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MOM1 and Pol-IV/V interactions regulate the intensity and specificity of transcriptional gene silencing

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It is commonly observed that onset or release of transcriptional gene silencing (TGS) correlates with alteration of repressive epigenetic marks. The TGS regulator MOM1 in *Arabidopsis* is exceptional since it regulates transcription in intermediate heterochromatin with only minor changes in epigenetic marks. We have isolated an enhancer of the *mom1* mutation that points towards regulatory interplay between MOM1 and RNA polymerase-V (Pol-V). Pol-V transcribes heterochromatic loci, which seems to be required for maintenance of their silencing; however, it is still not clear how Pol-V is targeted to heterochromatin. We now provide evidence that Pol-V is required for MOM1-mediated suppression of transcription at a subset of its chromosomal targets. Thus, Pol-V genetically interacts with MOM1 in the control of gene silencing. Interestingly, functional cooperation of MOM1 and Pol-V not only broadens the range of the controlled loci in comparison to each individual factor, but also determines the degree of TGS.

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Introduction

Mitotic heritability of gene expression patterns is crucial for differentiation of multicellular eukaryotes and involves epigenetic mechanisms based on covalent modifications of DNA and histone proteins. In plants, where the germline is formed late in development, epigenetic information that may change during somatic growth can be transmitted to the progeny in the form of semi-stable epialleles (Finnegan, 2002). Maintenance of cytosine methylation, especially at CG sequences (^{m}CGs) due to the activity of DNA methyltransferase MET1, appears crucial for transgenerational epigenetic inheritance (Ronemus *et al*, 1996; Saze *et al*, 2003; Mathieu *et al*, 2007). Alterations of DNA methylation patterns are carried out by Domains Rearranged Methyltransferase-2 (DRM2) and chromomethyltransferase-3 (CMT3), which seem to have partially redundant functions in *de novo* methylation (Cao and Jacobsen, 2002a, b). Reduction of methylation levels is accomplished by DNA glycosylases, such as Repressor of Silencing-1 (ROS1) or Demeter (DME), both having elevated affinity for ^{m}C removal (Choi *et al*, 2002; Gong *et al*, 2002; Agius *et al*, 2006; Morales-Ruiz *et al*, 2006).

Changes in DNA methylation pattern seem to involve small interfering RNAs (siRNAs) that provide target specificities for methyltransferases (for a recent review see Matzke *et al*, 2009) and possibly also ^{m}C-glycosylases (Zheng *et al*, 2008). Molecular components involved in RNA-directed DNA methylation (RdDM) include plant-specific RNA polymerases Pol-IV and Pol-V (Arabidopsis Genome Initiative, 2000; Matzke *et al*, 2009). Pol-IV is composed of NRPD1 and NRPD2/NRPE2 (herein NRPD2), and Pol-V contains NRPE1 and NRPD2 (Herr *et al*, 2005; Kanno *et al*, 2005b; Onodera *et al*, 2005; Pontier *et al*, 2005). NRPD1 and NRPE1 are the largest subunits and NRPD2 is the second largest subunit of the polymerase complexes. Numerous other subunits of Pol-IV and Pol-V have been reported recently (He *et al*, 2009; Huang *et al*, 2009; Lahmy *et al*, 2009; Ream *et al*, 2009). Pol-IV is required for biogenesis of the majority of 24-nt siRNAs and is supported by Pol-V, which is responsible for production of a subset of siRNAs (Mosher *et al*, 2008; Wierzbicki *et al*, 2008). Pol-V has been proposed to act mainly downstream of Pol-IV and siRNA biogenesis by producing RNAs at target loci subjected to RdDM (Wierzbicki *et al*, 2008, 2009). Current models suggest that Pol-IV and Pol-V both preferentially transcribe hypermethylated, heterochromatic templates, reinforcing their silencing through RdDM and thus securing the stability of heterochromatin (Matzke *et al*, 2009). However, it remains unclear how the two polymerases are guided to their target loci. Particular heterochromatin properties and DNA methylation levels may be implicated in this process (Pikaard *et al*, 2008; Matzke *et al*, 2009). Furthermore, although the principal components of RdDM and their functional interactions are known, the regulation and biological role of this process are still rather obscure. It is clear, however, that RdDM can act as a backup system in case of deficiency in ^{m}CG methylation (Mathieu *et al*, 2007; Reinders *et al*, 2009; Teixeira *et al*, 2009).

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A similar backup function for deficiencies in DNA methylation was suggested for the epigenetic regulator MOM1 (Mittelsten Scheid *et al*, 2002), which is required for silencing of loci associated with chromatin of bivalent properties residing between silent heterochromatin and active euchromatin (Habu *et al*, 2006). Activation of transcription in *mom1* mutants occurs with very little or no change in DNA methylation, histone modifications or chromatin condensation (Amedeo *et al*, 2000; Steimer *et al*, 2000; Probst *et al*, 2003; Habu *et al*, 2006). The predicted *MOM1* gene product is a 2001-amino-acid protein with similarities to CHD3 chromatin-remodelling ATPases (Amedeo *et al*, 2000; Caikovski *et al*, 2008). Recent deletion studies of MOM1 showed that only nuclear localisation and a C-terminal domain of 197 amino acids are necessary and sufficient for MOM1-mediated transcriptional suppression (Caikovski *et al*, 2008). Molecular mechanisms associated with MOM1-mediated regulation of transcriptional gene silencing (TGS) are not known. To define them, we set up a screen for genetic modifiers of *mom1*.

Here we describe *mom enhancer-1* (*moe1*), which is a new allele of *nrpe1*. This suggests an unexpected link between MOM1 and heterochromatin-based transcription, siRNAs and RdDM. We provide evidence that MOM1 reinforces NRPE1-mediated silencing at a subset of its targets, and that NRPE1 either together or independently of MOM1 controls transcription at loci residing also in gene-rich chromosome regions. Furthermore, transcription profiling showed that the functional relationship between *nrpe1* and *mom1* is more complex than simple enhancement of transcription at MOM1-regulated loci. We have documented enhancing, suppressing and independent activities of NRPE1 and MOM1, suggesting that the chromosomal targets themselves largely determine the nature of MOM1 and NRPE1 cooperation.

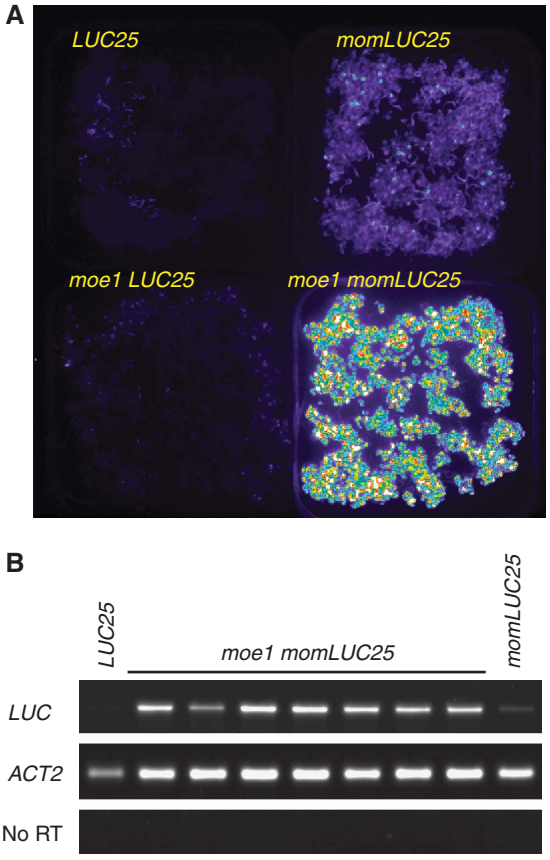

Figure 1 Characterisation of the *moe1*-mutant line. (**A**) Bioluminescence images of *LUC25*, *momLUC25*, *moe1 LUC25* and *moe1 momLUC25* seedlings. All plants are siblings derived from a backcross of *moe1 momLUC25* (M3) to *LUC25* producing BC1 plants that were self-fertilised and their progeny was genotyped at all three loci (MOE1, MOM1 and LUC25) as described later and depicted in Supplementary Figure 1B. Silencing of *LUC25* (upper left) is slightly released in *momLUC25* (upper right) but not in *moe1 LUC25* plants (lower left). The *moe1 momLUC25* plants show strong release of *LUC25* silencing (lower right). (**B**) Semi-quantitative RT–PCR of *LUC* transcript levels in *moe1 momLUC25* M2 individuals as compared with those in *LUC25* and *momLUC25* controls. *ACTIN-2* (*ACT2*) is shown as a control and no RT lacks RT.

