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# IDN2 has a role downstream of siRNA formation in RNA-directed DNA methylation

Andreas Finke, Markus Kuhlmann and Michael Florian Mette*

Leibniz Institute of Plant Genetics and Crop Plant Research; Gatersleben, Germany

**Keywords:** RNA-directed DNA methylation, transcriptional gene silencing, short interfering RNA, mutagenesis, *Arabidopsis thaliana*, IDN2, At3g48670, NRPD2a/NRPE2a, At3g23780

**Abbreviations:** *AGO*, *ARGONAUTE*; *AtCOPIA4*, *A. thaliana COPIA 4*; *AtMU1*, *A. thaliana MUTATOR 1*; *AtSN1*, *A. thaliana SINE 1*; *CMT3*, *CHROMOMETHYLASE 3*; Col-0, Columbia-0; *DCL3*, *DICER-LIKE 3*; *DRM2*, *DOMAINS-REARRANGED METHYLATRANSFERASE 2*; dsRNA, double-stranded RNA; EMS, ethyl methanesulfonate; *HEN1*, *HUA ENHANCER1*; *IDN2*, *INVOLVED IN DE NOVO 2*; *IR*, inverted repeat; L*er*, Landsberg *erecta*; *MEA-ISR*, *MEDEA-INTERGENIC SUBTELOMERIC REPEATS*; MET1, methyltransferase 1; *NPTII*, *NEOMYCIN PHOSPHOTRANSFERASE II*; *nrd*, *no rna-directed transcriptional silencing*; *NRPD2a/NRPE2a*, *NUCLEAR RNA POLYMERASE D 2a/NUCLEAR RNA POLYMERASE E 2a*; p4-RNAs, RNAP IV-dependent RNAs; p5-RNAs, RNAP V-dependent RNAs; RNAP IV, RNA polymerase IV; RNAP V, RNA polymerase V; *ProNOS*, *NOPALINE SYNTHASE promoter*; PTGS, post-transcriptional gene silencing; RdDM, RNA-directed DNA methylation; *RDM12*, *RNA-DIRECTED DNA METHYLATION 12*; RdTGS, RNA-directed transcriptional gene silencing; *RDR2*, *RNA-DEPENDENT RNA POLYMERASE 2*; siRNA, short interfering RNA; *SGS3*, *SUPPRESSOR OF GENE SILENCING 3*; TGS, transcriptional gene silencing



In plants, a particular class of short interfering (si)RNAs can serve as a signal to induce cytosine methylation at homologous genomic regions. If the targeted DNA has promoter function, this RNA-directed DNA methylation (RdDM) can result in transcriptional gene silencing (TGS). RNA-directed transcriptional gene silencing (RdTGS) of transgenes provides a versatile system for the study of epigenetic gene regulation. We used transcription of a nopaline synthase promoter (*ProNOS*)-inverted repeat (*IR*) to provide a RNA signal that triggers de novo cytosine methylation and TGS of a homologous *ProNOS* copy in trans. Utilizing a *ProNOS-NPTII* reporter gene showing high sensitivity to silencing in this two component system, a forward genetic screen for EMS-induced *no rna-directed transcriptional silencing* (*nrd*) mutations was performed in *Arabidopsis thaliana*. Three *nrd* mutant lines were found to contain one novel loss-of-function allele of *idn2/rdm12* and two of *nrpd2a/nrpe2a*. *IDN2/RDM12* encodes a XH/XS domain protein that is able to bind double-stranded RNA with 5′ overhangs, while *NRPD2a/NRPE2a* encodes the common second-largest subunit of the plant specific DNA-dependent RNA polymerases IV and V involved in silencing processes. Both *idn2/rdm12* and *nrpd2a/nrpe2a* release target transgene expression and reduce CHH context methylation at transgenic as well as endogenous RdDM target regions to similar extents. Nevertheless, accumulation of *IR*-derived siRNA is not affected, allowing us to present a refined model for the pathway of RdDM and RdTGS that positions function of IDN2 downstream of siRNA formation and points to an important role for its XH domain.

## Introduction

Methylation of cytosines at position 5 is a common modification of plant genomic DNA that is associated with epigenetic phenomena such as transgene silencing, transposon suppression, maternal/paternal imprinting and paramutation.[1,2] In the genome of the model plant *Arabidopsis thaliana*, approximately 7% of cytosines are methylated.[3] As in other plants, and in contrast to mammalian somatic cells, where 5-methyl-cytosine occurs in CG context only, DNA methylation in *A. thaliana* occurs in CG as well as CHG and CHH context (with H standing for C, A or T). Shotgun bisulfite sequencing studies revealed that around 24% of cytosines in CG context, but only 6.7% in CHG and 1.7% in CHH context are methylated.[4] Only unmethylated cytosines are incorporated during DNA replication. Thus, DNA methylated on both strands before semi-conservative replication will bear methylated cytosines just on one strand, the template strand, afterwards. This leads to the necessity to methylate cytosines on the newly synthesized strand in order to re-establish the initial DNA methylation pattern. The maintenance of methylation in CG context in *A. thaliana* is performed by the Dnmt1-class enzyme methyltransferase 1 (MET1) using the hemimethylated DNA as substrate,[5] whereas maintenance of CHG methylation is achieved by the activity of the plant specific

*Correspondence to: Michael Florian Mette; Email: mette@ipk-gatersleben.de
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CHROMOMETHYLASE 3 (CMT3) thought to be guided by histone H3 lysine 9 dimethylation.[6,7] A particular case is DNA methylation in CHH context, which is established de novo by DOMAIN REARRANGED METHYLTRANSFERASE 2 (DRM2) after each cell cycle in reaction to a sequence specific signal provided by a class of small RNAs.[8-10]

Small RNA-based pathways are important for genome stability over generations,[11] as well as for regulation of gene expression, the latter being performed either at the level of transcription, referred to as transcriptional gene silencing (TGS), or at the level of mRNA stability or translation, referred to as post-transcriptional gene silencing (PTGS).[12] The pathways involving small RNAs in plants share common biochemical features. A double stranded (ds)RNA is formed that is subsequently processed to 21–24 nt long dsRNA fragments exhibiting staggered ends with 3' overhangs. One strand of such a dsRNA fragment is then incorporated into an effector complex and guides this complex to partially or fully complementary RNA or DNA to conduct regulatory function. Genetic analysis has established a core pathway leading to siRNA-mediated DNA methylation and related TGS of endogenous as well as transgenic targets in *A. thaliana* (for a review see refs. 13–16). It is initiated by the production of single stranded RNA from target sequences by the plant specific DNA-dependent RNA polymerase IV (RNAP IV).[17,18] The resulting RNAP IV-dependent RNAs (p4-RNAs)[19] then serve as substrate of RNA-DEPENDENT RNA POLYMERASE 2 (RDR2), which synthesizes a complementary strand to generate long dsRNA.[20,21] This is then processed by DICER-LIKE 3 (DCL3) into small dsRNAs of predominantly 24 nt length.[20,21] The 3' termini of resulting dsRNA fragments are subsequently methylated by HUA ENHANCER1 (HEN1).[22,23] Single short interfering (si)RNA strands from the fragments are loaded onto ARGONAUTE4 (AGO4)[24,25] and to a lesser extent onto related AGO6 and AGO9.[26-28] A second plant specific DNA-dependent RNA polymerase V (RNAP V) is thought to produce different transcripts (p5-RNAs)[19] of target loci that serve as scaffold to attract the siRNA-AGO complexes,[17,29-32] which in turn guide DRM2 to the genomic loci to be methylated de novo.[10,33] DNA methylation at target sequences might then serve as a signal for further transcription by RNAP IV, allowing self-perpetuation of RdDM via a positive feedback loop.

