The EMBO Journal Vol. 21 No. 24 pp. 6832–6841, 2002

HDA6, a putative histone deacetylase needed to enhance DNA methylation induced by double-stranded RNA

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To analyze relationships between RNA signals, DNA methylation and chromatin modifications, we performed a genetic screen to recover *Arabidopsis* mutants defective in RNA-directed transcriptional silencing and methylation of a nopaline synthase promoter–neomycinphosphotransferase II (*NOSpro–NPTII*) target gene. Mutants were identified by screening for recovery of kanamycin resistance in the presence of an unlinked silencing complex encoding NOSpro double-stranded RNA. One mutant, *rts1* (RNA-mediated transcriptional silencing), displayed moderate recovery of *NPTII* gene expression and partial loss of methylation in the target NOSpro, predominantly at symmetrical C(N)Gs. The *RTS1* gene was isolated by positional cloning and found to encode a putative histone deacetylase, HDA6. The more substantial decrease in methylation of symmetrical compared with asymmetrical cytosines in *rts1* mutants suggests that HDA6 is dispensable for RNA-directed *de novo* methylation, which results in intermediate methylation of cytosines in all sequence contexts, but is necessary for reinforcing primarily C(N)G methylation induced by RNA. Because CG methylation in centromeric and rDNA repeats was not reduced in *rts1* mutants, HDA6 might be specialized for the RNA-directed pathway of genome modification.

Keywords: DNA cytosine methylation/double-stranded RNA/histone deacetylase/RNA-directed DNA methylation/transcriptional gene silencing

Introduction

RNA silencing refers to epigenetic gene silencing that is triggered by double-stranded RNA (dsRNA) (Matzke *et al*., 2001; Wang and Waterhouse, 2001). RNA silencing is usually considered with respect to post-transcriptional processes of RNA degradation that occur primarily in the cytoplasm, such as post-transcriptional gene silencing (PTGS) in plants (Vance and Vaucheret, 2001; Voinnet, 2001), quelling in *Neurospora crassa* (Cogoni, 2001) and RNA interference (RNAi) in animals (Hannon, 2002; Zamore, 2002). A central step in the PTGS/RNAi mechanism is processing of dsRNA into 21–24 nucleotide (nt) RNAs, which are believed to guide ribonuclease (RNase) cleavage of the complementary mRNA. Despite the current emphasis on post-transcriptional varieties of RNA silencing, there is increasing evidence that dsRNA can also induce transcriptional silencing and epigenetic changes at the genome level.

RNA-directed DNA methylation (RdDM) in plants provided the first example of an RNA-mediated epigenetic alteration of homologous nuclear DNA (Wassenegger *et al*., 1994; Wassenegger, 2000). RdDM involves the *de novo* methylation of almost all cytosines (Cs), not just those in symmetrical CG or CNG nucleotide groups, within a region of sequence identity between the triggering RNA and the target DNA (Pélissier *et al*., 1999). Experiments using Cre/*lox*-mediated recombination to convert a transcribed DNA direct repeat into an inverted repeat (IR), a step that initiates transcription of a hairpin RNA, confirmed that RdDM requires dsRNA (Mette *et al*., 2000). Moreover, if a dsRNA contains promoter sequences, *de novo* methylation and transcriptional gene silencing (TGS) of homologous promoters *in trans* can occur (Jones *et al*., 1999, 2001; Mette *et al*., 2000; Sijen *et al*., 2001). Similarly to the dsRNA involved in PTGS/RNAi, promoter dsRNAs are processed into short RNAs a little over 20 nt in length. It is not yet certain whether the short RNAs or the dsRNA precursors direct methylation of homologous DNA sequences, although the length of short RNAs is close to the minimal DNA target size of RdDM (~30 bp) (Pélissier and Wassenegger, 2000). In addition, experiments using a virus-induced gene silencing system suggested that RNAs as short as 33 nt are sufficient to direct *de novo* methylation (Thomas *et al*., 2001). Evidence for functionally distinct sub-populations of short RNAs in plants was reported recently: 'longer' short RNAs 24–26 nt in length were implicated in RdDM, whereas 'shorter' short RNAs (21–22 nt) were involved in initiating PTGS (Hamilton *et al*., 2002).

Although RdDM is well established in plants, the extent to which it occurs in other organisms is not yet clear. The unusual pattern of methylation induced by RNA, in which Cs in any sequence context become modified, contrasts to the distribution in animal genomes, where methylation is present predominantly in CG dinucleotides. In a recent study, however, significant non-CG methylation was detected in mammalian embryonic stem cells (Ramsahoye *et al*., 2000). This finding hints that RdDM might occur in mammals but be restricted to an early period of development. Even in organisms that do not substantially methylate their DNA, RNAs might direct chromatin modifications to specific regions of the genome. Genetic evidence from *Drosophila* (Pal-Bhadra *et al*., 2002), fission yeast (Hall *et al*., 2002; Volpe *et al*., 2002) and *Caenorhabditis elegans* (Dudley *et al*., 2002) indicates that TGS and/or chromatin modifications require components of the RNAi machinery. If RNA-guided genome modifications occur regularly in diverse

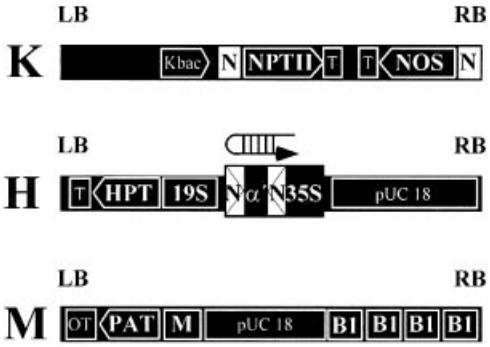

Fig. 1. Transgene constructs. The target construct K (Kanamycin resistance) contains a nopaline synthase (NOS) promoter (white N)–neomycin phosphotransferase (*NPTII*) gene and a *NOS* gene. The silencing construct H (Hygromycin resistance) contains a hygromycin phosphotransferase (*HPT*) gene driven by the 19S promoter and a NOSpro IR, separated by 260 bp spacer containing the α′ promoter of the soybean storage protein β-conglycinin gene (Chen *et al*., 1986), under the control of the 35S promoter. The M (Mutator) construct contains a phosphinothricin acetyltransferase (*PAT*) gene, encoding resistance to Basta, under the control of the mannopine synthase promoter (white M). Four copies of the B1 enhancer of tobacco endogenous pararetrovirus (Mette *et al*., 2002a) on the right allow for possible activation tagging (Weigel *et al*., 2000) in addition to insertional mutagenesis. A bacterial origin of replication (pUC18) can be used to clone 'M' inserts by plasmid rescue. Abbreviations: LB, RB: left and right T-DNA borders, respectively; Kbac, a Kan-resistance marker used in bacterial cloning steps; T, NOS terminator; OT, octopine synthase terminator.

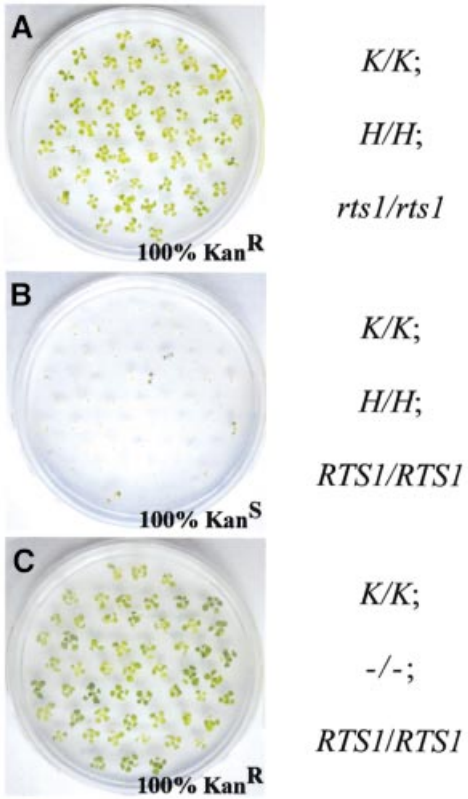

Fig. 2. Growth of *rts1* mutant seedlings on Kan. Seedlings that are triply homozygous for the target *K* locus, silencing *H* locus and the *rts1* mutation (**A**) grow almost as well on medium containing 40 mg/l Kan as wild-type seedlings that are homozygous for the unsilenced target *K* locus (**C**). Wild-type seedlings that are doubly homozygous for the target *K* locus and the silencing *H* locus are sensitive to Kan (**B**).

organisms, then RdDM would simply represent a special type that is prevalent in plants. Because RdDM occurs throughout plant development and produces a signature pattern of DNA methylation, it is amenable to analysis in plants.