Results

Forward genetic screen for enhancers of mom1

To identify factors that modulate the activity of MOM1, a luciferase-based reporter system was developed as a visual screen to show qualitative and quantitative differences in gene silencing. Transgenic *Arabidopsis* (Wassilewskija accession) plants were generated carrying a T-DNA insertion with the firefly D-luciferase gene (*LUC*) under the control of the *Arabidopsis ubiquitin-3* promoter (*UBQ3pro:LUC*). Two transgenic lines were chosen for further experiments: line *LUC26* containing a single copy of an active *LUC* transgene, and line *LUC25* carrying a single locus with multiple copies of a hypermethylated and silenced *LUC* transgene (Figure 1A and Supplementary Figure 1A). It has been shown that mutations in the *MOM1* gene lead to de-repression of the silenced transgenes as well as certain chromosomal loci (Amedeo *et al*, 2000; Steimer *et al*, 2000; Tariq *et al*, 2002; Habu *et al*, 2006; Vaillant *et al*, 2006). Introduction of the *mom1* (*mom1-1*, Zürich accession; Amedeo *et al*, 2000) mutation into *LUC25* (hence called *momLUC25*) resulted in partial release of silencing at the *LUC* transgene (Figure 1A). *momLUC25* plants show uniform expression of LUC, thus not showing variations in silencing maintenance due to random segregation of accession-specific traits. An experimental design can be found in Supplementary Figure 1B.

We chose an activation-tagging strategy (Weigel *et al*, 2000) for *momLUC25* mutagenesis and screened for both dominant and recessive mutations modifying the expression levels of *LUC*. For this purpose, an activation-tagging construct was generated with a selectable marker gene encoding the butafenacil-resistant form of *Arabidopsis* protoporphyrinogen oxidase (*PPO*) (see section Materials and methods; Hanin *et al*, 2001). After transformation and selection for butafenacil-resistant individuals, pools were created by combining seeds of primary transformants (M1). A visual screen, based on altered bioluminescence of LUC compared with the parental *momLUC25* strain, was applied at the M2 generation. We recovered five putative *mom1* enhancers, of which *moe1* (*mom1 enhancer1*) was further characterised and is discussed here.

The *moe1 momLUC25* seedlings showed significantly stronger LUC signals than *momLUC25* (Figure 1A). Individuals of the same M2 pool showing similarly enhanced LUC activity were analysed by RT–PCR. *LUC* transcript levels were clearly higher than those in *momLUC25* controls (Figure 1B). The enhanced *LUC* transcription was stable

throughout development in various tissues (data not shown). DNA of 13 M2 *moe1 momLUC25* plants was analysed by Southern blotting to determine whether the presence of tagging vector insertions was correlated with enhancement of LUC signals. Of these 13 plants, T-DNA insertion was not found in three *moe1 momLUC25* plants, showing that the *moe1* mutation was not tagged (data not shown). During T-DNA mediated mutagenesis, other mutations not linked to T-DNA have often been found due to ectopic deletions or DNA rearrangements (Krysan *et al*, 1999).

To determine the possible involvement of DNA methylation in controlling *LUC* expression, especially in *moe1 momLUC25*, we treated plants with 5-aza-2′-deoxycytidine (AzaC), which inhibits DNA methylation (Jones and Taylor, 1980). The lines *LUC25*, *LUC26*, *momLUC25* and *moe1 momLUC25* were germinated on control medium or medium supplemented with 4 μM AzaC. Luminescence measurements were performed 10 days after germination. In the absence of AzaC, a strong LUC signal was detected in *LUC26* and in *moe1 momLUC25* seedlings (Supplementary Figure 2A). After AzaC treatment, the LUC signal was significantly elevated in all the lines tested (Supplementary Figure 2A). In particular, AzaC treatment of *moe1 momLUC25* led to further enhancement of the LUC signal as compared with that in untreated *moe1 momLUC25* plants. Northern blots confirmed the bioluminescence data at the transcript level (Supplementary Figure 2B and C). This indicates a contribution of DNA methylation to the residual epigenetic suppression of transcription at the *LUC* locus, even in *moe1 momLUC25* plants.

NRPE1 is partially deleted in moe1

Since the *moe1* mutation was not tagged by a T-DNA insertion, we adopted a candidate gene-based approach to identify the causative mutation of *moe1*. We tested the transcription of genes involved in the control of TGS by semi-quantitative RT–PCR (Figure 2A).

As shown in Figure 2A, similar transcript levels of all tested DNA methyltransferases (MET1, DRM2 and CMT3) as well as histone methyltransferase KYP were observed in *LUC25*, *momLUC25* and *moe1 momLUC25*. This indicated that the *moe1* mutation has no influence on the transcription of these epigenetic regulators, and it is unlikely that they contribute to the *moe1* phenotype. Levels of *ROS1* RNA were significantly reduced in *moe1 momLUC25*. Thus the *ROS1* gene may have been affected in *moe1*. However, *ROS1* expression was reported to be under epigenetic control itself, and *ROS1* gene requires to be methylated for efficient transcription (Huettel *et al*, 2006; Mathieu *et al*, 2007). Consequently, changes in *ROS1* transcription in methylation-deficient mutants, but not in *mom1*, were observed by several authors (Huettel *et al*, 2006; Mathieu *et al*, 2007) and in this study. The absence of *ROS1* mRNA in *moe1 momLUC25* (Figure 2A), therefore, suggests direct or indirect involvement of MOE1 in epigenetic regulation through changes in DNA methylation, possibly due to impairment of RdDM.

RdDM involves a number of factors, among which are Pol-IV/V (Huettel *et al*, 2006; Pikaard *et al*, 2008). We decided to determine the transcript levels of the largest and second-largest Pol-IV and Pol-V subunit genes (*NRPD1*, *NRPE1* and *NRPD2*) in *moe1 momLUC25* and control plants. *NRPD1* and *NRPD2* had similar transcript levels in *moe1 momLUC25* as compared with those in *momLUC25* plants; however, *NRPE1* RNA was not detected in *moe1 momLUC25* (Figure 2A). This result suggested that either the *NRPE1* gene itself or a gene encoding its transcriptional regulator was mutated in *moe1*. To distinguish between these two options, we tested the integrity of all three Pol-IV/V genes in *moe1 momLUC25* plants. DNA from *NRPD1* and *NRPD2* could be amplified in both *momLUC25* and *moe1 momLUC25* plants. However, no *NRPE1* amplicon was obtained using *moe1 momLUC25* DNA and primers spanning the 3′-region of the *NRPE1* gene (Figure 2B). Detailed analysis of the locus showed a deletion

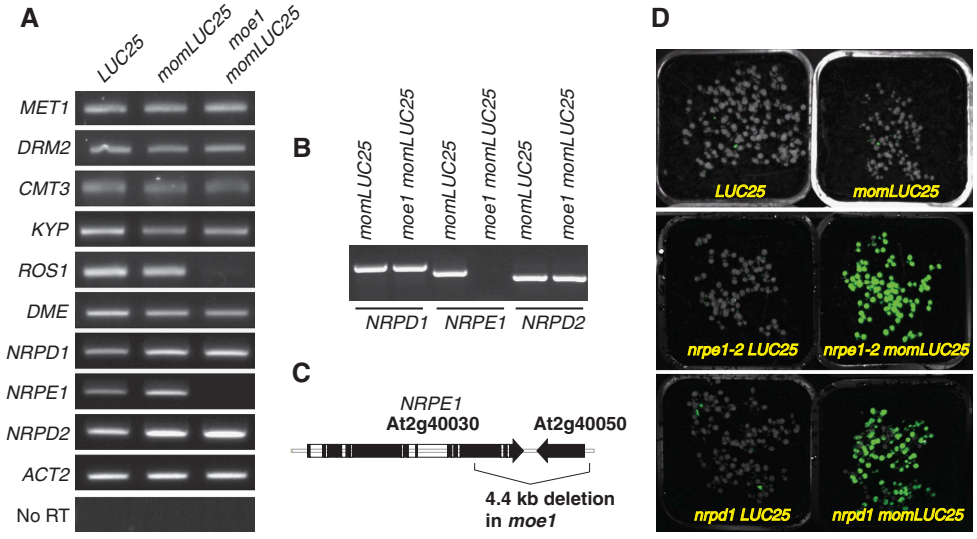

Figure 2 Identification of the *moe1* mutation and synergistic effects of the combination of *mom1* with mutations in *NRPD1* and *NRPE1*. (**A**) Semi-quantitative RT–PCR-based transcription analysis of selected genes involved in TGS. *ACT2* is shown as a control. (**B**) PCR on genomic DNA using Pol-IV/V subunit-specific primers as indicated below the image. (**C**) Genomic map of the 4.4-kb deletion in the *moe1* line. (**D**) Bioluminescence imaging showing the release of silent LUC expression in mutant combinations. In the upper row are *LUC25* and *momLUC25*. The middle and the lower row show *nrpe1-2*- and *nrpd1*-derived plants, respectively. Note that neither the *nrpd1* nor the *nrpe1* single mutant releases *LUC* silencing. All plants were photographed at the same time.