In order to extend the knowledge of factors involved in RNA-directed transcriptional gene silencing (RdTGS), we performed a forward genetic screen in *A. thaliana* using a transgene system showing particularly efficient silencing of a single T-DNA-copy transgene locus via RdTGS.[34,35] Thus, we identified new mutant alleles of *INVOLVED IN DE NOVO 2*/*RNA-DIRECTED DNA METHYLATION 12 (IDN2*/*RDM12)*, a recently identified component of the RdDM pathway,[36,37] with the ability to bind dsRNA with 5' overhangs,[38-40] and of *NUCLEAR RNA POLYMERASE D 2a*/*NUCLEAR RNA POLYMERASE E 2a* (*NRPD2a*/*NRPE2a*), the common second-largest subunit of RNAP IV and RNAP V.[30] Based on the effects of *idn2*/*rdm12* in comparison to *nrpd2a*/*nrpe2a* on target gene expression, RdDM of transgenic as well as endogenous target regions and siRNA accumulation, we present a refined model for RdDM and RdTGS that positions function of IDN2/RDM12 downstream of siRNA formation, with a potential role in siRNA-p5-RNA interaction.

## Results

**Forward-genetic screen for mutations abrogating RNA-directed transcriptional gene silencing.** To extend the knowledge of factors involved in RdDM and related RdTGS, we performed a screen for ethyl methanesulfonate (EMS)-induced *nrd* mutants that reactivate expression of a silenced reporter gene, according to the strategy outlined by Page and Grossniklaus.[41] The *A. thaliana* line (*K*/*K*;*H*/*H*) submitted to mutagenesis comprised a *SILENCER* (*H*) transgene residing on chromosome 4 containing an inverted repeat (*IR*) of the *NOPALIN SYNTHASE* promoter (*ProNOS*) sequence under control of the cauliflower mosaic virus 35S promoter (*Pro35S*)[34] and a *TARGET* ($K_{chr1-10}$) transgene on chromosome 1 containing a *NEOMYCIN PHOSPHOTRANSFERASE II* (*NPTII*) reporter gene controlled by a *ProNOS* conferring resistance to kanamycin (**Fig. 1A**), which was previously found to show efficient DNA methylation and silencing in the presence of the *SILENCER*.[35,42] Transcription of the *ProNOS-IR* in the *SILENCER* leads to formation of double-stranded RNA, which is processed to short interfering (si) RNAs with *ProNOS*-homology. These siRNAs can induce DNA methylation of homologous DNA sequences in trans and thus transcriptionally silence *ProNOS*-controlled genes.[34] As *TARGET* $K_{chr1-10}$ is highly sensitive to RdTGS, plants homozygous for *TARGET* and *SILENCER* (*K*/*K*;*H*/*H*) are sensitive to kanamycin (**Fig. 1B**). Seeds were incubated with EMS, sown and grown to $M_1$ plants, which were allowed to self-pollinate. The resulting $M_2$ seeds, the first generation in which a mutation can be homozygous and thus, if recessive, show its impact on the phenotype, were germinated on medium containing 200 mg/l kanamycin to screen for individuals that had reverted to kanamycin resistance. Presence and integrity of *TARGET* and *SILENCER* in resulting $M_2$ *nrd* candidates were verified via PCR using primer combinations specific for different parts of the transgenes. Accordingly, the symbol "*nrd*" is used to refer to mutant plants homozygous for *TARGET* and *SILENCER* transgene unless specified otherwise. $M_2$ plants were allowed to self-pollinate and kanamycin resistance was verified for the resulting $M_3$ generation (**Fig. 1B**). $M_3$ seedlings of independent mutant lines *nrd1* as well as *nrd2-1* and *nrd2-2* (for which intercrosses had revealed that they affect the same gene, see **Fig. S1A**) showed consistent resistance when grown on medium containing kanamycin, but did not have the same vigor as wild-type seedlings homozygous for the *TARGET* in the absence of the *SILENCER* (*K*/*K*).

As kanamycin resistance can arise in *A. thaliana* mutants by loss of chloroplast-localized transporter proteins required for kanamycin uptake,[43,44] rather than by reactivated *NPTII* expression, the amounts of NPTII protein in mutant plants were tested by ELISA (**Fig. 1C**). $M_3$ *nrd1*, *nrd2-1* and *nrd2-2* plants showed clearly more NPTII than (*K*/*K*;*H*/*H*) plants, indicating that their kanamycin resistance was due to a reactivation of *NPTII* expression. However, in particular in *nrd1*, NPTII levels stayed below that of (*K*/*K*;-/-) control plants containing the native *TARGET*