We have established a system in *Arabidopsis* for studying RNA-mediated promoter methylation and TGS of the constitutive nopaline synthase promoter (NOSpro). Silencing and methylation of the target NOSpro are initiated or substantially reversed within one generation after introducing or removing the silencing locus, which encodes a NOSpro dsRNA that is processed to short RNAs (Aufsatz *et al*., 2002). We are currently carrying out a genetic analysis of this system to identify components of the RdDM machinery and to assess the role of chromatin alterations in establishing and/or maintaining the distinctive pattern of methylation induced by RNA. As reported here, this mutant screen has identified a histone deacetylase, HDA6, that might act preferentially in the RNA-directed pathway of genome modifications.

Results

The NOSpro *trans*-silencing system has been described for tobacco (Mette *et al*., 2000) and *Arabidopsis* (Aufsatz *et al*., 2002). It consists of a target locus *K* (Kanamycin resistance), which contains NOSpro–*NPTII* and *NOS* genes (Figure 1, construct K), and an unlinked silencing complex *H* (Hygromycin resistance), which comprises a NOSpro IR downstream of a 35S promoter (35Spro) and a 19S-*HPT* gene (Figure 1, construct H). The target NOSpro–*NPTII* gene becomes transcriptionally silenced and methylated at symmetrical (CG and CNG) and asymmetrical (CNN) cytosines in the NOSpro region when NOSpro dsRNA is transcribed from the NOSpro IR. The NOSpro dsRNA is processed into short sense and antisense RNAs 21–24 nt in length (Aufsatz *et al*., 2002).

To obtain mutants impaired in NOSpro RNA-mediated TGS, T-DNA insertional mutagenesis was performed. Plants doubly homozygous for the target *K* locus and silencing *H* locus were transformed with the M (Mutator) T-DNA construct containing a *PAT* gene, which encodes resistance to the herbicide Basta, under the control of a mannopine synthase promoter (Figure 1, construct M). The 'M' T-DNA contains no sequence homology in regulatory or protein-coding regions to either the K or H construct and therefore has no potential to interfere with expression of genes at the *K* or *H* loci by homology-dependent silencing. Plants that had taken up the 'M' T-DNA were selected by spraying with Basta. Herbicide-resistant plants (M1 generation) were allowed to self-fertilize and pools of M2 seeds, which are still doubly homozygous for the target and silencing loci, were plated on medium containing kanamycin (Kan) to select for recovery of NOSpro–*NPTII* gene expression.

One Kan-resistant mutant line, *rts1* (RNA-mediated transcriptional silencing), exhibited genetic behavior consistent with a recessive mutation. Homozygous *rts1* progeny displayed moderate and relatively uniform resistance to Kan at a concentration of 40 mg/l (Figure 2A), appearing only slightly less resistant than wild-type

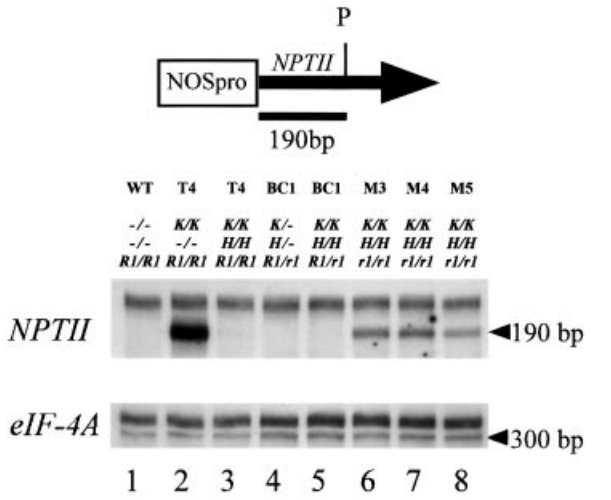

Fig. 3. RNase protection to analyze *NPTII* expression in *rts1* mutants. Top: the map shows the location of the protected 190 bp *NPTII* fragment with respect to the NOSpro–*NPTII* gene. As input RNA, *in vitro* transcribed antisense RNA (black line) from a subcloned *NPTII* fragment upstream of the internal *Pst*I (P) site was used. Bottom: *NPTII* expression was observed in wild-type T4 plants containing the unsilenced *K* locus (lane 2), but not detectable in T4 plants containing the silencing *H* locus (lane 3). Expression recovers 20–30% in *rts1* plants from the M3, M4 and M5 generations (lanes 6–8), which is sufficient for resistance at the relatively low concentration of Kan used (40 mg/l). Silencing is rapidly reinitiated in first generation backcross (BC1) progeny, which are heterozygous for the *rts1* mutation (lane 4, *K* and *H* loci are hemizygous; lane 5, *K* and *H* loci are homozygous). The percentage expression in *rts1* mutants was determined by scanning blots with a Pharmacia Image Master using the residual input RNA after RNase digest (top band) as a reference standard. Lower band: positive control showing constitutive expression of the *eIF-4A* gene indicated by a 300 bp protected RNA fragment. The top band represents residual input RNA after RNase digest. *R1* and *r1*: short versions of *RTS1* and *rts1*, respectively.

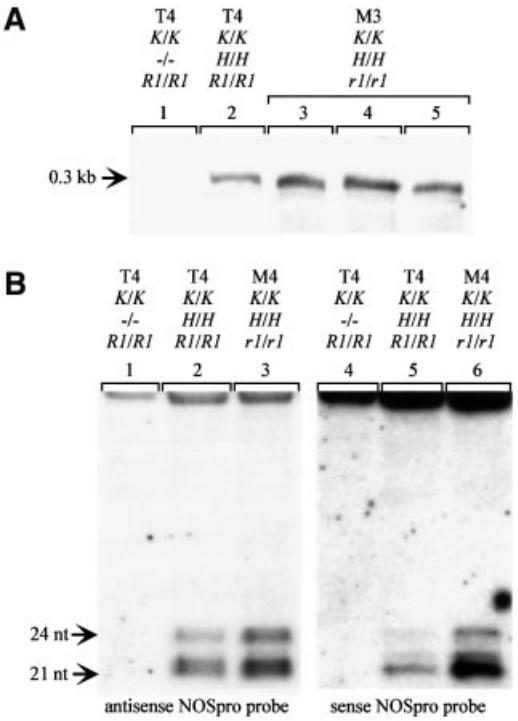

Fig. 4. NOSpro dsRNA and short RNAs in *rts1* mutants. (**A**) The 0.3 kb NOSpro dsRNA, which is not made in plants lacking the silencing *H* locus (lane 1), is synthesized from the *H* locus in *rts1* mutants (three shown, lanes 3–5) at levels comparable to those of wild-type plants (lane 2). (**B**) Sense and antisense NOSpro short RNAs 21–24 nt in length, which are not produced in plants lacking the silencing *H* locus (lanes 1 and 4) are somewhat enhanced in *rts1* mutants (lanes 3 and 6) compared with wild-type plants (lanes 2 and 5). T4, M3 and M4 indicate the plant generation tested. Genotypes of plants are indicated above each lane. *R1* and *r1* are shortened versions of *RTS1* and *rts1*, respectively. Arrows refer to the position of RNA size markers.

seedlings containing the target locus in the unsilenced state (Figure 2C). The moderate Kan resistance of *rts1* seedlings was accompanied by partial recovery of NOSpro–*NPTII* gene expression. RNase protection assays revealed that the steady-state level of *NPTII* RNA in *rts1* mutants is ~20–30% of that detected in the non-silenced target line in a wild-type background, and this value remained relatively stable into advanced generations (Figure 3, *NPTII* blot, lanes 6–8). Silencing is reinitiated in *rts1/RTS1* heterozygotes as indicated by a return to undetectable levels of *NPTII* RNA in first generation backcross progeny (Figure 3, *NPTII* blot, lanes 4 and 5). Accordingly, *rts1/RTS1* heterozygous seedlings are Kan sensitive (data not shown).

NOSpro dsRNA is synthesized at roughly wild-type levels in *rts1* mutants (Figure 4A), confirming that recovery of NOSpro–*NPTII* target gene expression is not due to a block in transcription of the NOSpro IR. Examination of NOSpro short RNAs in the *rts1* mutant revealed a slight enhancement of all size classes of sense and antisense short RNAs derived from the NOSpro dsRNA (Figure 4B), suggesting that the *rts1* mutation has a subtle influence on short RNA metabolism.