of 4.4 kb at the 3′-end of the *NRPE1* gene that extended into the neighbouring gene encoding a putative PHD domain-containing protein (At2g40050; Figure 2C).

Genetic interaction between MOM1 and the Pol-IV/V pathway

In the case of a modifier, the altered mutant phenotype should depend on the initial mutation. We determined the mutual relationship of the *moe1* and *mom1* mutations with respect to *LUC* transcription. M3 plants of *moe1 momLUC25* were backcrossed to *LUC25* and *momLUC25*. None of the F1 plants from either cross showed upregulation of the LUC signal as compared with that in the parental *LUC25* or *momLUC25* plants, suggesting that *moe1* is a recessive mutation (data not shown). Among the F2 segregating progenies, seedlings with elevated LUC signals in an approximate proportion of 1 in 16 (34 out of 484) and 1 in 4 (106 out of 485) were observed from crosses of *moe1 momLUC25* to *LUC25*, or to *momLUC25*, respectively. This indicates that the enhanced LUC signal requires the presence of both the *moe1* and *mom1* mutations, and that *moe1* alone does not release *LUC* silencing. Subsequently, we genotyped all plants showing elevated luciferase signals and confirmed that all were homozygotes for the *mom1* and *moe1* mutations. Segregants homozygous for *momLUC25* had a faint LUC signal and *moe1 LUC25* homozygous plants retained the *LUC* transgene in a silent state (Figure 1A). This result indicated that MOM1 and MOE1 act in parallel in maintaining silencing at the *LUC* locus.

To identify which of the two genes mutated in *moe1* (*NRPE1* or AT2g40050) was responsible for synergistic release of *LUC* silencing, another allele of *nrpe1* (*nrpe1-2*, formerly *nrpd1b-2*; Pontier *et al*, 2005) was introgressed into *momLUC25* and segregating F2 progenies were analysed. The single mutant for *nrpe1-2* did not reactivate the silent *LUC* transgene. Double mutant *nrpe1-2 momLUC25* individuals showed high LUC activity similar to that observed in *moe1 momLUC25* (Figure 2D). The same analysis performed with a strain with a T-DNA insertion in AT2g40050 did not show any synergistic release of *momLUC25* silencing in F2 segregating plants (data not shown). Therefore, the *moe1* mutation can be ascribed to a mutation in the *NRPE1* gene and hereafter we refer to *moe1* as *nrpe1* (allele *nrpe1-13*).

Next, we tested whether the cooperative effect observed in *mom1 nrpe1* is specific to Pol-V or if it also occurs in combination with Pol-IV. For this purpose, we crossed *momLUC25* with *nrpd1* (*nrpd1-3*; Herr *et al*, 2005) and measured the LUC signal in F2 segregants. Indeed, *nrpd1 momLUC25* double homozygous plants also showed increased levels of LUC, indicating a general synergistic effect of the MOM1 and the Pol-IV/V-silencing pathways (Figure 2D).

Arabidopsis nrpe1 and *mom1* single mutants (Col-0 accessions) show no obvious developmental abnormalities, apart from *nrpe1* having slightly delayed flowering under short-day conditions (7 days delay compared with the wild type; Amedeo *et al*, 2000; Pontier *et al*, 2005). Interestingly, *mom1 nrpe1* (Col-0 accessions) mutations show an enhanced late-flowering phenotype (12 days delay compared with the wild type) under short-day conditions when compared with wild-type plants (Supplementary Figure 2D). To determine the mechanism responsible for this delay, we examined *mom1 nrpe1* plants for ectopic activation of an epigenetically silenced gene encoding the flowering inhibitor FLOWERING WAGENINGEN (FWA), but its transcription remained suppressed (data not shown). Subsequent transcription profiling data (see below) did not show significant changes in the expression of other known regulators of flowering time. Thus, it is possible that an as yet unknown inhibitor of flowering is mis-expressed in the double mutant line.

MOM1 contributes to siRNA accumulation at the transgenic locus

The silent *LUC25* line has a single transgenic locus with three copies of the *LUC* gene and silencing at this locus is accompanied by DNA hypermethylation (Figure 3A and Supplementary Figure 1A). Therefore, it was of interest to examine the levels of DNA methylation in relation to transcriptional reactivation at the *LUC* transgene in the *momLUC25* and *nrpe1 LUC25* lines as compared with the *nrpe1 momLUC25* double mutant.

The following methylation-sensitive enzymes were used to analyse DNA methylation at *UBQ3pro*: *Hpa*II (recognises CCGG and is sensitive to $^{m}C^{m}CGG$, thus reporting on methylation in CG and CHG contexts), *Hae*III (recognises GGCC and is sensitive to $GG^{m}C^{m}C$) and *Nla*III (sensitive to ^{m}C ATG). Here, *Hae*III and *Nla*III both report on CHH methylation,

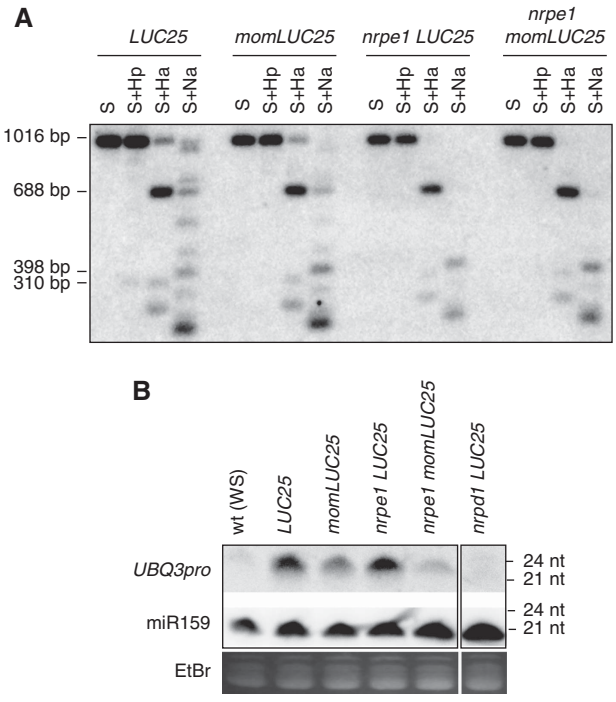

Figure 3 The levels of DNA methylation and siRNAs in the *UBQ3* promoter region that drives *LUC* expression. (**A**) Southern blot of genomic DNA digested with methylation-sensitive restriction enzymes and hybridised with a probe covering *UBQ3pro*. The digestion with the methylation insensitive *Sml*I enzyme produced a fragment of 1016 bp. S = *Sml*I, Hp = *Hpa*II, reports on CG/CHG methylation, Ha = *Hae*III and Na = *Nla*III, reports on CHH methylation. (**B**) Detection of siRNAs homologous to the transgenic *UBQ3pro* in *momLUC25*, *moe1 LUC25*, *moe1 momLUC25* and *nrpd1 LUC25*. Micro-RNA mir159- and ethidium bromide-stained gels (EtBr) are shown as loading controls.

which is the signature of RdDM (see Supplementary Figure 2E for restriction map of the *UBQ3pro*). The Southern blot in Figure 3A shows that, while methylation at CG sites remained unaffected in all mutant combinations, *nrpe1 LUC25* and *nrpe1 momLUC25* had strongly reduced levels of methylation at CHH sites, as expected for *nrpe1* mutation. In *momLUC*25, only slight reduction of CHH methylation was observed over the *LUC25* strain, suggesting that RdDM is not greatly affected by the *mom1* mutation.