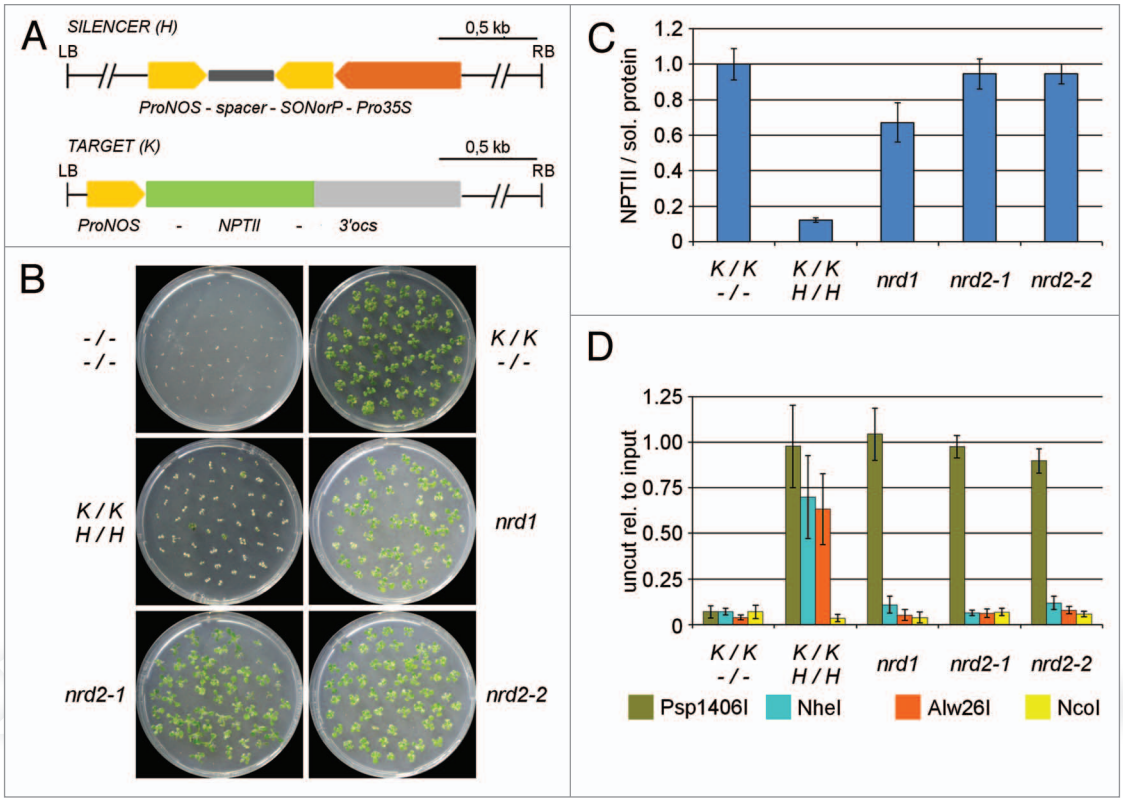


**Figure 1.** Mutations *nrd1*, *nrd2-1* and *nrd2-2* release RdTGS and RdDM of a *ProNOS-NPTII* reporter gene. (A) Transgene system: the *SILENCER* (*H*) transgene contains an inverted repeat (*IR*) of the *NOPALINE SYNTHASE* promoter (*ProNOS*) sequence under control of the strong constitutive cauliflower mosaic virus 35S promoter (*Pro35S*). Transcripts of the *ProNOS-spacer-SONorP* structure can fold to form double-stranded RNA with *ProNOS*-homology, which is then processed to short interfering (si)RNAs. These siRNAs serve as a signal for in trans DNA methylation and transcriptional silencing of a *ProNOS* copy that controls transcription of a *NEOMYCIN PHOSPHOTRANSFERASE II* (*NPTII*) (conferring kanamycin resistance if expressed) in the unlinked *TARGET* (*K*) transgene. In addition, the *SILENCER* contains a *HYGROMYCIN PHOSPHOTRANSFERASE* (*HPT*) gene conferring hygromycin resistance and the *TARGET* a GUS reporter gene (not shown). (B) Test for kanamycin resistance on medium containing 200 mg/l kanamycin. (C) Quantification of NPTII protein by ELISA: NPTII protein amouts in relation to total soluble protein were measured in extracts from leaves of 8-week-old plants. Results are displayed relative to the mean value for un-silenced expression in (*K/K;-/-*) plants (set to 1). Per genotype, five individual plants were tested. Column hights represent mean values; error bars represent standard deviations. (D) *TARGET ProNOS* cytosine methylation was determined by quantitative PCR after cleavage of genomic DNA from 8-week-old plants with methylation-sensitive restriction enzymes (C in recognition sequence underlined: methylation of cytosine blocks cleavage according to REBASE http://rebase.neb.com/rebase/rebase.html) Psp1406I (olive, symmetric CG context: AACGTT), NheI (blue, CHG and CHH context: GCTAGC), Alw26I (orange, asymmetric CHH context: GTCTC, GAGAC) and NcoI (yellow, asymmetric CHH context, control outside of the methylated region: CCATGG). Per genotype, five individual plants were tested. Results are displayed relative to the mean value for input DNA (set to 1). Column highs represent mean values; error bars represent standard deviations.

transgene. Consistent with the allelic status of *nrd2-1* and *nrd2-2*, high NPTII levels were found in their intercrosses (**Fig. S1B**). To address whether *nrd1*, *nrd2-1* and *nrd2-2* mutations release RdDM of the *TARGET* transgene, we analyzed *ProNOS* DNA methylation in the *ProNOS-NPTII* reporter gene by methylation sensitive restriction enzyme cleavage-qPCR (**Fig. 1D**). The results showed a reduction of cytosine methylation in non-CG context (NheI, Alw26I), but not CG context (Psp1406I), which is a hallmark of mutations affecting RdDM.[21,29,45,46] Cleavage with NcoI, whose recognition site lies outside the region targeted by RdDM and thus is not methylated, served as a control for the accessibility of genomic DNA.[35]

**Map-based cloning identifies *nrd1* as a new *idn2* allele and *nrd2-1* and *nrd2-2* as new *nrpd2a* alleles.** $M_3$ plants of *nrd1*, *nrd2-1* and *nrd2-2* were crossed with *A. thaliana* accession L*er* to establish mapping populations (**Fig. S2A**). $F_2$ generation seedlings obtained from these crosses by self-pollination were screened for individuals resistant to hygromycin ($Hyg^R$, *SILENCER* present) and kanamycin ($Kan^R$, *TARGET* present, homozygous for mutation releasing RdTGS). $Hyg^R$ $Kan^R$ frequencies were approximately 11% for *nrd1*, *nrd2-1* and *nrd2-2* (**Fig. S2B**), consistent with the 14% expected for single recessive mutations. To rule out possible false positives, $F_3$ progeny was obtained from $F_2$ by self-pollination and germinated in presence of hygromycin and kanamycin. Only $F_2$ that met the expected minimal 56% $Hyg^RKan^R$ in their $F_3$ progeny (data not shown) were included in mapping populations.

The *nrd1* mutation was mapped to a region on the lower arm of chromosome 3 (**Fig. S3A**) spanning ~555 kb physical distance as defined by the recombination events positioned between markers P613960 and P616207 as well as P617590 and IDMS3, respectively (**Fig. 2A**). Sequencing of gene loci known to be

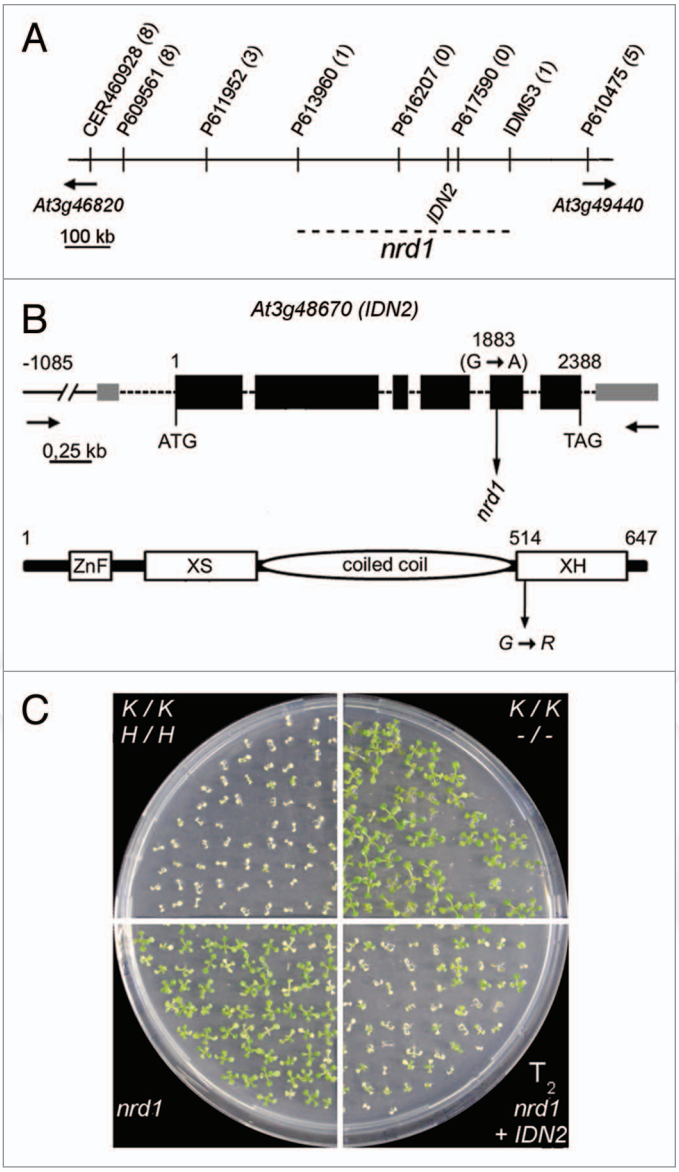


**Figure 2.** Map-based cloning of *nrd1*. (A) Physical map indicating markers and recombination events (numbers in parentheses, of 234 chromosomes in total) used to delineate the position of *nrd1* on the lower arm of chromosome 3. (B) Positions of the nucleotide (top) and related amino acid change (bottom) in *nrd1* in the *IDN2* gene model (according to TAIR 10). (C) Complementation of *nrd1* by transgenic *IDN2*. Seeds were germinated on medium containing 200 mg/l kanamycin. Approx. 75% of $T_2$ progeny obtained by self-pollination of (single locus) *nrd1* + *IDN2* $T_1$ transformants inherit a transgenic functional *IDN2* and thus are sensitive to kanamycin due to re-establishment of RdTGS of the *ProNOS-NPTII* gene.