Methylation-sensitive restriction enzymes and DNA blot analysis were used to examine methylation in the target NOSpro in *rts1* mutants. In wild-type plants, the target NOSpro is active and unmethylated in the absence of the silencing locus (Figure 5A, left, *K/K*;–/–; *R1/R1*). Following introduction of the silencing complex, the target NOSpro becomes heavily methylated at the sites recognized by *Bst*UI and *Sac*II, which are sensitive to CG and C(N)G methylation, respectively, and ~50% methylated at the site recognized by *Nhe*I, which is sensitive to methylation in an asymmetrical CNN on the top strand and a CNG on the bottom strand (Figure 5A, right, *K/K*;*H/H*;*R1/R1*) (Aufsatz *et al*., 2002). In *rts1* mutants, a unique pattern was reproducibly observed in all M3 plants tested: methylation was clearly reduced at the *Sac*II and *Bst*UI sites, but stayed essentially unchanged at the *Nhe*I site, resulting in similar proportions of methylated and unmethylated fragments for each of the three enzymes (Figure 5B, *K/K*;*H/H*;*r1/r1*; four plants). This pattern persisted into the M4 generation (Figure 5C, *K/K*;*H/H*;*r1/r1*; two plants), demonstrating that a stable methylation state had been attained. The reduction in methylation at the symmetrical Cs in the *Sac*II and *Bst*UI sites (~30–50%) in *rts1* mutants is consistent with the extent of recovery of *NPTII* gene expression (~20–30%). The *rts1* methylation pattern differed from those observed previously in *met1* and *ddm1* mutants, which are defective in a DNA methyltransferase (DMTase) and a putative chromatin remodeling protein, respectively. The effects of these mutations were non-uniform in our system, but some *met1*

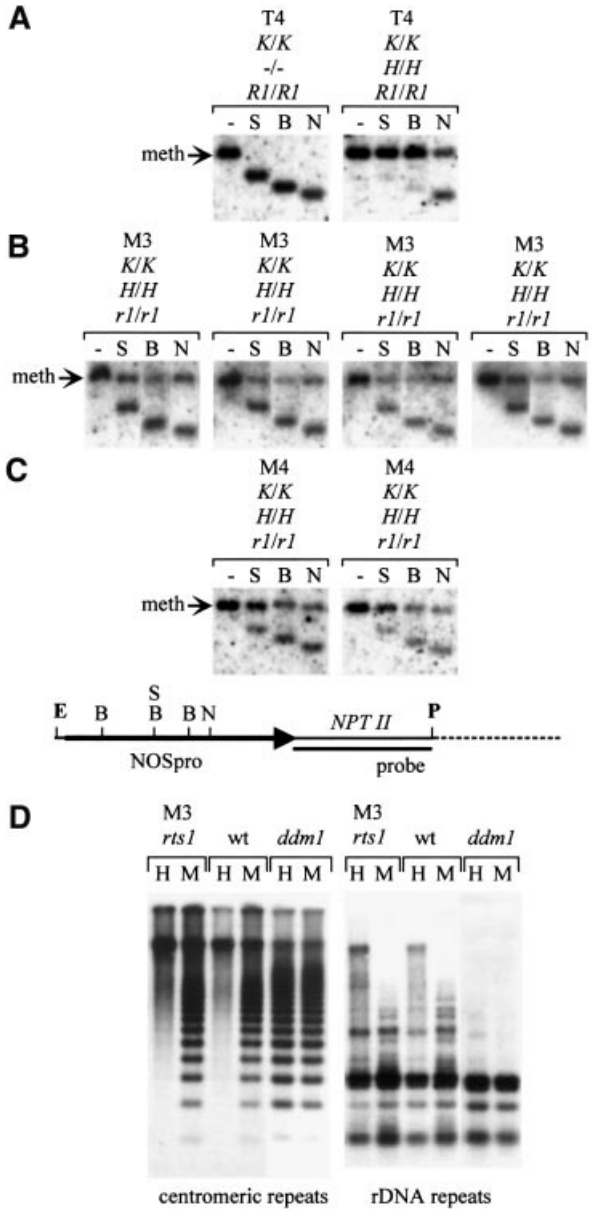

Fig. 5. DNA methylation analysis. As shown in the map below panel C, methylation in the target NOSpro was examined by performing an *Eco*RI/*Pst*I (E/P) double digest and adding as indicated a methylation-sensitive restriction enzyme: *Sac*II (S) ($^{m}C^{m}CGCGG$), *Bst*UI (B) ($^{m}CG^{m}CG$) or *Nhe*I (N) (top strand: GCTAG^{m}Caa; bottom: GCTAG^{m}Ctg). (A superscript 'm' indicates a methylated C that can inhibit cleavage.) The probe consisted of *NPTII*-coding sequences. (**A**) Left: the target NOSpro at the *K* locus is normally unmethylated in the absence of the silencing *H* locus, as indicated by shifts to the smaller fragments when S, B or N are used. Right: when the silencing *H* locus is introduced, heavy methylation at the S and B sites and ~50% methylation at the N site is observed. In individual *rts1* plants of the M3 (**B**) and M4 (**C**) generations, methylation at the S and B sites is reduced on average to ~50%. This is similar to the level of methylation at N sites, which remains unchanged in *rts1* mutants compared with the wild-type. (**D**) CG methylation at centromeric and rDNA repeats was examined in *rts1* and *ddm1* mutants and wild-type (wt) plants using the isoschizomers *Hpa*II (H) ($^{m}C^{m}CGG$) and *Msp*I (M) ($^{m}CCGG$). The patterns with H and M appear identical only in the *ddm1* mutants, indicating loss of CG methylation. Genotypes of individual plants are indicated above each panel. *R1* and *r1* are shortened versions of *RTS1* and *rts1*, respectively.

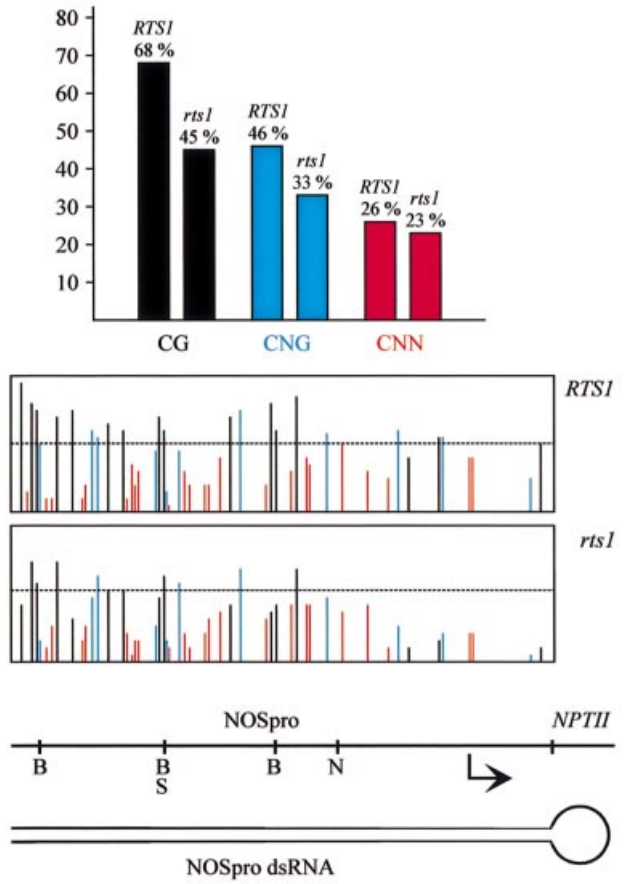

Fig. 6. Summary of bisulfite sequencing of the target NOSpro in wild-type plants and *rts1* mutants. Bottom: region of the NOSpro homologous to NOSpro dsRNA showing sites for methylation-sensitive restriction enzymes (Figure 5), the transcription start site (arrow) and start of the *NPTII*-coding region. Middle: cytosine methylation in the bottom DNA strand of the NOSpro (black: CG; blue: CNG; red: CNN). The height of the vertical lines shows the percentage of methylated Cs per total PCR clones sequenced. The dotted line indicates 50%. Top: bar graph showing reductions in methylation in the bottom strand for specific nucleotide groups in the *rts1* mutant. In the top DNA strand, methylation was reduced from 64 to 55% (14%) in CGs; from 47 to 46% (2%) in CNGs; and was slightly increased (from 49 to 51%) in CNNs (not shown). Approximately 20 cloned PCR fragments were sequenced from each strand for both wild-type and *rts1* mutant plants (see Supplementary data available at *The EMBO Journal* Online). DNA used for bisulfite treatment was isolated from pooled *rts1* plants of the M4 generation. The data represent an average of this population.

and *ddm1* mutant plants sustained substantial losses of methylation in both symmetric and asymmetric Cs (Aufsatz *et al*., 2002).