Since NRPE1 is involved in RdDM, we compared the influence of *nrpe1*, *mom1* and *mom1 nrpe1* on the accumulation of siRNAs derived from *UBQ3pro*. *LUC25* plants accumulated high levels of 24-nt-class siRNAs involved in directing DNA methylation and TGS (Figure 3B), and the *nrpe1* mutant showed similar levels of these siRNAs. Surprisingly, there was a reproducible reduction of siRNAs in *momLUC25* and the siRNA levels were even more reduced in the *nrpe1 momLUC25* double mutant. The levels of control micro-RNA (miR159) were not affected by any of the analysed mutations.

Even though biogenesis of *UBQ3pro* siRNAs was entirely dependent on NRPD1 (Figure 3B), the transgene remained silent in *nrpd1* and only in *nrpd1 momLUC25* double mutants was LUC expression activated (Figure 2D). Therefore, MOM1 is able to suppress transcription at this locus independently of siRNA biogenesis.

Properties of chromosomal targets silenced by MOM1 and/or NRPE1

To distinguish the loci where TGS is controlled by MOM1 and/or NRPE1 and loci with silencing simultaneously regulated by both activities, we compared transcript levels using *Arabidopsis* tiling arrays in wild-type, *mom1-2* (Habu *et al*, 2006), *nrpe1-2* (Pontier *et al*, 2005) and newly generated *nrpe1-2 mom1-2* double mutant plants. All profiled strains were in Col-0 background.

Comparison of *mom1* to wild type showed increased accumulation in the mutant of RNAs derived from hypermethylated loci residing adjacent to centromeres that are enriched in transposon-derived repeats (Supplementary Figure 3; Repbase, www.girinst.org; Zhang *et al*, 2006). In contrast, loci activated in *nrpe1* were more evenly distributed along chromosomal arms (Supplementary Figure 3), which is consistent with a previous description of a subset of transcripts that accumulated in *nrpe1* (Huettel *et al*, 2006). At first glance, the transcriptional profiling of the double mutant resembles the sum of the transcription profiles of *mom1* and *nrpe1* (Supplementary Figure 3).

However, a refined evaluation of the expression profiling data also showed non-additive changes of specific mRNAs. These were identified when we assessed the expression of unique annotated genes using TAIR7 genome annotation (Naouar *et al*, 2009). Activated genes were divided into three groups, with 67 and 22 activated in *mom1* and *nrpe1*, respectively, and 33 genes that were only significantly activated in *mom1 nrpe1* double mutants (Figure 4A). This last and most interesting category encompasses novel TGS targets silenced in a cooperative manner by MOM1 and NRPE1. It should be noted that the number of activated genes found by these analyses is highly undervalued since non-annotated and repetitive loci were not considered.

Genes activated by both single mutants and genes specifically activated in *mom1 nrpe1* double mutants reside predominantly in the pericentromeric regions of all five chromosomes (Figure 4B). Therefore, there is no drastic change in the spatial distribution of TGS targets requiring cooperation between MOM1 and NRPE1 for their silencing, as compared with the locations of targets regulated by each single mutant. This is consistent with observations of particular, bivalent chromatin properties of silencing targets controlled by MOM1 (Habu *et al*, 2006).

To supplement data obtained using the TAIR7 genome annotation (Naouar *et al*, 2009) by transposon sequences, we generated a chip definition file (CDF) containing all TAIR8-annotated transposons (www.arabidopsis.org). To limit errors due to cross hybridisation of similar transposons, we directed our assessment to transcriptional activities of different transposon families within one of the 17 super-families (Figure 4C; see Supplementary data for details). We applied the criteria for 'significant activation of transcription' similar to those for genes listed when using the CDF of Naouar *et al* (2009). However, for a family of transposons to be called 'activated', at least one member of the family needed to meet the criteria of the 'significant activation of transcription' (compare Figure 4C and Supplementary Table II). Our data for *mom1* showed preferential activation of LTR/Gypsy transposons, consistent with a previous observation that MOM1 mediates TGS of pericentromeric loci encoding repeats related to the *Athila* LTR/Gypsy transposon (Steimer *et al*, 2000; see Supplementary Figure 4 for details on retrotransposons). In comparison with *mom1*, a broader spectrum of transposon superfamilies was activated in *nrpe1*, with less preference for activation of a particular superfamily (with the possible exception of RC/Helitron). The *mom1 nrpe1* double mutant shows a clear additive effect of the two mutations with respect to the number of re-activated transposon families and also the level of their activation.

To better understand the properties of chromatin associated with MOM1- and NRPE1-regulated loci, we examined the levels of siRNAs and DNA methylation using data available for the corresponding target loci in wild-type plants (Zhang *et al*, 2006; Lister *et al*, 2008). For this purpose, we considered genes that are upregulated in each single mutant (67 for *mom1*, 22 for *nrpe1*) and the 33 synergistically upregulated genes in the *mom1 nrpe1* double mutant (Figure 4D). Interestingly, MOM1 and NRPE1 targets have slightly different properties. MOM1-controlled genes are hypermethylated and enriched in siRNAs throughout the entire gene regions (5′ upstream sequence, CDS and 3′ downstream sequence; Figure 4D). In contrast, NRPE1-controlled genes are hypermethylated and show siRNA accumulation mostly in the promoter area, with a maximum at about 700 bp upstream of the transcription start site. In general, all loci regulated by MOM1, NRPE1 or a combination of them had higher levels of siRNAs and DNA methylation than a randomly selected subset of genes (Figure 4D). These data are consistent with the observation that MOM1 targets are preferentially localised in pericentromeric heterochromatin enriched in repressive epigenetic marks (Habu *et al*, 2006), whereas NRPE1 targets tend to be located in more gene-rich euchromatic regions (Huettel *et al*, 2006). Interestingly, genes simultaneously regulated by MOM1 and NRPE1 have proper-