involved in RdDM within this region revealed a G→A mutation at position 1883 in exon 5 of *IDN2* (*At3g48670*, **Fig. 2B**). To verify that the mutation in *IDN2* is causative for the release of RdDM and RdTGS in *nrd1*, we complemented *nrd1* by introducing the wild-type *IDN2* gene via Agrobacterium-mediated transformation (**Fig. S4A**). Batches of $T_2$ generation seedlings obtained from five independent $T_1$ transformants by self-pollination were tested on kanamycin-containing medium (**Fig. 2C** and data not shown). Approximately 75% of $T_2$ progeny showed sensitivity to kanamycin, a proof of successful complementation of *nrd1* by a single *IDN2* transgene locus. $T_2$ plants of two of these lines that were further analyzed showed decreased NPTII levels (**Fig. S4B**) as well as re-established DNA methylation at the endogenous RdDM target *AtSN1* (**Fig. S4C**). Thus, *nrd1* is a new *idn2* allele and was renamed *idn2-8* according to *idn2* mutant alleles described previously.[36-40] The G→A transition in *idn2-8* leads to the exchange of a glycine (G) residue for an arginine (R) at protein level (**Fig. 2B**). Mutant *idn2-8* transcripts accumulate to similar levels as *IDN2* transcripts (**Fig. S5A**), pointing to an effect of the *idn2-8* mutation at the protein rather than the mRNA level. Sequence alignment of XH-domain containing proteins of *A. thaliana* and *Oryza sativa* revealed that the affected G residue is highly conserved (**Fig. S5B**). Hence, the replacement of the glycine might compromise XH domain function.

Mutations *nrd2-1* and *nrd2-2* were mapped to a region at the upper arm of chromosome 3 (**Fig. S3B and C**, respectively) spanning ~829 kb physical distance as defined by recombination events between markers C3AB015474 and C3P0484614 as well as MN38693286 and CER456071, respectively (**Fig. 3A**). Sequencing of gene loci known to be involved in RdDM within this region revealed G→A mutations in exon 2 at position 1590 (*nrd2-2*) and exon 7 at position 5977 (*nrd2-1*) of *NRPD2a/NRPE2a* (*At3g23780*, **Fig. 3B**) encoding the common, second-largest subunit of DNA-dependent RNA polymerase IV and V.[30] As *nrd2-1* and *nrd2-2* were shown to be allelic (**Fig. S1**) and *NRPD2a/NRPE2a* is well established to be required for transgene RdTGS,[29] it is very likely that these nucleotide changes are causative for the release of RdTGS in *nrd2-1* and *nrd2-2*, respectively. Thus, *nrd2-1* was renamed *nrpd2a-54* and *nrd2-2* was renamed *nrpd2a-55* following the *nrpd2a* allele counting by Lopez et al.[47] The G→A transition in *nrpd2a-54* leads to an exchange of a glutamate for a lysine at position 1079 of the protein, while the G→A transition in *nrpd2a-55* leads to a substitution of a glycine for an aspartate at position 174. Both affected amino acids are highly conserved among the second largest subunits of DNA-dependent RNA polymerases.[48-50]

**CHH and CHG-context DNA methylation at RdDM targets is similarly reduced in *idn2* and *nrpd2a* mutants.** As methylation sensitive restriction cleavage-qPCR (**Fig. 1D**) can test methylation only at the few restriction sites available, the overall methylation of the *ProNOS* in the *TARGET ProNOS-NPTII* reporter gene in wild-type and in $M_3$ generation of *idn2-8*, *nrpd2a-54* and *nrpd2a-55* plants was determined by bisulfite sequencing (**Fig. 4**; **Fig. S6**). The results showed that cumulative cytosine methylation in the *ProNOS* region undergoing RdDM that is close to 70% in wild-type (*K/K*;*H/H*) plants is reduced to 25% and below in all three mutants (**Fig. 4A**). This reduction is primarily due to an extensive loss of methylation in CHH context, with a somewhat less pronounced effect in *idn2-8* than in *nrpd2a-54* and *nrpd2a-55*. Methylation in CG context is hardly altered in *idn2-8* and only slightly reduced in *nrpd2a-54* and *nrpd2a-55* plants compared with wild-type. The analysis of the spatial distribution of the cytosine methylation along the *ProNOS* shows an almost uniform reduction at CHH context sites, while

the partial loss of methylation in CG and CHG context seems more prominent toward the transcription start site (TSS) at the 3' end of the promoter (**Fig. 4B**).

To ensure that methylation loss was not limited to the *ProNOS* of the *TARGET*, DNA methylation at the endogenous RdDM targets *AtSN1*,[24] *MEA-ISR*[9] and *AtMU1*,[51] and at *AtCOPIA4* as a RdDM independent control,[52] was examined by bisulfite sequencing (**Fig. 5**; **Figs. S7–10**). In $M_3$ *idn2-8*, *nrpd2a-54* and *nrpd2a-55* plants, DNA methylation is obviously reduced at *AtSN1* and *MEA-ISR* in CHH and CHG context and at *AtMU1* in CHH context only. Methylation in CG context resembles wild-type levels in all three mutants. Methylation at *AtCOPIA4* is unaltered in all contexts in *idn2-8*, *nrpd2a-54* and *nrpd2a-55*. Thus these mutations affect methylation patterns by inhibition of de novo methylation in a similar way at transgenic and endogenous RdDM targets.

***ProNOS-IR* derived siRNAs are not affected in *idn2* and *nrpd2a* mutants.** As there was extensive loss of CHH context *ProNOS* methylation, we analyzed the amount of *ProNOS-IR* derived 24nt siRNAs in wild-type, *idn2-8* and *nrpd2a-55* plants (**Fig. 6**). Northern blots showed no differences in *SILENCER* transgene-derived 24 nt, 22 nt and 21 nt siRNAs between wild-type and mutant plants. This indicates the requirement of IDN2/RDM12 and NRPD2a/NRPE2a for *ProNOS-NPTII* silencing in the used transgene system in steps downstream of siRNA formation.