A bisulfite sequencing analysis of the target NOSpro in wild-type and *rts1* mutant plants generally confirmed the results obtained with methylation-sensitive restriction enzymes. In wild-type plants, methylation was heavier in symmetric Cs than in asymmetric Cs, and in the *rts1* mutant, methylation was partially reduced primarily at CGs and CNGs (Figure 6). The fractional decreases in C(N)G methylation must be considered in the context of continuous *de novo* methylation induced by NOSpro dsRNA/short RNAs (Aufsatz *et al*., 2002). Indeed, it is noteworthy that the reductions occur primarily in symmetric Cs where methylation can be maintained by MET1 (CG) (Jones *et al*., 2001; Kishimoto *et al*., 2001) and CMT3 (CNG) (Bartee *et al*., 2001; Lindroth *et al*., 2001;

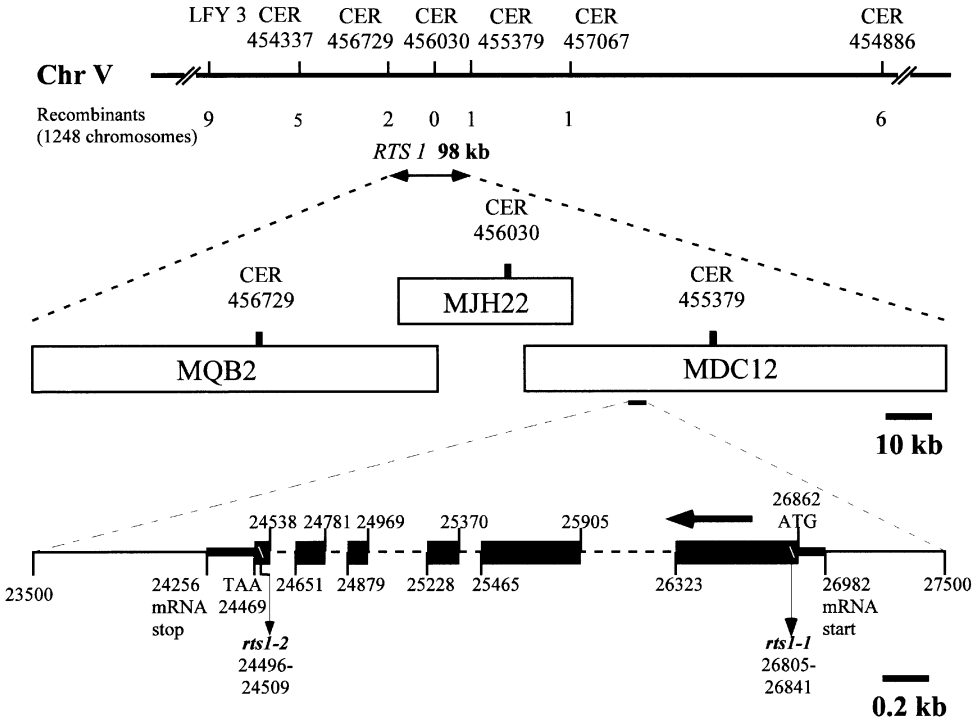

Fig. 7. Mapping of *RTS1*. The *rts1* phenotype (Kan resistance in the presence of the *H* silencing locus) cosegregated with markers LFY3 and CER454886 at the bottom of chromosome 5. In a mapping population of 624 plants there were nine recombination events between LFY3 and *RTS1* and six between *RTS1* and CER454886. Five additional markers were created within the LFY3/CER454886 genetic interval for fine mapping of *RTS1*. There were two recombination events between CER456729 and *RTS1* and one between *RTS1* and CER455379, locating *RTS1* on a 98 kb physical interval. This sequence is represented in overlapping BAC clones MBQ2, MJH22 and MDC12. The bottom part shows organization of the *RTS1/HDA6* gene. The black boxes represent exons, the dashed lines denote introns. Coordinates of translational start and stop, exon–intron boundaries and the positions of the mutation in the *rts1-1* and *rts1-2* alleles are shown with respect to the numbering of BAC clone MDC12 (accession No. AB008265). The start and end of the mRNA are demarcated according to accession Nos AF195548 and AY072201. The direction of the black arrow indicates that *RTS1/HDA6* is in complementary orientation to MDC12.

Papa *et al*., 2001). In contrast, methylation at asymmetric Cs is not thought to be maintained by any known DMTase (Pélissier *et al*., 1999) and this view is consistent with the observation that asymmetric C methylation in the NOSpro TGS system requires the continuous presence of NOSpro dsRNA (Aufsatz *et al*., 2002). Therefore, the level of asymmetric C methylation, which ranges from ~25–50% on the two strands of the target NOSpro (Figure 6), can be taken as an indication of ongoing *de novo* methylation catalyzed by a *de novo* DMTase(s) in the presence of NOSpro RNAs. Both the restriction enzyme analysis and the bisulfite sequencing experiments showed that the major change in *rts1* mutants is a shift downward in the amount of C(N)G methylation to a level approaching that in CNN nucleotide groups. This suggests that the *rts1* mutation does not severely perturb *de novo* methylation but impedes primarily the reinforcement of C(N)G methylation above the '*de novo*' level of ~25–50% resulting from continuous RdDM.

To ascertain the effects of the *rts1* mutation on global methylation patterns, CG methylation in centromeric and rDNA repeats was examined using the isoschizomers *Hpa*II and *Msp*I. Again in contrast to the effects of the *ddm1* (Figure 5D, *ddm1*) and *met1* (not shown) mutations, the *rts1* mutation did not reduce CG methylation of either repeat family, as indicated by a pattern of digestion identical to that observed in wild-type plants (Figure 5D, *rts1* and wt). This suggests that RTS1 activity is targeted to the NOSpro.

Attempts to isolate the *RTS1* gene by screening λ genomic clones for inserts containing 'M' T-DNA–plant DNA junctions were unsuccessful. PCR analysis revealed that several unlinked 'M' T-DNAs were integrated into the genome of the *rts1* mutant, but none of these cosegregated with the Kan-resistant phenotype of *rts1* plants. Therefore, the *RTS1* gene was isolated by positional cloning using codominant PCR-based markers; these detect polymorphisms between ecotype Columbia, which was used to set up the NOSpro silencing system, and the mapping ecotype Landsberg (Konieczny and Ausubel, 1993).

The *rts1-1* mutation was mapped within a 98 kb physical interval on chromosome 5 represented in BAC clones MQB2, MJH22 and MDC12 (Figure 7). This genetically defined interval contains 28 annotated open reading frames (ORFs) (www.arabidopsis.org). PCR amplification of sets of overlapping fragments from *rts1-1* mutant and wild-type plants identified a PCR fragment of MDC12.7 that showed a length polymorphism between mutant and wild-type plants. MDC12.7 encodes HDA6 (protein accession No. BAB10553.1), which belongs to the RPD3 family of histone deacetylases (Murfett *et al*., 2001; Plant Chromatin database; www.chromdb.biosci.arizona.edu).

Two *rts1* alleles were recovered in separate M2 pools: *rts1-1* had a 37 bp deletion 21 bp downstream of the start codon; *rts1-2* sustained a 14 bp deletion in the last exon (Figure 7). The *rts1-1* mutation is presumably a null allele since it disrupts the HDA6-coding region after the first

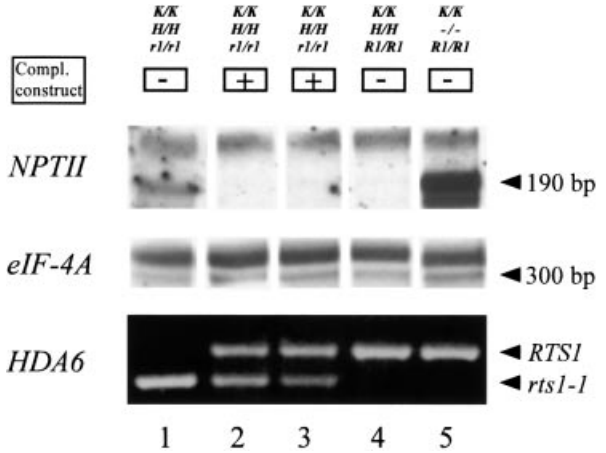

Fig. 8. Complementation of the *rts1* mutation with the wild-type *HDA6* gene analyzed by RNase protection. Top: transformation of *rts1-1* plants, which express the NOSpro–*NPTII* target gene, as evidenced by a 190 bp protected fragment (lane 1), with a 3.5 kb genomic *HDA6* fragment results in re-silencing of the *NPTII* gene (lanes 2 and 3) comparable to wild-type plants (lane 4). Two representatives of ten complemented (+) T1 plants are shown. Wild-type expression of the *NPTII* gene in plants containing the unsilenced target *K* locus is shown in lane 5. Middle: positive control showing constitutive expression of the *eIF-4A* gene (300 bp protected fragment). Bottom: *HDA6* genotypes. The *rts1-1* allele contains a 37 bp deletion resulting in higher mobility of a PCR-amplified fragment on agarose gels (lane 1) compared with the PCR fragment from wild-type plants (lanes 4 and 5). Both bands can be detected in *rts1* plants complemented with wild-type *HDA6* (lanes 2 and 3). The *K*, *H* and *RTS1* genotypes for individual plants are shown above each lane. *R1* and *r1* are shortened versions of *RTS1* and *rts1*, respectively.