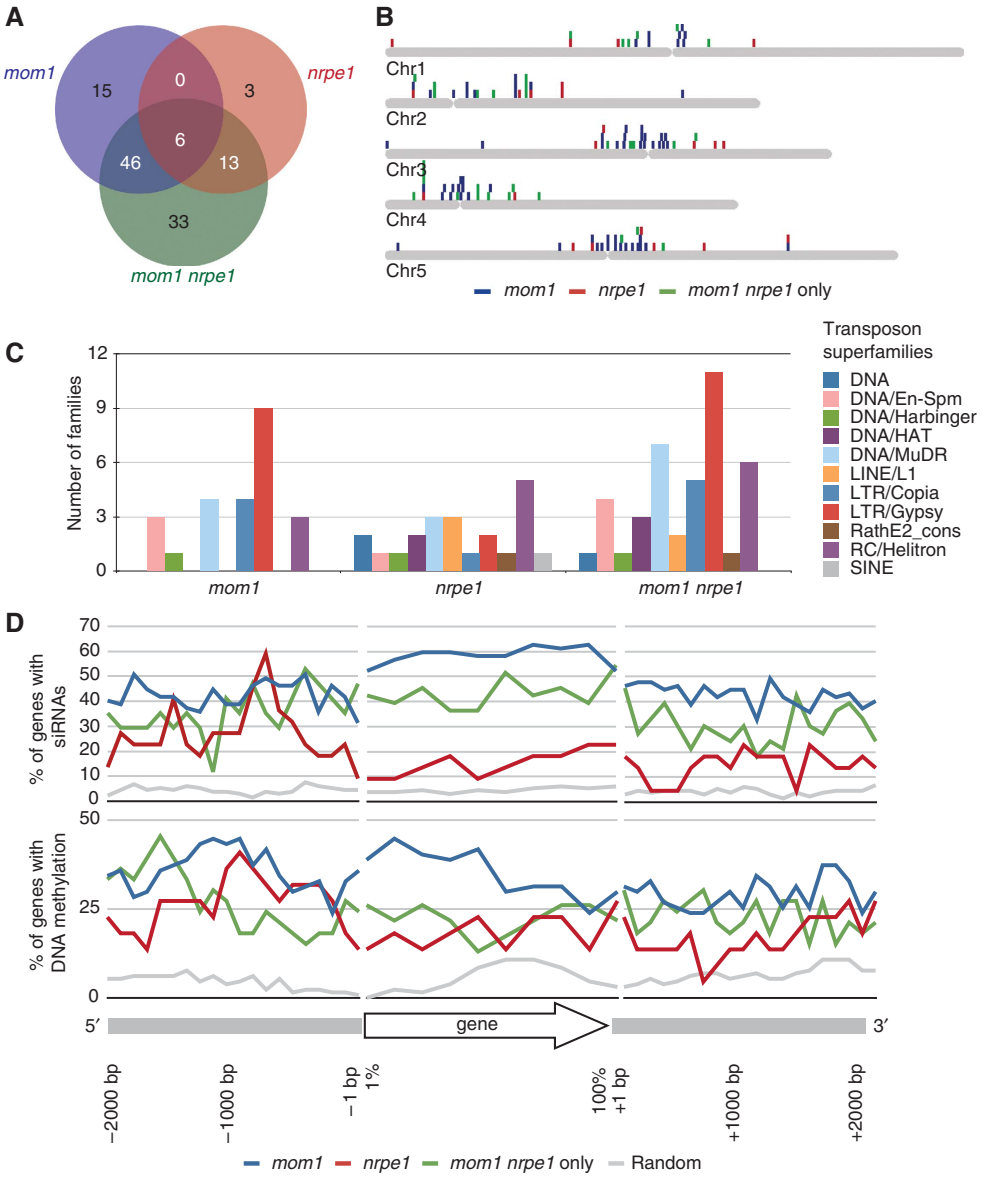

Figure 4 A genome-wide overview of transcripts upregulated in the different mutants and their epigenetic characteristics. (**A**) Venn diagram showing the number of upregulated genes. This diagram shows the target overlaps between *mom1*, *nrpe1* and *mom1 nrpe1*. (**B**) Genomic overview of the chromosomal localisation of genes activated in the mutants described in this study. Genes upregulated by *mom1* are presented as blue squares, *nrpe1* in red and genes synergistically upregulated only in the double mutant are shown in green. (**C**) The number of transposon families transcriptionally upregulated in the mutants analysed in this study. Each superfamily is represented by a different colour, as indicated to the right. (**D**) siRNA and DNA methylation levels (from wt plants; Zhang *et al* (2006) and Lister *et al* (2008)) at genes upregulated by the different mutant combinations (blue = *mom1*, red = *nrpe1*, green = *mom1 nrpe1* only, grey = random). The 2-kb 5′ and 3′ regions were divided into 100-bp segments and the gene body into 10 even-sized segments covering the whole gene. The data represent percentages of genes having siRNAs and DNA methylation in the corresponding segments. Percentages for randomly selected genes are presented in grey.

ties intermediate between those of genes regulated by a single silencing component (Figure 4D).

Locus-specific regulatory interplay between MOM1 and NRPE1

We isolated *nrpe1* in a screen for *mom1* enhancers and found in fact that transcript levels of the transgenic locus used for screening were higher in *mom1 nrpe1* double mutants than in *mom1* (Figure 1B). However, subsequent transcription profiling results suggested that several chromosomal loci showing TGS controlled by *mom1* were not transcriptionally enhanced in *mom1 nrpe1*. Thus, cooperation between MOM1 and NRPE1 in the maintenance of TGS is more complex than previously thought and is possibly locus-specific. To test this hypothesis, northern blot analysis was performed to determine transcript levels from previously well-characterised loci activated in *mom1* mutants, such as *Athila*-related Transcriptionally Silent Information (TSI) and *Mutator*-like element (*MULE-F19G14*) located upstream of the *Cyclophilin40* gene (*AtCyP40*) (Figure 5A; Steimer *et al*, 2000; Habu *et al*, 2006). With the exception of the 5-kb band for TSIA15, there was no difference between *mom1*

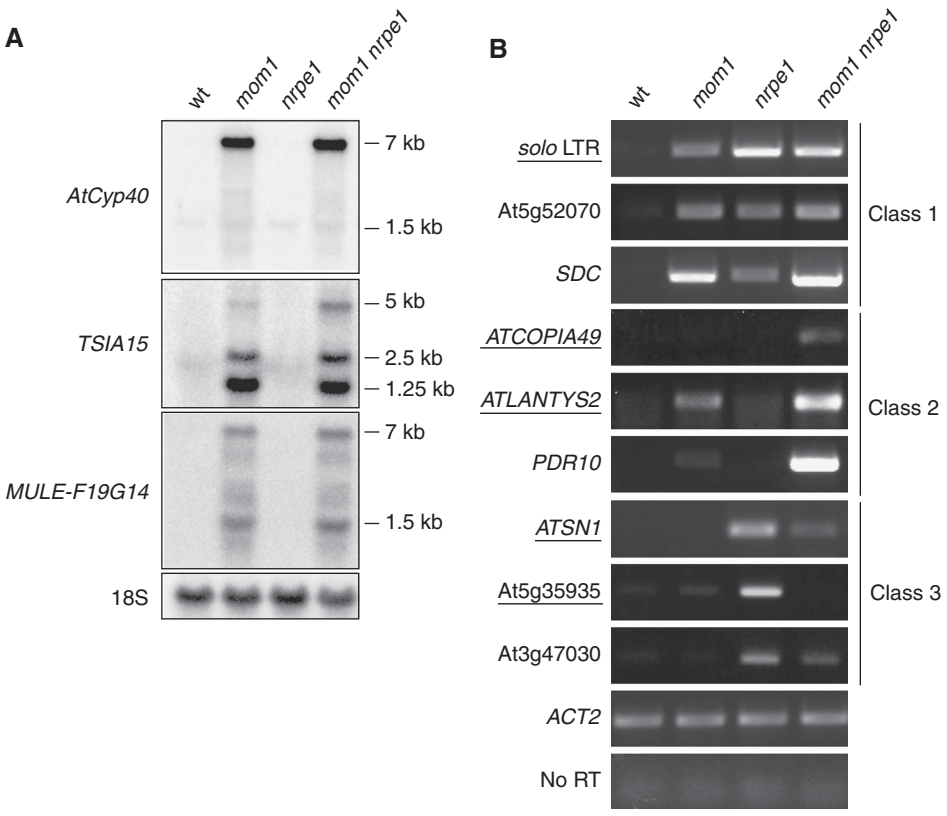

Figure 5 The effects of single and double mutants on selected epigenetically controlled loci. (**A**) Northern blots of targets previously shown to be upregulated in *mom1*. Upper panel: AtCyp40 (cyclophilin-40); middle panel: TSIA15 and lower panel: MULE-F19G14. The bottom panel shows 18S RNA as a loading control. (**B**) Semi-quantitative RT–PCRs of representative endogenous loci. The underlined names indicate the transposon-derived targets. *ACT2* is shown as a control.

and *mom1 nrpe1* in the levels of transcripts from these loci (Figure 5A). Therefore, enhancement of transcript accumulation at MOM1-regulated loci due to depletion of NRPE1 seems not to be a general rule.

Further detailed analysis identified three classes of TGS targets that were differently expressed in single as compared with double mutants (Figure 5B). Class 1 encompasses loci that are transcriptionally activated similarly in *mom1* and *nrpe1* and *mom1 nrpe1* (e.g., loci shared by all three circles in Figure 4A). The existence of this class suggests that for these targets MOM1 and NRPE1 act non-redundantly in maintenance of TGS, and each factor is individually required for silencing. Class 1 includes previously described RdDM targets such as soloLTR (Huettel *et al*, 2006) and SDC (Henderson and Jacobsen, 2008).

Class 2 are loci that are synergistically activated in double *mom1 nrpe1* mutants as compared with each single mutant, as well as loci that retain silencing in *nrpe1,* as observed for *LUC* (Figure 5B and Figure 1A). Transcription profiling identified 24 targets of class 2 (Supplementary Table I), eight of which were associated with members of a specific LTR/Copia retrotransposon family, *ROMANIAT5*.