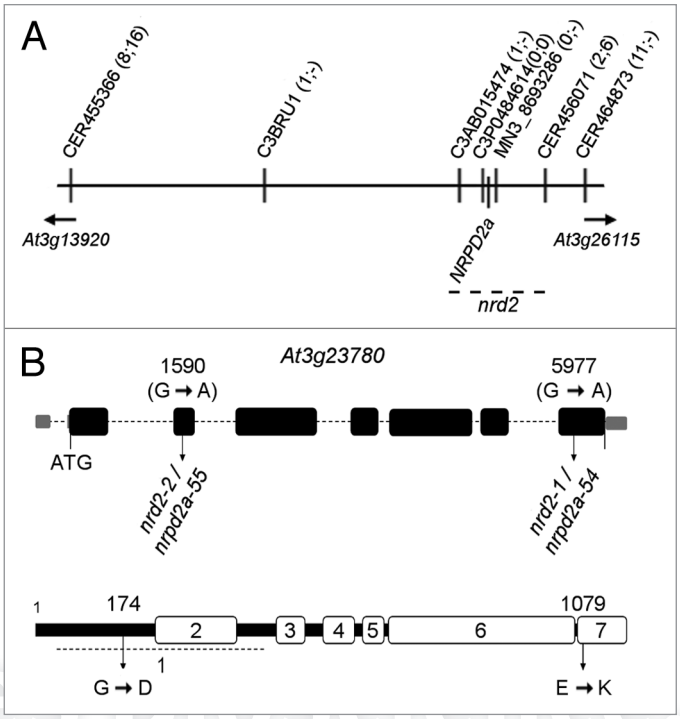


**Figure 3.** Map-based cloning of *nrd2-1* and *nrd2-2*. (A) Physical map indicating markers and recombination events (numbers in parentheses separated by semicolon, of 172 chromosomes in total for *nrd2-1* and 134 for *nrd2-2*, respectively) used to delineate the position of *nrd2* on the upper arm of chromosome 3. (B) Positions of the nucleotide (top) and related amino acid change (bottom) in *nrd2-1* and *nrd2-2* in the *NRPD2a/NRPE2a* gene model (according to TAIR10).

## Discussion

The nearly complete loss of CHH context methylation at *AtSN1* and CHH and CHG context methylation at *MEA-ISR* in *idn2-8* is similar to results presented by Ausin et al.[38,53] for deletion allele *idn2-1* and by Zhang et al.[40] for T-DNA insertion allele *idn2-5/rdm12-2*, respectively. This suggests that *idn2-8* is a loss-of-function allele. The observation that a single amino acid exchange in the XH domain severely compromises IDN2/RDM12 function points to an important role of this domain. And as the extend of reduction of CHH context methylation at *ProNOS*, *AtSN1*, *MEA-ISR* and *AtMU1* in *nrpd2a-54* and *nrpd2a-55* is similar to that in *idn2-8*, *nrpd2a-54* and *nrpd2a-55* can be considered loss-of-function alleles as well.

A core pathway for RdDM in *A. thaliana* has emerged from genetic analysis (**Fig. 7**). Both, NRPD2a/NRPE2a and IDN2/RDM12 have been previously reported to be required for RdDM at endogenous[17,18,38,53] and transgenic target sequences.[29,36,37] For NRPD2a/NRPE2a as common second-largest component of RNAP IV and RNAP V,[30] the placement in the circular RdDM pathway is well established.[15,54] Less clear is where in the pathway IDN2/RDM12 is localized. Similar to SUPPRESSOR OF GENE SILENCING 3 (SGS3), its counterpart from PTGS,[55] IDN2/RDM12 has the potential to bind dsRNA with blunt ends and 5' overhangs via its XS domain in vitro.[38-40] However, as dsRNA occurs more than once in the RdDM pathway,[15] alternative positions of IDN2/RDM12 action have been suggested. In analogy to the cooperation of SGS3 and RDR6 in generating dsRNA in PTGS,[55] IDN2/RDM12 has been proposed to team up with RDR2 in the production of dsRNA from p4-transcripts.[36] Alternatively, IDN2/RDM12 could act in stabilizing siRNAs-p5-transcript complexes in the process of guiding DRM2-mediated DNA methylation.[38] In our transgene system *ProNOS* dsRNA is generated by RNAP II-dependent transcription of the promoter-*IR* in the *SILENCER* transgene. Thus, RdDM in this experimental system works according to a linear pathway in which dsRNA and subsequent primary siRNA formation are independent from RNAP IV and V and are not affected by *nrpd2a-55*. Similar observations have been made for *nrpd2a*[29] and *rdr2* mutants,[21] in a comparable transgene setup. *ProNOS* siRNAs in *idn2-8* plants were also not reduced. As *ProNOS* CHH context DNA methylation was markedly reduced at the same time, IDN2/RDM12 needs to have a role downstream of siRNA formation in RdDM. Consistently, siRNAs derived from endogenous RdDM-target *AtSN1* are only partially reduced in *idn2/rdm12* mutants, while a strong impact of *idn2/rdm12* on CHH and CHG context methylation as seen by us and others for *AtSN1*, *MEA-ISR* and *AtMU1*.[36,38,40]

*IDN2/RDM12* is a member of a large gene family in plants characterized by the presence of zinc finger, XS, coiled-coil and XH domains.[61] Somewhat contradicting results have been reported on the possible involvement of further members of this gene family in RdDM. Two independent studies by Zhang et al.[40] and Ausin et al.[53] combining genetic and biochemical approaches