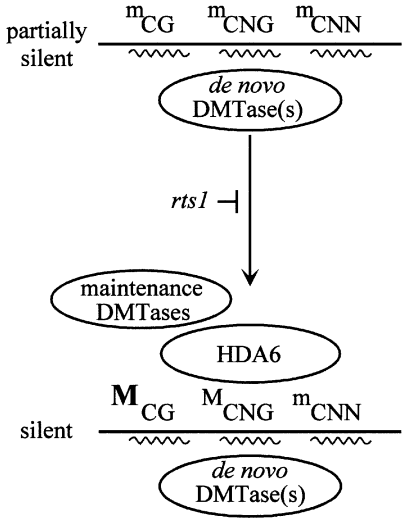

Fig. 9. Hypothetical model for RTS1 activity. One or more *de novo* DMTases in conjunction with NOSpro RNAs (wavy lines) catalyze an intermediate level of uniform methylation (m) of Cs in all sequence contexts in the target NOSpro, resulting in partial silencing. Although a known *de novo* DMTase in *Arabidopsis*, DRM (Domain-Rearranged Methyltransferase; Cao and Jacobsen, 2002), remains a good candidate for this step, other candidate DMTases (MET1, CMT3, Dnmt2; Finnegan and Kovac, 2000) cannot yet be ruled out. HDA6 is envisioned to recognize the partially methylated DNA and to recruit maintenance (or reinforcement) DMTases such as MET1 and CMT3, which enhance CG (bold capital M) and to a lesser extent CNG (capital M) methylation. This step helps to lock in the silent state, probably in conjunction with additional histone modifications.

seven amino acids. Although the *rts1-2* allele only lacks the last 13 amino acids of the HDA6 protein, the strength of Kan resistance in *rts1-2* and *rts1-1* plants is comparable, suggesting that *rts1-2* might also be a null allele.

To confirm the correspondence between HDA6 and the *rts1* mutation, the wild-type *HDA6* gene containing ~1 kb 5′ and ~160 bp 3′ flanking sequences was placed on a T-DNA encoding resistance to Basta and reintroduced into *rts1* plants. RNase protection experiments performed using RNA prepared from Basta-resistant seedlings indicated that *NPTII* RNA was no longer detectable (Figure 8, lanes 2 and 3), demonstrating reinitiation of silencing. The wild-type *HDA6* gene was thus able to complement the *rts1* mutation.

The *hda6*^{rts1} mutant plants display a very mild phenotype. Even in plants of the M5 generation, when the *rts1-1* mutation has been homozygous for four generations, the only visible defect is somewhat reduced fertility of the first flowers (data not shown).

Discussion

In a genetic screen designed to recover mutants impaired in TGS and RdDM of a NOSpro–*NPTII* target gene, we have identified a mutant, *rts1*, that is deficient in a putative Rpd3-like histone deacetylase, HDA6. Analysis with methylation-sensitive restriction enzymes and bisulfite sequencing revealed that the primary methylation defect in *rts1* mutants is a reduction in methylation at symmetric CGs and CNGs, while levels of CNN methylation remain relatively unchanged. The partial loss of C(N)G methylation in *rts1* mutants is associated with a moderate recovery of NOSpro–*NPTII* target gene expression, indicating that enhanced C(N)G methylation occurring as a consequence of HDA6 activity contributes to full silencing. The fact that the *hda6*^{rts1} mutation does not affect CG methylation in centromeric or ribosomal DNA repeats suggests that HDA6 might be specialized for the RNA-directed pathway of chromatin modification. If so, HDA6 would be the first unique component of the RdDM pathway to be identified.

In mammals, histone deacetylases (HDACs) and DMTases act together to repress transcription through the action of methyl-binding proteins, which are components of, or recruit, HDAC complexes to methylated DNA (Burgers *et al*., 2002; for a dissenting view, see Goll and Bestor, 2002). There is evidence for similar links between DNA methylation and histone deacetylation in plants (Chen and Pikaard, 1997; Li *et al*., 2002). Nevertheless, knowledge about how DMTases and HDACs are targeted to specific regions of the genome is limited. In our system, it is clear that *de novo* methylation at symmetrical and asymmetrical Cs in the target NOSpro is directed by NOSpro RNAs (Aufsatz *et al*., 2002). This information allows us to propose a hypothetical sequence of events in which HDA6 acts in between the *de novo* and C(N)G maintenance (or reinforcement) steps (Figure 9).

We assume that the degree of methylation in asymmetrical Cs (~25–50%), which is not maintained following cessation of dsRNA synthesis (Aufsatz *et al*., 2002), reflects continuous RdDM catalyzed by one or more *de novo* DMTase(s), which remain to be identified, in the presence of NOSpro RNAs. The *rts1* mutation does not

appear to interfere substantially with this step, which leads to roughly comparable, intermediate levels of methylation in symmetric and asymmetric Cs and to partial silencing. Instead, the *rts1* mutation appears to inhibit the enhancement of methylation, predominantly in C(N)G nucleotide groups, to a degree in excess of the '*de novo*' level. The augmentation of C(N)G methylation is apparently necessary to lock in the silent state, probably in conjunction with additional histone modifications. In this hypothetical model, HDA6 is envisaged to recognize the partial methylation at most Cs resulting from the *de novo* step of RdDM; it then recruits MET1 and CMT3, the traditional CG and CNG maintenance activities, respectively (Bartee *et al*., 2001; Kishimoto *et al*., 2001; Lindroth *et al*., 2001; Papa *et al*., 2001), to reinforce preferentially C(N)G methylation. This scenario would be consistent with reported associations between Dnmt1, the mammalian homolog of MET1, and HDACs in mammals (Burgers *et al*., 2002). Furthermore, a role for HDA6 in maintenance rather than establishment of transcriptional repression is consistent with a similar maintenance role proposed for an Rpd3-like HDAC in *Drosophila* (Wheeler *et al*., 2002).

HDA6 was recovered in a separate mutant screen that was initially based on deregulation of an auxin-responsive transgene (Murfett *et al*., 2001). It turned out, though, that the *hda6* mutation was not interfering with an auxin response, but was alleviating silencing of a multicopy transgene locus. It is interesting to consider why *hda6* mutations were repeatedly recovered in our screen and in the one carried out by Murfett and coworkers, particularly since this HDAC has not been identified in other genetic screens to find mutants defective in DNA methylation (Vongs *et al*., 1993; Bartee *et al*., 2001; Lindroth *et al*., 2001) or *cis*-TGS (Amedeo *et al*., 2000). The NOSpro *trans*-TGS system relies on promoter dsRNA produced by transcribing a NOSpro IR. Intriguingly, the transgene construct used by Murfett and colleagues also contained a promoter IR of ~260 bp. While the authors attributed the spontaneous silencing of their transgene locus to its repetitive nature, it is conceivable that a promoter dsRNA was unknowingly transcribed from the promoter IR in their system.

The *Arabidopsis thaliana* genome encodes 15 HDACs; four of these, including HDA6, are similar to the *Saccharomyces cerevisiae* Rpd3 family. The amino acid sequences of all four Rpd3-like HDACs in *Arabidopsis* are highly similar except in the C-termini, which diverge significantly. This suggests that the four enzymes have distinct functions. Two of the genes, *HDA1* and *HDA6*, are expressed in seedlings, which is the stage at which both we and Murfett and coworkers selected for mutants. *HDA1* is expressed more highly than *HDA6* in seedlings (Plant Chromatin Database; http://chromdb.biosci.arizona.edu), indicating that the frequent recovery of *hda6* mutants in each genetic screen was not simply due to selection for the HDAC expressed most strongly at this stage of development. Although the enzymatic activity of HDA6 has not yet been confirmed, the *HDA6* gene encodes all of the amino acids that are important for HDAC activity and/or transcriptional repressor function (Hassig *et al*., 1998; Kadosh and Struhl, 1998). Antisense suppression of the *HDA1* gene leads to pleiotropic effects on *Arabidopsis* development, owing in some cases to ectopic expression of tissue-specific genes (Tian and Chen, 2001). In contrast, $hda6^{rts1}$ mutants appear essentially normal, even into advanced generations.