In class 2, two genes in particular caught our attention. Pumilio-related *APUM9* (At1g35730) and *PLEIOTROPIC DRUG RESISTANCE-10* (*PDR10*, At3g30842) showed very high accumulation of transcripts in the double mutant (Figures 5B and 6A and B, and Supplementary Figure 5). Furthermore, this accumulation was correlated to strongly activated transcription of *ROMANIAT5* elements residing in the vicinity of these genes (Figure 6A and B, and Supplementary Figure 5). For example, northern blot analysis of *APUM9* transcripts showed accumulation in *mom1 nrpe1* of RNAs with estimated sizes of 3.1 and 4.1 kb (Figure 6B). This result is unexpected since the annotated cDNA length of *APUM9* is 1695 bp (www.arabidopsis.org). According to tiling array data, the transcription start site was located almost 1 kb upstream of the predicted ORF of *APUM9* and ended about 200 bp downstream of the gene model (Figure 6A). This could account for the 3.1-kb band observed on the northern blot. The longer 4.1-kb transcript may result from incomplete splicing, as again suggested by the tiling array analyses (Figure 6A, signal present in the introns of *APUM9*; Alló *et al*, 2009).

Consistently, *ROMANIAT5* transcripts also synergistically accumulated in *mom1 nrpe1* (Figure 6A and B). The northern blot data not only indicate transcription of the *ROMANIAT5* members flanking *APUM9* and *PDR10*, but also ectopic copies of *ROMANIAT5*-related transposon sequences resulting in transcripts about 2 kb smaller than expected for a full-length transposon and also very long RNAs (>6 kb of gel resolution). According to the tiling array data these long transcripts may reach up to 28 kb (Figure 6B and Supplementary Figure 5).

To better understand the molecular mechanism involved in synergistic activation of the *ROMANIAT5* family, we estimated their transcript levels in *drm2*, *mom1 drm2*, *ddm1* and in *met1* mutants. The double *mom1 drm2* mutant showed synergistic effects similar to those observed for the *mom1 nrpe1* (Figure 6B). Therefore, a further component of

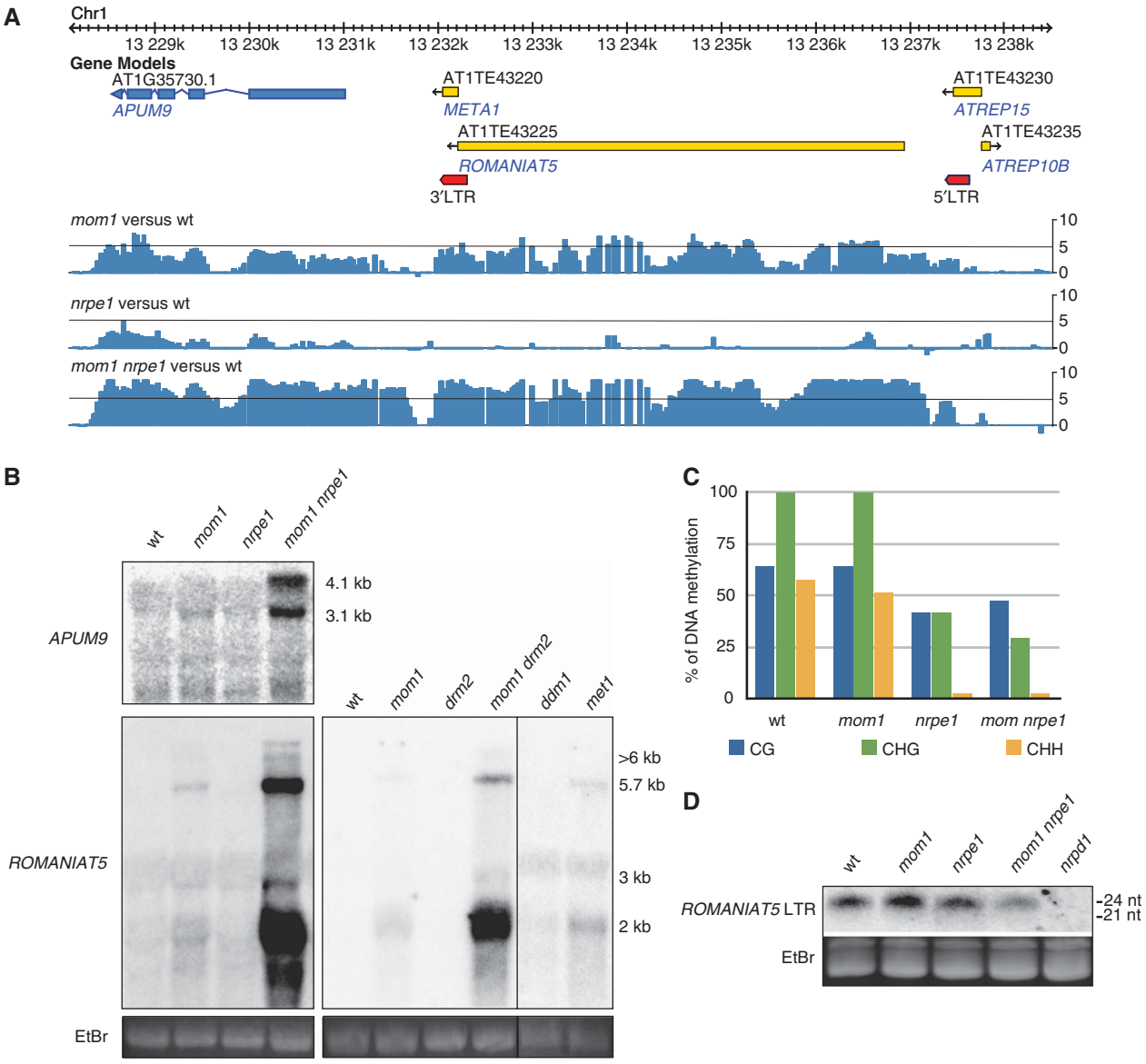

Figure 6 The synergistic transcriptional effects of functional loss of MOM1 and factors involved in RdDM. (**A**) A 10-kb region around *APUM9* showing the gene and the *ROMANIAT5* transposon that controls its expression. Data were visualised with our online genome browser software, EpiExpress. The expression levels of the different mutants as compared with the wild type are shown in the blue histograms below. Intensities are shown on a log2 scale. LTRs as annotated by Peterson-Burch *et al* (2004) are represented as red arrows. (**B**) Qualitative and quantitative confirmation of the tiling array data by northern blotting. Both *APUM9* and *ROMANIAT5* show strong synergistic transcriptional reactivation in *mom1 nrpe1* double mutants (upper and lower panels, respectively). Synergistic effects were also observed in the combination of *mom1* with *drm2* (right panel). Neither *ddm1* nor *met1* released *ROMANIAT5* transcription to a comparable level (far right). An EtBr-stained gel is shown as loading control. (**C**) Bisulphite sequencing of the *ROMANIAT5* LTR residing in front of *APUM9*. The bars represent the percentage of cytosines methylated in the corresponding sequence context. (**D**) siRNA blots detecting 24-nt siRNAs that target the *ROMANIAT5* LTRs. An EtBr-stained gel is shown as loading control.

the RdDM pathway shows cooperative silencing together with MOM1 of the *ROMANIAT5* transposon family. This further supports a functional link between MOM1 and Pol-V, and more general to RdDM. It is notable that neither the *ddm1* nor *met1* mutations were capable of strong reactivation of the transcription of this transposon family.

To search for possible mechanisms involved in activating the *ROMANIAT5* element flanking *APUM9*, we determined the distribution of DNA methylation at the 3′LTR neighbouring the *APUM9* promoter (Figure 6C and Supplementary Figure 6A). The only significant reduction in DNA methylation was correlated with the NRPE1 deficiency. In *nrpe1* and *mom nrpe1* this reduction was observed in all sequence contexts, with predominant methylation depletion in CHH sequences (Figure 6C). Since *ROMANIAT5* elements remain silent in *nrpe1*, it can be concluded that significant reduction of DNA methylation and almost complete loss of CHH methylation is not sufficient for release of TGS, although it may be effective for transcriptional activation of the *ROMANIAT5* family when MOM1 function is compromised.

