (c) obtained by the $2^{-\Delta\Delta Ct}$ method, and they represent the mean + SD of three technical replicates using *UBQ5* as a reference gene and Col-0 as control. Different letters indicate significant differences among samples (p < 0.05; by ANOVA using Tukey's multiple comparison test).

Figure 4 - MOM1 does not target TEs placed in the proximity of *PRR/NLR* genes induced in *mom1* plants, nor does it regulate basal *ADR1* H3K9me2 content in seedlings. (a) Venn diagram showing the number of genes adjacent to MOM1-TEs (TEs repressed by MOM1), *PRR/NLR* genes, and biotic stress genes (TAIR10). (b) Number of RC/Helitron and DNA/MuDR TEs proximal to the *PRR/NLR* genes induced *mom1*. Five windows covering the ORF and proximal 5 kb toward 5' and 3' gene ends were analyzed. n= 25 *PRR/NLR* genes induced in *mom1* (black bars) or 10 sets of 30 randomly selected *PRR/NLR* genes (white bars). Poisson distribution (p<0.05) was applied for statistical analysis. (c) Scheme of TEs (striped boxes) present in the *ADR1* gene. The LTR/Gypsy *AtGP8* element is highlighted. Dotted lines show the genomic regions analyzed by ChIP with the indicated primers (arrows). The histogram representing the H3K9me2 content (Z-score) in non-infected

wild type plants was obtained from a previous study (Bernatavichute *et al.*, 2008). (d) Top: Abundance of H3K9me2 at the *ADR1* promoter (ChIP-qPCR) in wild type (Col-0) and *mom1-5* seedlings (left), non-treated and *Pst*-infected wild type plants (middle) and adult non-infected wild type and *mom1-5* plants (right). Values represent the mean + SD of three technical replicates. One representative experiment from two biological replicates is shown. Different letters indicate significant differences among samples ($p < 0.05$; by ANOVA using Tukey's multiple comparison test). Bottom: *ADR1* expression in samples used to analyze H3K9me2 by ChIP-qPCR. *GapC* was used as a reference gene.

Figure 5 - The CMM2 MOM1 motif rescues constitutive repression of pathogen defenses. (a) *PR1* expression in *miniMOM1* and *mom1-1* plants at different developmental stages. (b) *RMG1*, *ADR1*, *RPS4* and *RLK7* expression in adult plants (15-20 leaves). *UBQ5* was used as a reference gene. Values indicate differences with *mom1-1* at 4, 8 or 20-30 leaf (a) or adult (b) stages, obtained by the $2^{-\Delta\Delta Ct}$ method and they represent the mean + SD of three technical replicates. Different letters indicate significant differences among samples ($p < 0.05$; by ANOVA using Tukey's multiple comparison test in [a] and *t* test $p<0.05$ [b]). (c) Pathogen content in infected Col-0, *mom1-1* and *miniMOM1* leaves at 1 and 3 dpi, determined as in Fig 3a.

Figure 6 - Possible co-regulation of *PRR*/*NLR* genes and unlinked TEs by common sRNAs. Different mechanisms maintain repressed pericentromeric TEs and *PRR*/*NLR* genes in non-treated wild type tissues. At early stages of *Pst*-infection TEs are expressed probably due to DNA demethylation (Pavet *et al.*, 2006). Later, sRNAs matching TEs and genes are increased, and TEs are re-silenced by RdDM apparently replenishing pericentromeric DNA methylation (Dowen *et al.*, 2012). Interestingly, these sRNAs do not silence homologous *PRR*/*NLR* genes in infected tissues. Similarly, sRNAs coexist with active homologous *PRR*/*NLR* genes in adult non-treated *mom1* plants that keep pericentromeric TEs active although they contain repressive 5mC marks (Vaillant *et al.*, 2006; Habu, 2010; Numa *et al.*, 2010). Both examples suggest that common sRNAs do not silence *PRR*/*NLR* genes when TEs are expressed.