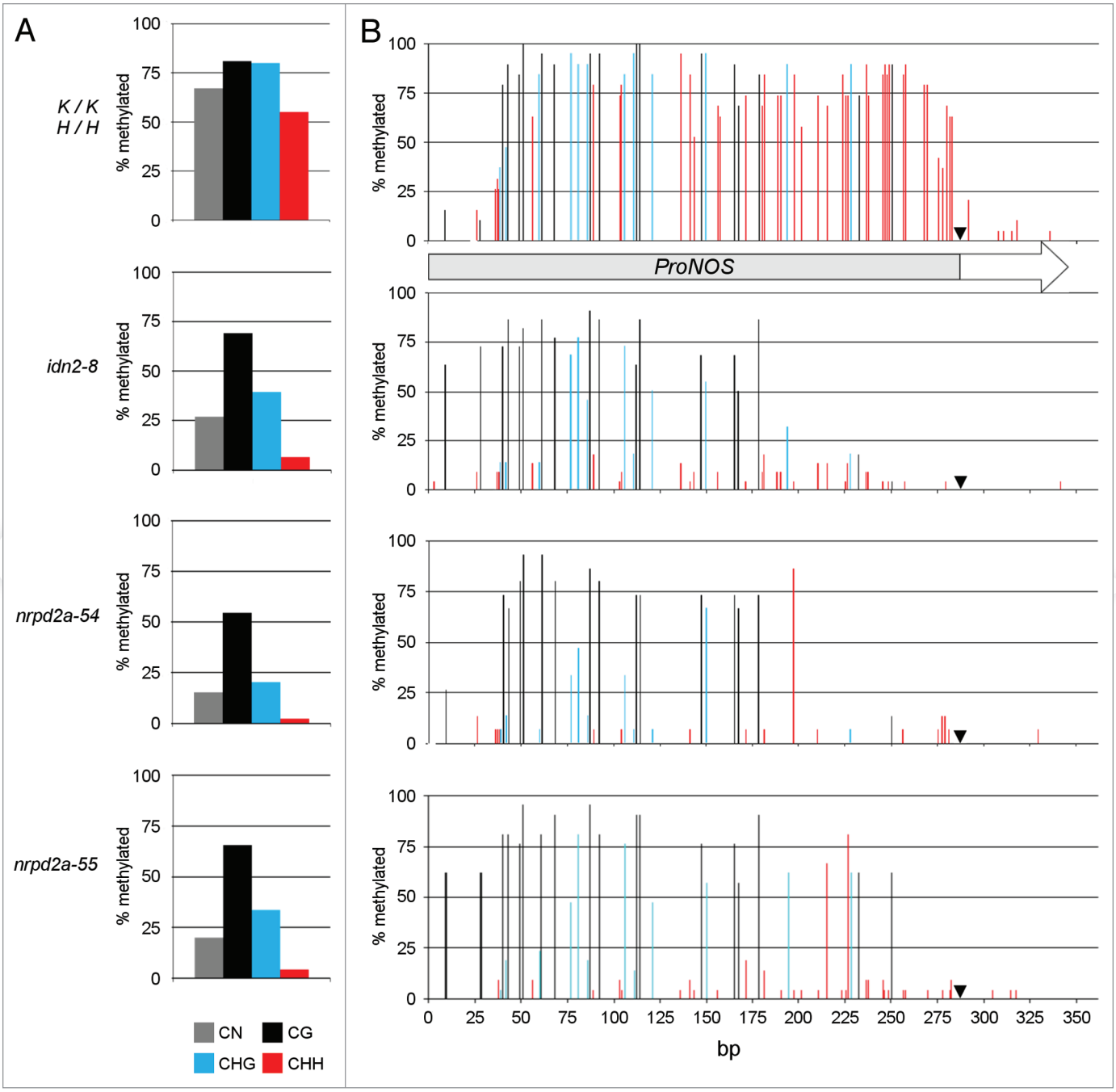


**Figure 4.** Detailed TARGET *ProNOS* DNA methylation analysis in *idn2-8*, *nrpd2a-54* and *nrpd2a-55*. DNA methylation patterns in the *ProNOS* of the *ProNOS-NPTII* reporter gene were analyzed in detail by bisulfite sequencing. (A) Cumulative methylation levels at all cytosines in the analyzed region (gray columns), cytosines in CG context (black columns), CHG context (blue columns; H stands for A, C or T) and CHH context (red columns). (B) Methylation levels at individual cytosines in CG context (black columns), CHG context (blue columns) and CHH context (red columns). A black arrowhead marks the *ProNOS* transcription start site. Numbers of clones sequenced per target and genotype were: 15 (*K/K -/-*), 19 (*K/K;H/H*), 20 (*idn2-8*), 15 (*nrpd2a-54*), 21 (*nrpd2a-55*)

did not find functional redundancy between *IDN2*/*RDM12* and gene family members *FDM1*/*IDP1*/*IDNL1* and *FDM2*/*IDP2*/*IDNL2*. Rather, FDM1/IDP1/IDNL1 and FDM2/IDP2/IDNL2 were reported to be functionally redundant and to form a complex with IDN2/RDM12 dimers via the XH domains. In contrast, Xie et al.[39] claimed based on analysis of double mutants that up to five gene family members, *FDM1*/*IDP1*/*IDNL1*, *FDM2*/*IDP2*/*IDNL2*, *FDM3*, *FDM4* and *FDM5* act partially redundant to *IDN2*/*RDM12* in RdDM. However, their observation that double mutants showed stronger loss of DNA methylation than the respective single mutants might be due to the used alleles that carried T-DNA insertions in the 5' UTR or in

introns and thus could still have conferred some gene function. Our data showing extensive loss of CHH context DNA methylation in *idn2-8* similar to *nrpd2a-54* and *nrpd2a-55* at the *ProNOS* and endogenous RdDM targets. This rather argues against the interpretation that *IDN2*/*RDM12* function in RdDM can be replaced by other gene family members.

Thus, albeit a core pathway of RdDM in *A. thaliana* as a model plant is known, genetic analysis in a transgene-based experimental system shortcutting part of the pathway has provided important information. We expect that ongoing characterizing and mapping of mutations releasing transgene RdTGS will continue to produce valuable insight into gene silencing pathways.

## Materials and Methods

**Plant material and cultivation.** The transgenic *A. thaliana* line double homozygous for *TARGET* and *SILENCER* transgenes ($K_{chr1-10}/K_{chr1-10}$;*H*/*H*) has been described in Fischer et al.[35] *A. thaliana* was cultivated on soil at 21°C under a 16 h light/8 h dark (long day) regime for propagation and seed production; and at 21°C under a 8 h light/16 h dark (short day) regime for generation of rosette leaf material for molecular analysis. For kanamycin resistance tests, seeds were surface-sterilized (10 min, 8% NaClO) and germinated under long day regime on agar-plates with germination medium (½ strength MS salts, 10 g/l sucrose) containing 200 mg/l kanamycin. Resistance was evaluated according to root growth and primary leaf development after 3 weeks.

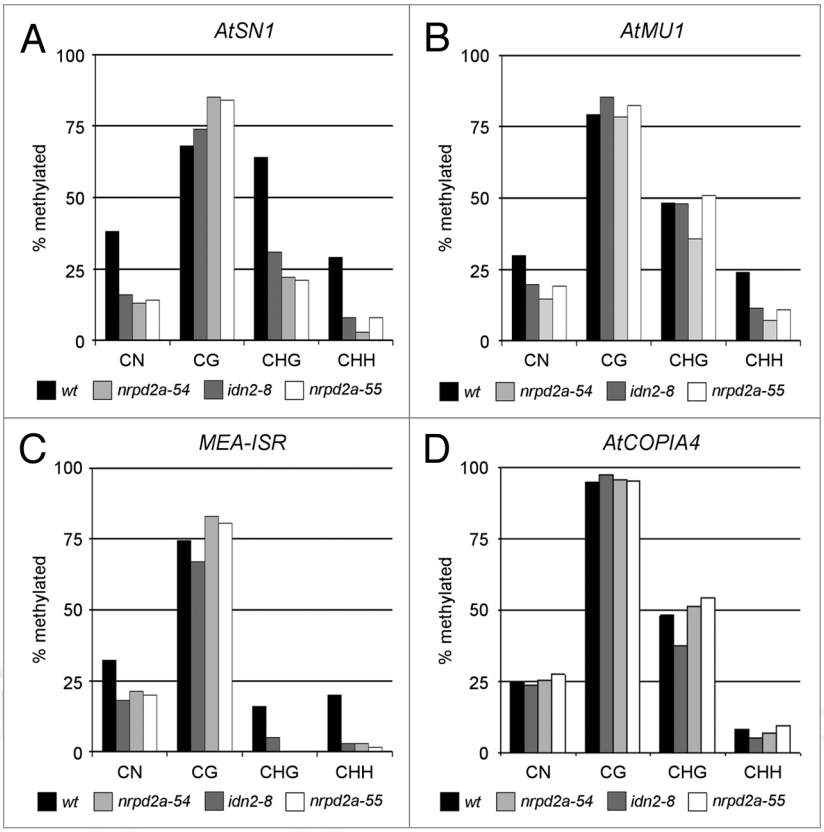


**Figure 5.** Detailed DNA methylation analysis at endogenous *AtSN1*, *MEA-ISR*, *AtMu1* and *AtCOPIA4* sequences in *idn2-8*, *nrpd2a-54* and *nrpd2a-55*. DNA methylation patterns of endogenous sequences (A) *AtSN1*, (B) *AtMU1*, (C) *MEA-ISR* and (D) *AtCOPIA4* were analyzed in detail by bisulfite sequencing in non-mutagenized control plants (black columns), *idn2-8* (dark gray columns), *nrpd2a-54* (light gray columns) and *nrpd2a-55* (white columns). A minimum of 12 clones was sequenced per target and genotype. Exact numbers are indicated in **Figures S8–10**.