Pol-IV and to some extent Pol-V are involved in the biogenesis of 24-nt siRNAs that are believed to guide TGS (Mosher *et al*, 2008). To elucidate the roles of these two polymerases in siRNA biogenesis in the area of the

ROMANIAT5 LTR neighbouring the *APUM9* promoter, we determined the local levels of siRNAs in *mom1*, *nrpe1*, *mom nrpe1* and *nrpd1* (Figure 6D). Interestingly, siRNA levels decreased when the *mom1* and *nrpe1* mutations were combined, but were unaltered in each single mutant. This suggests that NRPD1-mediated siRNA biogenesis or the subsequent stability of siRNAs depend on MOM1 and NRPE1. Alternatively, involvement of RNA Pol-II transcription may restrict the access of the Pol-IV complex to this area. Noticeably, although the *nrpd1* mutation abolished siRNA production (Figure 6D), it did not release the silencing of *APUM9* or *ROMANIAT5* (Supplementary Figure 6B). This suggests that removal of siRNAs is not sufficient for TGS release at this locus, mirroring the observations for the *LUC* transgene (Figures 2D and 3B).

The very intriguing class-3 silencing targets include loci at which TGS is compromised in *nrpe1* but restored in *mom1 nrpe1* double mutants (Figure 5B). Therefore, the *mom1* mutation acts as an *nrpe1* suppressor at these loci. This may be explained by the activation of a further TGS mechanism(s) in double mutant plants or by a requirement for MOM1 for transcriptional activation or transcript stability in the absence of NRPE1.

The RT–PCRs in Figure 5B validate the transcription profiling data and add information on ATCOPIA49 and ATLANTYS2 not present on the tiling array, and on AtSN1, which produces a non-polyadenylated Pol-III transcript (Wierzbicki *et al*, 2008). These additional data further support the existence of all three classes of silencing targets and confirm the intriguingly complex pattern of silencing functions shared between MOM1 and NRPE1, which vary surprisingly at the different target loci.

Discussion

The molecular mechanisms of epigenetic regulation of transcription are tightly associated with changes in DNA methylation and changes in covalent modifications of histones, both of which provide epigenetic marks influencing the transcriptional states of chromatin (Henderson and Jacobsen, 2007; Pfluger and Wagner, 2007; Vaillant and Paszkowski, 2007; Pikaard *et al*, 2008; Matzke *et al*, 2009). Although correlations between specific combinations of epigenetic marks and transcriptional activity have been well established, it is still not understood well how these marks are targeted to individual loci, or with the exception of DNA methylation at CG dinucleotides (Saze *et al*, 2003), how they are propagated through DNA replication. Furthermore, it has been observed that the marks can be overridden by certain epigenetic regulators that are able to activate silent loci without significant changes in their epigenetic signatures. Several such regulators have been described in *Arabidopsis*, including MOM1 (Amedeo *et al*, 2000; Vaillant *et al*, 2006), Brushy1 (BRU1) (Takeda *et al*, 2004) and Replication protein-A2 (RPA2) (Elmayan *et al*, 2005), but the molecular mechanisms contributing to their transcriptional silencing activities are not known. Among these factors, MOM1 most clearly contributes to transcriptional silencing and its depletion results in the activation of transcription at transgenic and endogenous silent loci (Amedeo *et al*, 2000; Steimer *et al*, 2000; Habu *et al*, 2006; Vaillant *et al*, 2006).

The identification of a new mutant allele of *nrpe1* as a *mom1* enhancer implies a functional interplay between MOM1 and the RdDM pathway. Interestingly, although release of silencing at the transgenic locus used for the mutant screen was enhanced in the *mom1 nrpe1* double mutant, it could be further boosted by chemical inhibition of DNA methylation (Supplementary Figures 2A–C). This result points towards yet another level of silencing in addition to MOM1 and RdDM that probably involves CG or CHG methylation mediated by MET1 or CMT3, respectively (Ronemus *et al*, 1996; Lindroth *et al*, 2001). This suggests multilayer, overlapping control of transcriptional silencing at this transgenic locus, as also observed for other loci for which silencing is regulated by MOM1 (Mittelsten Scheid *et al*, 2002). Enhanced release of silencing has been documented previously for *mom1 ddm1* double mutants (Mittelsten Scheid *et al*, 2002). DNA methylation is compromised in *ddm1* as in *nrpe1*. Thus, MOM1 seems to provide a silencing backup linked to DNA methylation deficiency. In the case of the interaction between the *mom1* and *ddm1* mutations, *mom1* acted as a *ddm1* enhancer, but it was not possible to determine the influence of the *ddm1* mutation on *mom1*, since all MOM1-controlled loci were strongly activated in the *ddm1* mutant, reaching transcripts levels much higher than those observed for *mom1*. Therefore, *nrpe1* is the first genetic modifier of the *mom1* mutation.

As NRPE1 participates in the RdDM process, we assessed whether mutations in other components of this pathway, such as NRPD1 (Pol-IV) or DRM2, also behave as genetic modifiers of *mom1*. When either the *nrpd1* or the *drm2* mutants were combined with *mom1*, there was enhanced transcript accumulation at MOM1-regulated targets (Figure 6B and Supplementary Figure 6B). Therefore, it can be concluded that MOM1 genetically interacts not only with one selected component of RdDM the pathway, but is functionally linked to the RdDM process as such.

Comparing the transcription profiles of single *nrpe1* and *mom1* to double *mom1 nrpe1* mutants showed complex regulatory interactions between MOM1 and RdDM in the genome-wide control of TGS. Genes targeted for silencing by MOM1 and NRPE1 seem to have similar but not identical properties. MOM1 targets have elevated DNA methylation and siRNA levels across the entire loci (Figure 4D). NRPE1-upregulated genes also have higher levels of siRNAs and DNA methylation, but this occurs mostly in their promoter regions (Figure 4D). On reactivation in *mom1*, DNA methylation levels seem to be only slightly reduced. In the case of *UBQ3pro* slight reduction of CHH methylation in *mom1* correlates with reduction of siRNA levels corresponding to this locus. This is in contrast to *nrpe1* where DNA methylation in the CHH sequence context is significantly reduced. Decrease of CHH methylation was also observed in the *mom1 nrpe1* double mutant, where levels of transcripts were very high. Most MOM1-regulated loci remained silent in *nrpe1*, although they likely have reduced CHH methylation (Kanno *et al*, 2005b; Pontier *et al*, 2005); thus loss of CHH methylation contributes to high levels of transcription only when MOM1 function is compromised. As a consequence, the *mom1 nrpe1* mutant showed a number of novel RdDM-controlled chromosomal loci that are hidden in the wild type and *nrpe1* by MOM1-mediated TGS.

To better illustrate the interplay between MOM1 and NRPE1 in the regulation of chromosomal TGS, we screened the tiling array transcriptomes of the wild type, *mom1*, *nrpe1* and the *mom1 nrpe1* double mutant for activation of transcription. At several MOM1-regulated chromosomal loci, we observed very high transcript levels in *mom1 nrpe1* as expected for a mutation selected as a *mom1* enhancer (Figures 5B, 6A and B). A subset of these loci was located in the vicinity of an LTR/Copia retrotransposon *ROMANIAT5* that is silent in the wild type, and in the *nrpe1, drm2* mutants (Figure 6A and B). Transcription of *ROMANIAT5* was only slightly activated in *mom1*, but reached very high transcript levels in *mom1 nrpe1*. Interestingly, depletion of DNA methylation in the *drm2, ddm1* or *met1* mutant was not sufficient to cause significant alleviation of *ROMANIAT5* silencing. This is rather unusual, since most of the loci silenced by MOM1, which reach low transcript levels in *mom1* mutants, are highly transcribed in the *met1* and *ddm1* mutants (Vaillant *et al*, 2006). Thus, the combination of epigenetic mechanisms involved in TGS maintenance at *ROMANIAT5* seems to be exceptional.

Reduction of siRNA levels in *mom1 nrpe1* has been observed for the transgenic locus and for *ROMANIAT5* LTR. siRNA biogenesis at these loci is linked to NRPD1 activity, but complete removal of siRNAs in the *nrpd1* mutant did not reactivate their transcription. Therefore, it is not clear whether reduction in siRNAs levels in *mom1 nrpe1* is required for hyperactivation of transcription. It was notable in the case of the transgenic *LUC* locus, that siRNA levels were also slightly reduced in the *mom1* single mutant. Therefore, MOM1 seems to influence the biogenesis and/or stabilisation of siRNAs, although the functional significance of this in the release of silencing in *mom1* and *mom1 nrpe1* mutants is obscure. It is possible that common MOM1–RdDM targets maintain particular silencing and chromatin properties that promote NRPD1 activity over other RNA polymerases, thus contributing to high levels of siRNAs (Wierzbicki *et al*, 2008). The chromatin properties of MOM1 target loci were previously characterised and classified as intermediate heterochromatin (Habu *et al*, 2006). From this study, it is apparent that RdDM also contributes to regulation in this intermediate heterochromatin and, therefore, very likely influences its properties. Nevertheless, MOM1 seems to have a decisive role in their initial, low transcriptional activation and only later does RdDM control the levels of transcripts. Thus MOM1 and NRPE1 form a double lock preventing transcription of certain genes and transposons.