NOSpro short RNAs were slightly but reproducibly enhanced in the $hda6^{rts1}$ mutant. Until the RNA species involved in RdDM is clarified, it is difficult to judge the significance of this observation. Nevertheless, an influence on NOSpro short RNA metabolism is consistent with the involvement of guide RNAs in NOSpro silencing and methylation, which in turn is compatible with the recruitment of HDA6 to the target NOSpro. The idea that HDA6 might function preferentially in an RNA-directed pathway of genome modifications to reinforce primarily C(N)G methylation implies a degree of HDAC specialization that is in agreement with recent results from yeast. The five HDACs in *S.cerevisiae* are targeted to different areas of the genome and overlap only slightly in function: Hos1/Hos3 and Hos2 affect rDNA and ribosomal protein genes, respectively; Sir2 represses subtelomeric heterochromatin; and Rpd3 and Hda1 are involved mainly in deacetylating specific promoters by recruitment mechanisms that remain to be discovered (Robyr *et al*., 2002). Our observation that Rpd3-like HDA6 does not act on centromeric or rDNA repeats but appears to be recruited to the NOSpro as part of an RNA-directed pathway of silencing opens up the possibility that guide RNAs might similarly target specific HDACs to cognate promoters in yeasts and other eukaryotes. Promoter regions are not generally thought to be transcribed. However, further analysis of the numerous microRNAs (miRNAs) derived from dsRNAs encoded by imperfect IRs in intergenic regions in *Arabidopsis* (Llave *et al*., 2002a, b; Mette *et al*., 2002b; Park *et al*., 2002; Reinhart *et al*., 2002; Rhoades *et al*., 2002) and animals (McManus and Sharp, 2002) might reveal that some of these contain promoter sequences. Indeed, seven miRNAs that possibly target promoters of endogenous genes were identified recently in *Arabidopsis* (Park *et al*., 2002). Given the extraordinary specificity that can be achieved through RNA–DNA base pairing, even with very short RNAs (Eddy, 2002; Storz, 2002), the potential for guide RNAs to play a major role in directing epigenetic modifications to identical sites in eukaryotic genomes is considerable.

Materials and methods

Transgenic plants

K and H constructs and the production of the NOSpro-silenced K/K; H/H plants have been described previously (Aufsatz *et al*., 2002). The silencer H construct is integrated on chromosome 4, BAC clone F10M10 (accession No. AL035521). Kan resistance in the original target K line segregated as a monogenic trait, which indicated a single insertion of a (functional) NOSpro–*NPTII* gene. Recloning and sequence analysis revealed that the *K* locus contains several complete and incomplete sequences of the T-DNA. The *K*-locus T-DNA was found to be associated with chromosome 5 BAC clone K9I9 (accession No. AB013390; Aufsatz *et al*., 2002). Sequencing also identified flanking *Arabidopsis* DNA from chromosome 3 BAC clone F21O3 (accession No. AC009853) indicating a rearrangement of plant DNA during T-DNA integration (Nacry *et al*., 1998; Tax and Vernon, 2001). The location of a functional gene encoding Kan resistance on BAC clone K9I9, however, was already evident from previous work (Aufsatz *et al*., 2002) and was further confirmed during positional cloning of the *rts1-1* mutant (described below).

Mutagenesis

T4 plants that were doubly homozygous for the target and silencing loci (genotype *K/K*;*H/H*) were mutagenized by transformation with T-DNA construct 'M' (Mutator) according to published protocols (Clough and Bent, 1998). With the exception of pUC18 sequences included to allow cloning of the 'H' and 'M' T-DNA inserts via plasmid rescue, the 'M' construct was assembled to contain no sequence homology to either the target *K* or silencing *H* locus. The 'M' construct has a MASpro (mannopine synthase promoter)–PAT (phosphinothricin acetyltransferase)–OT (terminator from octopine synthase gene) cassette (derived from vector pSKI015; Weigel *et al*., 2000) at the left border to allow the detection of transformed plants by selection with the herbicide Basta (Aufsatz *et al*., 2002). At the right border, construct 'M' harbors four copies of the B1 enhancer of tobacco endogenous pararetrovirus (Mette *et al*., 2002a). This provides the possibility of activation tagging in addition to insertion mutagenesis (Weigel *et al*., 2000). M2 seeds were pooled from 50–100 Basta-resistant primary transformants. Collections of 5000–10 000 seeds per M2 pools were screened for reactivation of Kan resistance on MS agar containing 40 mg/l Kan (Sigma, St Louis, MO). The progeny of Kan-resistant (Kan^R) plants were checked for inheritance of Kan resistance and for hygromycin (Hyg) resistance, which is the selection marker for the *H* locus (Aufsatz *et al*., 2002). The integrity of the NOSpro IR encoded by the silencer *H* locus was checked by genotyping PCR for the presence of both repeat units (IR1; IR2): primers for IR1 were: F, 5′-AAGATGCCTCTGCCGACA-3′; R, 5′- CGTCAAACTGC-ATGCCAC-3′. Primers for IR2 were: F, 5′-TTGGGCTGCTCGATT-GAC-3′; R, 5′-ATTACCGCCAATGTCTCT-3′.

Genetic mapping and positional cloning

The *rts1-1* mutant was identified as a monogenic loss-of-function mutant which showed 100% Kan-sensitive seedlings in the BC1 progeny from a backcross to the K/K; H/H non-mutagenized parental line and ~25% Kan^R seedlings in the BC1F2 generation. The original M2 *rts1-1* plant harbored four insertions of construct 'M', the exact coordinates of which were identified by recloning *M* loci from a λ genomic library [prepared according to procedures described by Jakowitsch *et al*. (1999) and screened with *PAT* and *B1* probes]. The *rts1-1* mutant was found to be non-tagged by 'M' T-DNA through the lack of cosegregation of insertion-site derived PCR markers with Kan resistance in populations segregating for the mutation. The *rts1-2* allele, which was non-tagged as well, was identified by a failure to complement *rts1-1* in the F1 of an intercross, which yielded 100% Kan^R seedlings.

To obtain a mapping population, M3 *rts1-1* plants (genotype *K/K*; *H/H*; *rts1-1/rts1-1*; ecotype Columbia) were crossed to ecotype Landsberg and the F2 seeds of this cross were selected on MS agar containing 40 mg/l Kan and 15 mg/l Hyg (Calbiochem, La Jolla, CA). Only plants homozygous for the *rts1-1* mutation should show Kan resistance in the presence of the silencer *H* locus (identified by Hyg selection). Using an initial mapping population of 30 F2 plants and a set of cleaved amplified polymorphic sequence (CAPS) markers which detect polymorphisms between the Columbia and Landsberg ecotypes (Konieczny and Ausubel, 1993), the *rts1-1* mutation was found to be linked to the LFY3 marker on chromosome 5 (116.9 cM; 0 recombinants per 30 F2 plants). LFY3 is ~2.2 Mb north of BAC clone K9I9 harboring the *K* locus (see above), which indicates linkage of *rts1-1* to the *K* locus. With an increased mapping population of 624 F2 plants, the *rts1-1* mutation was found to reside within a ~2.2 Mb interval between CAPS marker LFY3 (nine recombinants) and INDEL (insertion/deletion) marker CER454886 (Cereon Genomics; six recombinants). Marker CER454886 locates on BAC clone K9I9 south of the integration site identified for the K construct. This region was not homozygous for Landsberg sequences in any of the 624 Kan^R F2 plants tested, which again demonstrates that the functional Kan resistance encoded by the moderately complex *K* locus resides on chromosome 5 BAC clone K9I9 (see above). For fine mapping, additional PCR-based markers, which were all derived from the collection of DNA polymorphisms provided by Cereon Genomics, were generated within the LFY3/CER454886 physical interval (CER454337, CER456729, CER456030, CER455379, CER457067). The *rts1-1* mutation was mapped within a 98 kb physical interval between markers CER456729 (two recombinants) and CER455379 (one recombinant). This region is represented in BAC clones MQB2, MJH22 and MDC12, which are sequenced and annotated by the *Arabidopsis* Sequencing Project (www.arabidopsis.org). No recombinant was obtained for marker CER456030 that locates within this 98 kb interval.