**EMS mutant screen.** EMS (ethyl-methanesulfonate) mutagenesis of seeds homozygous for *TARGET* and *SILENCER* transgenes ($K_{chr1-10}/K_{chr1-10}$;*H*/*H*) in the accession Col-0 was performed by Lehle Seeds. From the obtained 32 batches of $M_2$ seeds, (each batch representing the progeny from approximately 1500 $M_1$ plants), 20,000 seeds per batch were germinated on medium containing kanamycin (200 mg/l). Resistant $M_2$ plants were transferred to soil and allowed to set seeds by selfing. The suppression of TGS was confirmed by germinating resulting $M_3$ seeds on kanamycin containing selective medium (200mg/l). Lines showing more than 90% resistant $M_3$ plants as judged by good root growth and development of primary leaves were considered true candidate lines for *no rna-directed transcriptional silencing* (*nrd*) mutations.

**Protein analysis.** Amount of NPTII protein was determined using Agdia PathoScreen Kit for NPTII (Agdia cat. no. PSP73000/0288). Rosette leafs of 8-week-old short-day-grown plants were flash frozen in liquid nitrogen, grinded using a swingmill (Retsch, MM301) and resuspended in protein extraction buffer. All further procedures were performed according to manufacturers' recommendations. Per genotype, leaves from five individual plants were assayed in technical duplicates. Total protein in the same extracts was determined using a Pierce BCA Protein Assay kit (Pierce, cat. no. 23225). Twenty-five microliters of the NPTII ELISA raw extract were added to 500 µl of BCA working solution and incubated for 30 min at 37°C in a water bath. After incubation, 500 µl of bi-distilled water were added to every sample and absorbance at 592 nm was determined using an Ultrospec 3100pro UV/Vis spectrophotometer (Amersham Bioscience, cat. no. 80–2112–38) and converted to protein concentration using a BSA serial dilution in concentration range between 0.125 and 2 µg/ml as standard.

**DNA methylation analysis using Bisulfite sequencing.** Approximately 0.15 µg of DNA extracted from leaves of 8 week old plants grown under short day regime were bisulfite-treated using Qiagen Epitect Bisulfite Kit (Qiagen cat. no. 59104) following the manufacturer's instructions. One microliter of treated DNA solution was used to amplify *ProNOS, AtSN1, AtMU1, AtCOPIA4* and *MEA-ISR* in a 50 µL reaction using GoTaq Flexi DNA polymerase (Promega, cat. no. M8308). Primers used for amplification were bitop2f, bitop3r (*ProNOS*);[35] JP1821, JP1822 (*AtSN1*);[24] JP3100, JP3101 (*AtCOPIA4*);[52] JP1026, JP1027 (*MEA-ISR*);[38] JP1387, JP1388 (*AtMU1*).[51] Amplified fragments

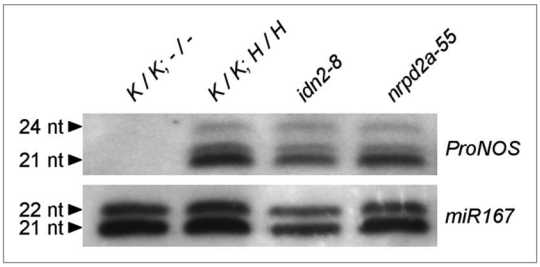


**Figure 6.** *ProNOS*-derived and endogenous siRNAs in *idn2-8* and *nrpd2a-55*. Northern blot for siRNA derived from transcription of *ProNOS* IR in the *SILENCER*. (A) Blots were hybridized with a RNA probe specific for sense *ProNOS* siRNAs. (B) Equal loading was confirmed by re-hybridization with miR167-specific probe after stripping.

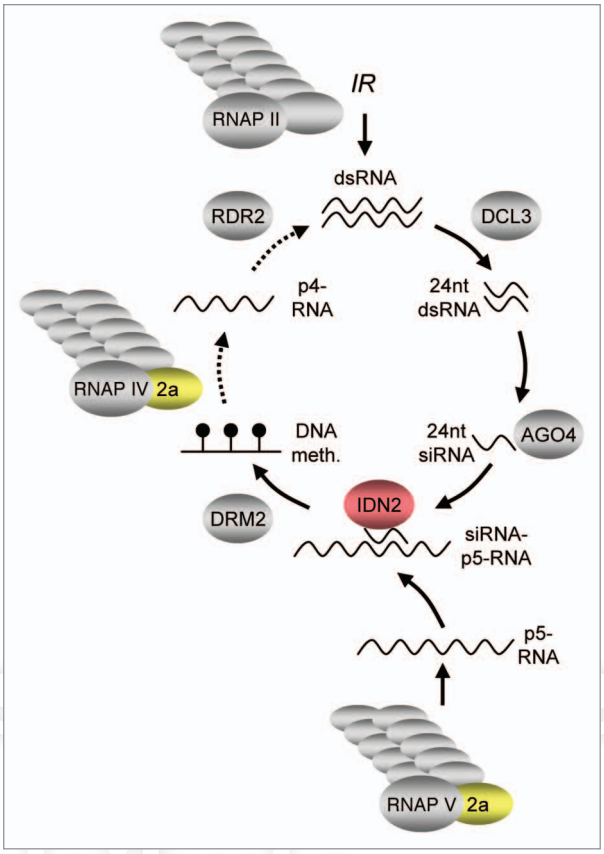


**Figure 7.** Genetic model of RdDM. The core pathway leading to RdDM is initiated by the production of single stranded RNA from target sequences by multi-subunit RNAP IV. The resulting p4-RNA then serves as substrate of RDR2, which synthesizes a complementary strand to generate dsRNA. This dsRNA is then processed by DCL3 into 24 nt dsRNA fragments and single strands of 24 nt short interfering (si)RNA are incorporated mainly into AGO4. Multi-subunit RNAP-V is thought to transcribe RdDM target loci, with the resulting p5-RNA serving as scaffold to attract the siRNA-AGO4 complexes, which in turn guide DRM2 to the genomic loci to be methylated de novo. Transcription of an inverted repeat (*IR*) by multi-subunit RNAP II provides a shortcut in the pathway, as dsRNA as a substrate for DCL3 action is produced independently of RNAP IV and RDR2 (solid arrows). NRPD2a/NRPE2a (golden) is a subunit common to RNAP IV and RNAP V, but is not required for RNAP II function. IDN2 (red) has a role downstream of siRNA formation, possibly by stabilizing a siRNA-p5-RNA complex.

were cloned into vector pSC-A using a Strataclone PCR cloning kit (Agilent Technologies cat. no. 240207). Plasmids were isolated using QuiaPrep Spin Miniprep Kit (Qiagen, cat. no. 27104) and checked for insert size via restriction cleavage using EcoRI. Positive clones were sequenced using M13 forward and M13 reverse primer. Sequences from at least 12 individual clones per locus and genotype were obtained and DNA methylation patterns were analyzed using CyMATE software.[56]

**DNA methylation analysis using methylation sensitive restriction enzymes.** Approximately 50 ng of DNA extracted from adult leaves of 8-week-old plants grown under short day regime dissolved in 400 µl of distilled water were added to 50 µl of 10-times Tango buffer (Fermentas) and 50 µl distilled water to reach a final volume of 500 µl. Aliquots of 100 µl were taken and combined with 10 U of restriction enzymes Psp1406I, NheI, Alw26I and NcoI (Fermentas), respectively. One control was kept without restriction enzyme. Aliquots were incubated at 37°C for 16 h and subsequently for 5 min at 85°C. After inactivation, 399 µl of bi-distilled water were added (final volume: 500 µL). Quantitative PCR was performed in 25 µl volume in an iCycler IQ™ PCR device (Biorad cat. no. 170-8740). 12.5 µL SYBR green Supermix (Biorad cat. no. 170-8882) and 1.25 µL of ProNOS-top-F and ProNOS-top-rev primers (final concentration 0.25 µM each) were added to 10 µL of cleaved and control templates, respectively. The following temperature regime was used for the PCR: 5 min 95°C, 40× (15 sec 95°C, 30 sec 62°C and 30 sec 72°C). PCR was calibrated using a logarithmic dilution series from $10^{-2}$ to $10^{-5}$ of genomic DNA. Data analysis was performed using ΔΔCt method according to Pfaffl.[57] Results are presented relative to the mean signal obtained for the control samples without restriction enzyme.