In this respect, it is interesting to mention the regulatory role of transposons that are able to influence the expression of neighbouring genes (Kashkush *et al*, 2003). In our studies, genes residing in the vicinity of *ROMANIAT5* accumulated high levels of transcripts in *mom1 nrpe1* (class 2; Figures 5B, 6A and B). However, this is unlikely to be due to simple transcriptional read-through, since we did not detect hybrid RNAs spanning the transposon and the neighbouring gene (Figure 6B). The tiling array data showing low to no hybridisation signals just downstream of the *ROMANIAT5* 3′LTR supports this observation (Figure 6A). This is further consistent with the transcript patterns on the tiling array that suggest high transcriptional activation of the regions surrounding *ROMANIAT5* transposons, not resulting in one long transcript, but rather producing several smaller transcripts covering the activated area. Long transcripts deriving from *ROMANIAT5* were, however, observed on northern blots that might originate upstream of *PDR10* (Figure 6B and Supplementary Figure 5). Thus, it can be envisaged that chromatin properties may have initially changed at activated *ROMANIAT5* transposons, and that the active chromatin marks subsequently spread to neighbouring genes (Talbert and Henikoff, 2006; Kanno *et al*, 2008). Alternatively, the 5′LTR of *ROMANIAT5* may drive the expression of the transposon itself and the 3′LTR drives *APUM9* expression. It has been reported that LTRs can have both strong promoter and enhancer activities (Kashkush *et al*, 2003). In the case of *APUM9*, an additional transcript longer than the correctly processed *APUM9* mRNA over-accumulated in *mom1 nrpe1*. According to the tiling array analyses, this transcript resulted from an upstream alternative transcription start and aberrant mRNA processing. This is consistent with accumulating evidence for a link between chromatin properties and these crucial steps in mRNA biogenesis (Alló *et al*, 2009).

The *nrpe1* mutation was found in a screen for *mom1* enhancers using the overexpression of an initially silent transgenic locus as the readout (Figure 1A). Subsequent RNA profiling data confirmed largely synergistic release of silencing at many endogenous loci (Figure 4A and B, and Supplementary Table I). However, the data also showed more complex and also unanticipated interactions between *mom1* and *nrpe1*. For example, class-3 silencing targets with transcription activated in the *nrpe1* mutant and suppressed in the *mom1 nrpe1* double mutant best illustrate this complexity, showing that genetic interactions between epigenetic factors or pathways can drastically differ at different loci (Figure 5B). It is paradoxical that factors involved in suppressing transcription can also have a function in promoting transcription. Obviously, an indirect effect through activation of a suppressor cannot be excluded.

In summary, the data presented here show the existence of a complex interplay between the MOM1- and NRPE1-silencing pathways. They further show that NRPE1 also contributes to the suppression of transcription of targets residing not only in euchromatin, but also in intermediate heterochromatin. Furthermore, we show that certain transposon-derived loci are under the tight control of two epigenetic pathways, creating a backup system for the suppression of potentially deleterious elements.

Materials and methods

Plant material and mutagenesis

The *LUC25* and *LUC26* lines were generated by transforming a modified pPH108 vector in which the *NOS* promoter, that drives *LUC*$^{+}$, was replaced by the *UBQ3* promoter (P Heifetz, unpublished data, and O Mittelsten Scheid, personal communication) into *Arabidopsis* plants of Wassilewskija accession (WS). *momLUC25* was obtained by crossing *mom1-1* mutant (Amedeo *et al*, 2000) with *LUC25* followed by selection for the homozygous *mom1-1* mutation.

The activation-tagging vector used for the mutagenesis of *momLUC25* was obtained by replacing the Basta-resistance gene in pSKI015 (kindly provided by Dr Weigel; Weigel *et al*, 2000) with the butafenacil-resistance marker (Hanin *et al*, 2001), resulting in pAT-PPO. *momLUC25* plants that were transformed with the *Agrobacterium* GV3101 pMP90RK strain carrying the pAT-PPO using the floral dip method (Clough and Bent, 1998). To select

transformants, T1 plants were treated with butafenacil and pools of 10 individual resistant T1 plants were made.

To obtain *mom1 nrpe1* double mutants in a pure Columbia accession background for transcription profiling, a *mom1-2* (Habu *et al*, 2006) plant was crossed with *nrpe1-2* (formerly *nrpd1b-2*; Pontier *et al*, 2005).

drm2-2 seeds were obtained from SALK (SALK_150863; Chan *et al*, 2006) and *ddm1-2* seeds were provided by E Richards (Vongs *et al*, 1993).

Northern and siRNA blots

RNAs and siRNAs were extracted using the Ambion *mir*Vana miRNA isolation kit as described previously (Kanno *et al*, 2008). siRNAs were analysed as reported earlier (Kanno *et al*, 2008), with the difference that cross-linking of RNA to the membrane was performed using carbodiimide (Pall *et al*, 2007). Northern blots were performed with 10 μg of RNA, radioactively labelled double-stranded cDNA probes (Megaprime DNA labelling system; Amersham) and Perfecthyb plus (Sigma) as the hybridisation solution. Transcript sizes were estimated using the peqGOLD High Range RNA ladder (Peqlab, Germany). TSIA15 was cloned as an *Spe*I/*Kpn*I fragment into pBSK and vector-specific primers were used to produce the probe.

Transcription profiling, RT–PCRs and bioinformatic data analysis

Hybridisation data were processed with the R statistical software (r-project.org) and BioConductor 2.1 (www.bioconductor.org), applying the chip definition file (CDF) kindly provided by Naouar *et al* (2009). These analyses compared the expression of each gene between the mutants and the wild type (Supplementary Table I). In this approach, we suppressed repetitive tiles present on the tiling array, thereby reducing possible cross-hybridisation artefacts (Naouar *et al*, 2009). The following criteria were applied in the selection of loci with significant activation of transcription in the mutants: a twofold or higher increase in transcript level and a *P*-value below 0.01. All profiling data are available online through our genome browser EpiExpress (gbrowse.vital-it.ch/cgi-bin/gbrowse/epiexpress/). Detailed descriptions are included in the Supplementary data.

Southern blots and bisulphite sequencing

Southern blots were carried out as described previously (Kanno *et al*, 2005a). Bisulphite sequencing was performed as reported by Kanno *et al* (2008). Twelve individual clones were sequenced for each condition (see Supplementary Figure 6A for detailed analysis).

Luciferase imaging

Plants were sprayed with an aqueous luciferin (Biosynth, Switzerland) solution (31.5 mg per 100 ml water) and incubated for 15 min in the dark. Luminescence imaging was performed using an Aequoria dark box with a mounted ORCAII CCD camera (Hamamatsu, Japan). Brightfield and luminescence image overlays were created using the Wasabi software package (Hamamatsu, Japan). After taking the brightfield image, plants were left in the darkbox without light for 1 min to prevent background autofluorescence signals in the following luminescence image.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Author contributions: CY constructed the tagging vector, created the activation tagging population, performed the mutant screen, found and identified *moe1* as *nrpe1-13*, showed its dependence on *mom1* and performed RT–PCRs and northern analyses. EB prepared the Southern, northern and siRNA blots, and performed bisulphite sequencing, RT–PCRs, tiling array experiments, bioinformatic treatment of the data and set up the genome browser. MC performed RNA extractions, designed assays to detect transcription of repetitive loci and created mutant combinations. IV performed northern blots. JN performed genotyping and assisted in the mutant screen. OMS created the *LUC25*, *momLUC25* and *LUC26* lines. JP supervised the work and wrote the paper together with CY and EB.

Conflict of interest

The authors declare that they have no conflict of interest.

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