This genetically defined interval harbors 28 annotated ORFs (www.arabidopsis.org). By annotation, three of these ORFs are *bona fide* candidates for encoding the *RTS1* gene: MBQ2.26 ('similarity to dof zinc finger protein'), MDC12.7 ('histone deacetylase') and MDC12.8 ('ATP-dependent RNA-helicase-like protein'). PCR amplification of sets of overlapping fragments from *rts1-1* mutant and wild-type plants identified a PCR fragment of MDC12.7 which showed a length polymorphism between mutant and wild-type plants. MDC12.7 encodes HDA6 (protein accession No. BAB10553.1), which belongs to the RPD3 family of histone deacetylases (The Plant Chromatin database; www.chromdb.biosci.arizona.edu). Three overlapping fragments of the *HDA6* gene were PCR-amplified (HDA6-1 to 6-3) from mutant (*rts1-1* and *rts1-2*) and wild-type Columbia plants and subjected to direct sequencing using Thermo Sequenase (Amersham, St Paul, MN) and dye-labeled primers (Biolegio, Malden, The Netherlands). The sequencing reactions were resolved on a LICOR sequencer (LICOR, Lincoln, NE). Primers for PCR amplification were as follows: HDA6-1F, 5′-TCAAGTCAACCGTAACACTG-3′; HDA6-1R, 5′-ATGGAAACAT-CGTTCCACAC-3′; HDA6-2F, 5′-TAGGTCAAGTGCATCAGGTG-3′; HDA6-2R, 5′-ATAAGTCGCAGTTCCACTCC-3′; HDA6-3F, 5′-ATC-CTTAGCCGGAAGATGAC-3′; HDA6-3R, 5′-CTTTCGAGCTTAGA-GAGACC-3′. The *rts1-1* allele causes a 37 bp deletion of coordinates 26805–26841 in exon 1; the *rts1-2* allele has a 14 bp deletion of coordinates 24496–24509 in exon 6. The coordinates given are according to those of BAC clone MDC12 (accession No. AB008265).

Complementation

Mutant *rts1-1* plants were cured of Basta resistance resulting from 'M' T-DNA insertions by analyzing the F2 progeny of a backcross to K/K; H/H plants for seedlings that were Kan^R and did not harbor PAT sequences. PAT-negative plants were identified by genotyping with BAR primers (Aufsatz *et al*., 2002). F3 progeny of Kan^R, PAT-negative plants (genotype *K/K*; *H/H*; *rts1-1/rts1-1*) were transformed with one of two T-DNA constructs (construct 12 and 31, respectively) harboring an ~3.5 kb fragment of the wild-type *HDA6* gene (coordinates 24313–27842 in accession No. AB008265). This fragment contains ~1 kb of sequences upstream of the ATG translational start codon and ~160 bp of sequences downstream of the translational stop codon; it does not overlap with annotated neighboring ORFs. Both constructs harbored a MASpro–PAT–OT cassette for selecting transformants with Basta. In construct 12, the genomic *HDA6* fragment was terminated with the NOSter (terminator of the *NOS* gene). Basta-selected T1 plants were genotyped with primer pair RTS1-F/R, which distinguishes the 37 bp deletion in *rts1-1* from the wild-type fragment. Primers were 5′-GATTCTGAGTGAGAGACGGAG-3′ (RTS1-F) and 5′-AGC-CATACGGATCCGGTGAGG-3′ (RTS1-R). It was shown that the wild-type HDA6 fragment is encoded by the newly introduced T-DNAs 12 or 31 by genotyping with T-DNA-anchoring primer 5′-GCAGAA-CCGGTCAAACCTAAAAGACTG-3′ (OT-F) and construct-specific primers that discriminate the different orientation of the *HDA6* fragment in constructs 12 and 31. Construct-specific primers were as follows: 12, 5′-ATCCTTAGCCGGAAGATGAC-3′ and 31, 5′-ATG-GAAACATCGTTCCACAC-3′.

DNA isolation and methylation analysis

Plant genomic DNA was extracted as described (Mette *et al*., 2000). Methylation analysis of the target NOSpro with methylation-sensitive restriction enzymes and bisulfite sequencing was performed according to previously published protocols (Mette *et al*., 2000; Aufsatz *et al*., 2002). Degenerate primers for amplification after bisulfite treatment were: 5′-YATGAGYGGAGAATTAAGGGAGT-3′ and 5′-CCRAATARCCTC-TCCACCCAA-3′ for the top strand and 5′-CACRTTATRACC-CCCRCC-3′ and 5′-AGIAGIIGATTGTITGTTGTG-3′ for the bottom strand. The size of the amplified top fragment was 380 bp; the bottom fragment 389 bp. Abbreviations: I, inosine; R, purine; Y, pyrimidine. All data are derived from plants containing the *rts1-1* allele.

RNA analysis

NOSpro dsRNA and short RNAs were isolated and analyzed as described previously (Mette *et al*., 2000). RNase protection experiments were performed using total RNA extracted from *Arabidopsis* leaves according to published protocols (Mette *et al*., 2000; Aufsatz *et al*., 2002). For *NPTII* RNA analysis, antisense *in vitro* transcripts were synthesized from a subcloned 0.19 kb *NPTII* DNA fragment (Matzke *et al*., 1989) and used as input RNA in protection experiments. As a loading control, antisense *in vitro* transcripts generated from a subcloned ~0.3 kb fragment of eukaryotic protein synthesis initiation factor 4A (*eIF-4A*) were used as input RNA (Metz *et al*., 1992). The subcloned *eIF-4A* fragment corresponds to coordinates 22–319 of accession No. NM_112246.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

Acknowledgements

We thank Dr Tatsuo Kanno for critical reading of the manuscript; Adriana Danielopol for technical assistance; Ortrun Mittelsten Scheid for the *som8* allele of *ddm1*; and Eric Richards for *met1* and probes for centromeric and rDNA repeats. We are grateful to Cereon Genomics for marker information and the Nottingham Arabidopsis Stock Center for ecotype Landsberg seeds. Work in our laboratory is supported by the Austrian FWF (grant No. Z21-MED).

References

Amedeo,P., Habu,Y., Afsar,K., Mittelsten Scheid,O. and Paszkowski,J. (2000) Disruption of the plant gene *MOM* releases transcriptional silencing of methylated transgenes. *Nature*, **405**, 203–206.

Aufsatz,W., Mette,M.F., van der Winden,J., Matzke,A.J.M. and Matzke,M. (2002) RNA-directed DNA methylation in *Arabidopsis*. *Proc. Natl Acad. Sci. USA*, in press.

Bartee,L., Malagnac,F. and Bender,J. (2001) *Arabidopsis cmt3* chromomethylase mutations block non-CG methylation and silencing of an endogenous gene. *Genes Dev.*, **15**, 1753–1758.

Burgers,W., Fuks,F. and Kouzarides,T. (2002) DNA methyltransferases get connected to chromatin. *Trends Genet.*, **18**, 275–277.

Cao,X. and Jacobsen,S. (2002) Role of the *Arabidopsis* DRM methyltransferases in *de novo* DNA methylation and gene silencing. *Curr. Biol.*, **12**, 1138–1144.

Chen,Z.J. and Pikaard,C.S. (1997) Epigenetic silencing of RNA polymerase I transcription: a role for DNA methylation and histone modification in nucleolar dominance. *Genes Dev.*, **11**, 2124–2136.

Chen,Z.L., Schuler,M. and Beachy,R. (1986) Functional analysis of regulatory elements in a plant embryo-specific gene. *Proc. Natl Acad. Sci. USA*, **83**, 8560–8564.

Clough,S.J. and Bent,A.F. (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.*, **16**, 735–743.

Cogoni,C. (2001) Homology-dependent gene silencing mechanisms in fungi. *Annu. Rev. Microbiol.*, **55**, 381–406.

Dudley,N., Labbé,J. and Goldstein,B. (2002) Using RNA interference to identify genes required for RNA interference. *Proc. Natl Acad. Sci. USA*, **99**, 4191–4196.

Eddy,S. (2002) Computational genomics of noncoding RNA genes. *Cell*, **109**, 137–140.

Finnegan,E.J. and Kovac,K. (2000) Plant DNA methyltransferases. *Plant Mol. Biol.*, **43**, 189–201.

Goll,M.G. and Bestor,T.H. (2002) Histone modification and replacement in chromatin activation. *Genes Dev.*, **16**, 1739–1742.

Hall,I., Shankaranarayana,G., Noma,K., Ayoub,N., Cohen,A. and Grewal,S. (2002) Establishment and maintenance of a heterochromatin domain. *Science*, **297**, 2232–2237.

Hamilton,A., Voinnet,O., Chappell,L. and Baulcombe,D. (2002) Two classes of short interfering RNA in RNA silencing. *EMBO J.*, **21**, 4671–4679.

Hannon,G.J. (2002) RNA interference. *Nature*, **418**, 244–251.

Hassig,C., Tong,J., Fleischer,T., Owa,T., Grable,P., Ayer,D. and Schreiber,S. (1998) A role for histone deacetylase activity in HDAC1-mediated transcriptional repression. *Proc. Natl Acad. Sci. USA*, **95**, 3519–3524.

Jakowitsch,J., Mette,M.F., van der Winden,J., Matzke,M. and Matzke,A.J.M. (1999) Integrated pararetroviral sequences define a unique class of dispersed repetitive DNA in plants. *Proc. Natl Acad. Sci. USA*, **96**, 13241–13246.

Jones,L., Hamilton,A., Voinnet,O., Thomas,C., Maule,A. and Baulcombe,D. (1999) RNA–DNA interactions and DNA methylation in post-transcriptional gene silencing. *Plant Cell*, **11**, 2291–2301.