**Mapping of mutations.** $M_3$ *nrd1*, *nrd2-1* and *nrd2-2* (all *K/K*;*H/H*, respectively) mutant plants derived from accession Col-0 were crossed with wild-type mapping partner Landsberg *erecta* (L*er*) by manual pollination of emasculated L*er* flowers. Success of crosses was confirmed by GUS staining of leaf discs of the resulting $F_1$ progeny. GUS positive plants were allowed to self-pollinate. The resulting $F_2$ progeny was germinated on ½ strengh MS medium supplied with 10 g/l sucrose containing hygromycin (20 mg/l) and kanamycine (200 mg/l). The resistance phenotype was evaluated after 3 weeks and should only appear if the plant is homozygous for the respective mutation. Plants resistant to hygromycin and kanamycin ($Hyg^R$ $Kan^R$) were transferred to soil and allowed to reproduce by self-pollination. Individual DNA preparations were derived from leaf material. Potential "false positive" $F_2$ plants erroneously scored $Hyg^R$ $Kan^R$ were ruled out by checking segregation of resistance in their $F_3$ progeny. A control population was established by crossing non-mutagenized wild-type (*K/K*;*H/H*) with L*er*. The resulting $F_1$ generation was checked by GUS assay and allowed to self-pollinate. Resulting $F_2$ seeds were germinated on germination medium containing hygromycin (20 mg/l). $Hyg^R$ plants were transferred to soil and presence of the *TARGET* was confirmed by GUS assay. $Hyg^RGUS^+$ plants were checked for presence of the *SILENCER* transgene via specific PCR.

**Construction of complementation vector and transformation procedure.** To generate a minimal pCAMBIA without functional genes between left (LB) and right (RB) border, pCAMBIA1300 was cut with PdmI/XmnI (GAANN NNTTC; blunt end; three sites) and PvuII (CAG CTG; blunt end; two sites) and self-ligated. The resulting minimal pCAMBIA contained a single EcoRI restrictions site between LB and RB. A ProMAS-BAR-35Ster resistance cassette flanked by EcoRI-sites was amplified from genomic DNA of a SAIL line containing a T-DNA derived from pDAP101 into the EcoRI site of the minimal pCAMBIA to generate pCAMBIA-proMAS-BAR-35St (pCMBAR). LacZ and MCS of plasmid pGEM7f(+) were amplified using primers pGEM7Z-MCS-for (5'-AAC CTG CAG GGC GCG TCC ATT CGC CAT TC-3') and pGEM7Z-MCS-rev (5'-ATT CTG CAG CGG AAG AGC GCC CAA TAC GC-3') and introduced into pCMBAR at a unique PstI restriction site. The resulting vector named pCMBL contains unique AatII, ZraI, PspXI, SciI, XhoI, XmaI, SmaI, BstBI, HindIII, BspEI and BstXI restriction sites for the insertion of DNA fragments. The wild-type *IDN2* ORF (including 3'UTR) and a fragment of around 1300 bp upstream of the transcriptional start site was amplified from *A. thaliana* accession Col-0 genomic DNA using primers IDN2-clone-for (5'-CTT GAC TCG AGA CTT GCC TTG TGT CAG CG-3') and IDN2-clone-rev (5'-ACG CTC GAG GGG TCA ATA TCA AAT TTG AC-3') to introduce a XhoI restriction sites and cloned into pSC-A vector using a Strataclone PCR-cloning kit (Agilent Technologies, cat. no. 240205). After propagating the vector, the functional *IDN2* gene was excised by XhoI digestion and cloned into the XhoI restriction site of pCMBL2 yielding the binary vector pCMBL2+IDN2. pCMBL2+IDN2 was propagated in *E. coli* DH5α cells, purified using a Qiagen Plasmid Midi Kit (Qiagen, cat. no. 12143) and introduced into *Agrobacterium tumefaciens* strain pGV2260 by electroporation.

**Small RNA analysis.** For analysis of *ProNOS*-derived siRNAs, a RNA preparation enriched in small RNAs was extracted from leaves of 8-week-old plants grown under short day regime. Leaves were harvested, frozen in liquid nitrogen and stored at -80°C. Approximately 500 mg of frozen plant material were ground to powder and resuspended in 15 ml of TRIzol reagent by vigorous vortexing for 1 min at room temperature. The suspension was transferred to a 30 ml Corex-tube and 3 ml of chloroform were added. After vortexing for 1 min, centrifugation for 30 min at 4°C in a Sorvall RC5B Plus centrifuge using rotor Sorvall HB-6 was performed. The upper phase was transferred into a 50 ml disposable vessel, 1 volume of 80% ethanol was added and the total volume was applied to an RNeasy Maxi (Qiagen, cat. no. 75162) column. After centrifugation for 5 min at 3166 × g at room temperature, the flow-through was transferred to a new vessel and 1.4 volumes of 100% ethanol were added. Four ml of this solution were applied to a RNeasy Midi column (Qiagen, cat. no. 75142) followed by centrifugation for 5 min at 3166 × g. This step was repeated until the whole volume had passed the column. The RNeasy Midi column was washed twice with RPE buffer (Qiagen) and subsequently the small RNA fraction was eluted by adding 250 µl of RNase-free water to the dried membrane followed by a final centrifugation step for 5 min at 3166 × g. After elution, the concentration of RNA was determined using an Ultrospec 3100pro UV/Vis spectrophotometer (Amersham Bioscience, cat. no. 80–2112–38). The eluted small RNAs were precipitated over night at -20°C by adding 3 M sodium acetate solution (pH 5.2) to a final concentration of 0.3 M and 2.5 volumes of 100% ethanol. After centrifugation at 18640 × g for 20 min the sediment was washed with 70% ethanol and dried under vacuum for 30 min. The pellet was resuspended in 25 µl of RNase-free water and 25 µl of gel loading buffer II (Ambion, cat.no. AM8546G) were added. The mixture was incubated at 95°C for 5 min, separated using a 15% polyacrylamid, 7 M urea gel, blotted and probed for detection of *ProNOS*-sense siRNAs according to established protocols.[58,59] For re-hybridization with a miR167 probe, the membrane was stripped at 95°C in 0.1× SSC containing 0.5% SDS. The miR167 probe (**Table S1**) was labeled with $^{32}$P using T4 POLYNUCLEOTIDE KINASE (Fermentas, cat.no. EK0031) according to manufacturer's protocol. The stripped membrane was pre-hybridized with hybridization buffer according to Church and Gilbert for 16 h at 42°C.[60] Subsequently the labeled probe was added and allowed to hybridize for 24 h at 42°C. The blot was washed in 2xSSC containing 0.2% SDS and exposed to an X-ray film with intensifier screen for 3 d at -80°C.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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### Supplemental Material

Supplemental materials may be found here:
www.landesbioscience.com/journals/epigenetics/article/21237

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