Jones,L., Ratcliff,F. and Baulcombe,D. (2001) RNA-directed transcriptional gene silencing in plants can be inherited independently of the RNA trigger and requires Met1 for maintenance. *Curr. Biol.*, **11**, 747–757.

Kadosh,D. and Struhl,K. (1998) Histone deacetylase activity of Rpd3 is important for transcriptional repression *in vivo*. *Genes Dev.*, **12**, 797–805.

Kishimoto,N., Sakai,H., Jackson,J., Jacobsen,S., Meyerowitz,E., Dennis,E. and Finnegan,E.J. (2001) Site specificity of the *Arabidopsis* MET1 DNA methyltransferase demonstrated through hypermethylation of the *superman* locus. *Plant Mol. Biol.*, **46**, 171–183.

Konieczny,A. and Ausubel,F. (1993) A procedure for mapping *Arabidopsis* mutations using co-dominant ecotype-specific PCR-based markers. *Plant J.*, **4**, 403–410.

Li,G., Hall,T.C. and Holmes-Davis,R. (2002) Plant chromatin: development and gene control. *BioEssays*, **24**, 234–243.

Lindroth,A., Cao,X., Jackson,J., Zilberman,D., McCallum,C., Henikoff,S. and Jacobsen,S. (2001) Requirement of CHROMOMETHYLASE3 for maintenance of CpXpG methylation. *Science*, **292**, 2077–2080.

Llave,C., Kasschau,K., Rector,M. and Carrington,J. (2002a) Endogenous and silencing-associated small RNAs in plants. *Plant Cell*, **14**, 1605–1619.

Llave,C., Xie,Z., Kasschau,K. and Carrington,J. (2002b) Cleavage of *Scarecrow-like* mRNA targets directed by a class of *Arabidopsis* miRNA. *Science*, **297**, 2053–2056.

Matzke,M., Primig,M., Trnovsky,J. and Matzke,A.J.M. (1989) Reversible methylation and inactivation of marker genes in sequentially transformed tobacco plants. *EMBO J.*, **8**, 643–649.

Matzke,M., Matzke,A.J.M. and Kooter,J. (2001) RNA guiding gene silencing. *Science*, **293**, 1080–1083.

McManus,M. and Sharp,P. (2002) Gene silencing in mammals by small interfering RNAs. *Nat. Rev. Genet.*, **3**, 737–747.

Mette,M.F., Aufsatz,W., van der Winden,J., Matzke,M. and Matzke,A.J.M. (2000) Transcriptional silencing and promoter methylation triggered by double-stranded RNA. *EMBO J.*, **19**, 5194–5201.

Mette,M.F., Kanno,T., Aufsatz,W., Jakowitsch,J., van der Winden,J., Matzke,M.A. and Matzke,A.J.M. (2002a) Endogenous viral sequences and their potential contribution to heritable virus resistance in plants. *EMBO J.*, **21**, 461–469.

Mette,M.F., van der Winden,J., Matzke,M. and Matzke,A.J.M. (2002b) Short RNAs can identify new candidate transposable element families in plants. *Plant Physiol.*, **130**, 6–9.

Metz,A., Timmer,R. and Browning,K. (1992) Sequences for two cDNAs encoding *Arabidopsis thaliana* eukaryotic protein synthesis initiation factor 4A. *Gene*, **120**, 313–314.

Murfett,J., Wang,X.-J., Hagen,G. and Guilfoyle,T. (2001) Identification of Arabidopsis histone deacetylase HDA6 mutants that affect transgene expression. *Plant Cell*, **13**, 1047–1061.

Nacry,P., Camilleri,C., Courtial,B., Caboche,M. and Bouchez,D. (1998) Major chromosomal rearrangements induced by T-DNA transformation in *Arabidopsis*. *Genetics*, **149**, 641–650.

Pal-Bhadra,M., Bhadra,U. and Birchler,J. (2002) RNAi related mechanisms affect both transcriptional and posttranscriptional transgene silencing in *Drosophila*. *Mol. Cell*, **9**, 315–327.

Papa,C., Springer,N., Muszynski,M., Meeley,R. and Kaeppler,S. (2001) Maize chromomethylase *Zea methyltransferase2* is required for CpNpG methylation. *Plant Cell*, **13**, 1919–1928.

Park,W., Li,J., Song,R., Messing,J. and Chen,X. (2002) CARPEL FACTORY, a Dicer homolog and HEN1, a novel protein, act in microRNA metabolism. *Curr. Biol.*, **12**, 1484.

Pélissier,T. and Wassenegger,M. (2000) A DNA target size of 30 bp is sufficient for RNA-directed DNA methylation. *RNA*, **6**, 55–65.

Pélissier,T., Thalmair,S., Kempe,D., Sanger,H. and Wassenegger,M. (1999) Heavy *de novo* methylation at symmetrical and non-symmetrical sites is a hallmark of RNA-directed DNA methylation. *Nucleic Acids Res.*, **27**, 1625–1634.

Ramsahoye,B., Biniszkiewicz,D., Lyko,F., Clark,V., Bird,A. and Jaenisch,R. (2000) Non-CpG methylation is prevalent in embryonic stem cells and may be mediated by DNA methyltransferase 3a. *Proc. Natl Acad. Sci. USA*, **97**, 5237–5242.

Reinhart,B., Weinstein,E., Rhoades,M., Bartel,B. and Bartel,D. (2002) MicroRNAs in plants. *Genes Dev.*, **16**, 1616–1626.

Rhoades,M., Reinhart,B., Lim,L., Burge,C., Bartel,B. and Bartel,D. (2002) Prediction of plant microRNA targets. *Cell*, **110**, 513–520.

Robyr,D., Suka,Y., Xenarios,I., Kurdistani,S., Wang,A., Suka,N. and Grunstein,M. (2002) Microarray deacetylation maps determine genome-wide functions for yeast histone deacetylases. *Cell*, **190**, 437–446.

Sijen,T., Vijn,I., Rebocho,A., van Blokland,R., Roelofs,D., Mol,J. and Kooter,J. (2001) Transcriptional and posttranscriptional gene silencing are mechanistically related. *Curr. Biol.*, **11**, 436–440.

Storz,G. (2002) An expanding universe of noncoding RNAs. *Science*, **296**, 1260–1263.

Tax,F.E. and Vernon,D.M. (2001) T-DNA-associated duplication/translocations in *Arabidopsis*. Implications for mutant analysis and functional genomics. *Plant Physiol.*, **126**, 1527–1538.

Thomas,C., Jones,L., Baulcombe,D. and Maule,A. (2001) Size constraints for targeting post-transcriptional gene silencing and for RNA-directed methylation in *Nicotiana benthamiana* using a potato virus X vector. *Plant J.*, **25**, 417–425.

Tian,L. and Chen,Z.J. (2001) Blocking histone deacetylation in *Arabidopsis* induces pleiotropic effects on plant gene regulation and development. *Proc. Natl Acad. Sci. USA*, **98**, 200–205.

Vance,V. and Vaucheret,H. (2001) RNA silencing in plants—defense and counterdefense. *Science*, **292**, 2277–2280.

Voinnet,O. (2001) RNA silencing as a plant immune system against viruses. *Trends Genet.*, **17**, 449–459.

Volpe,T., Kidner,C., Hall,I., Teng,G., Grewal,S. and Martienssen,R. (2002) Regulation of heterochromatin silencing and histone H3 lysine-9 methylation by RNAi. *Science*, **297**, 1833–1837.

Vongs,A., Kakutani,T., Martienssen,R. and Richards,E. (1993) *Arabidopsis thaliana* DNA methylation mutants. *Science*, **260**, 1926–1928.

Wang,M. and Waterhouse,P. (2001) Application of gene silencing in plants. *Curr. Opin. Plant Biol.*, **5**, 146–150.

Wassenegger,M. (2000) RNA-directed DNA methylation. *Plant Mol. Biol.*, **43**, 203–220.

Wassenegger,M., Heimes,S., Riedel,L. and Sanger,H. (1994) RNA-directed *de novo* methylation of genomic sequences in plants. *Cell*, **76**, 567–576.

Weigel,D. *et al*. (2000) Activation tagging in *Arabidopsis*. *Plant Physiol.*, **122**, 1003–1013.

Wheeler,J., VanderZwan,C., Xu,X., Swantek,D., Tracey,W. and Gergen,J. (2002) Distinct *in vivo* requirements for establishment versus maintenance of transcriptional repression. *Nat. Genet.*, **32**, 206–210.

Zamore,P.D. (2002) Ancient pathways programmed by small RNAs. *Science*, **296**, 1265–1269.

Received August 2, 2002; revised and accepted October 21, 2002