The RNA Silencing Enzyme RNA Polymerase V Is Required for Plant Immunity

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Abstract
RNA-directed DNA methylation (RdDM) is an epigenetic control mechanism driven by small interfering RNAs (siRNAs) that influence gene function. In plants, little is known of the involvement of the RdDM pathway in regulating traits related to immune responses. In a genetic screen designed to reveal factors regulating immunity in Arabidopsis thaliana, we identified NRPE2 as the OVEREXPRESSOR OF CATIONIC PEROXIDASE 1 (OCP1). NRPE2 encodes the second largest subunit of the plant-specific RNA Polymerases IV and V (Pol IV and Pol V), which are crucial for the RdDM pathway. The cpc1 and nrpd2 mutants showed increases in disease susceptibility when confronted with the necrotrophic fungal pathogens Botrytis cinerea and Plectosphaerella cucumerina. Studies were extended to other mutants affected in different steps of the RdDM pathway, such as nrpd1, nrpe1, ago4, drd1, rdr2, and drm1drm2 mutants. Our results indicate that all the mutants studied, with the exception of nrpd1, phenocopy the nrpd2 mutants; and they suggest that, while Pol V complex is required for plant immunity, Pol IV appears dispensable. Moreover, Pol V defective mutants, but not Pol IV mutants, show enhanced disease resistance towards the bacterial pathogen Pseudomonas syringae DC3000. Interestingly, salicylic acid (SA)–mediated defenses effective against PsDC3000 are enhanced in Pol V defective mutants, whereas jasmonic acid (JA)–mediated defenses that protect against fungi are reduced. Chromatin immunoprecipitation analysis revealed that, through differential histone modifications, SA–related defense genes are poised for enhanced activation in Pol V defective mutants and provide clues for understanding the regulation of gene priming during defense. Our results highlight the importance of epigenetic control as an additional layer of complexity in the regulation of plant immunity and point towards multiple components of the RdDM pathway being involved in plant immunity based on genetic evidence, but whether this is a direct or indirect effect on disease-related genes is unclear.

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Introduction
RNA-directed DNA methylation (RdDM) is an epigenetic modification mechanism driven by noncoding small interfering RNAs (siRNAs) [1,2]. siRNAs are present in most eukaryotic organisms, are highly developed in plants and regulate gene expression at the transcriptional and posttranscriptional level in a sequence-specific manner. In contrast to microRNAs (miRNAs) that are derived from the transcripts of miRNA genes generated by RNA Polymerase II, production of RdDM-associated siRNAs requires RNA Polymerase IV (Pol IV) complex activity which includes, among other constituents, the largest and second largest subunits, NRPD1 and NRPD2, respectively [3–5]. Upon the action of Pol IV, the resulting single-stranded RNAs are used as templates for RNA-dependent RNA polymerase 2 (RDR2) generating double-stranded RNAs, which are processed by DICER-LIKE 3 (DCL3) [6,7]. Subsequently, RNA methyltransferase HUA ENHANCER-1 (HEN1) generates functional siRNAs that are recruited by ARGONAUTE4 (AGO4) to form the AGO4-RISC multiprotein complex guided to siRNA-complementary genome sequences [8–10]. AGO4-siRNA complexes interact with the RNA Polymerase V (Pol V) complex, which includes the largest and second largest subunits, NRPE1 and NRPD2, respectively. Pol V is somehow required to recruit DRM2 methyltransferase as well as histone-modifying complexes to finally establish the methylation pattern in the siRNA-complementary genome sequences; however, the details of this recruitment are unknown. This process results in the methylation of certain genome repeat regions and their subsequent transcriptional silencing [2]. Among the different classes of siRNA, the 24 nt in length heterochromatic siRNAs (hc-siRNAs) and repeat-associated siRNAs (ra-siRNAs), primarily derived from transposons, repeated elements and heterochromatin regions, are those functioning in the RdDM pathway by mediating DNA methylation and/or histone modification at the target sites [2].

Small RNAs regulate a multitude of biological processes in plants, including sustaining genome integrity, development, metabolism and responses to changing environmental conditions and abiotic stress [11]. Increasing evidences also indicate that plant endogenous small RNAs, including miRNAs and siRNAs, are integral regulatory components of plant defense machinery against microbial pathogens [12]. The Arabidopsis miR393 imparts basal resistance to the bacterial pathogen Pseudomonas syringae DC3000 by targeting the auxin receptors TIR1, ABF2 and ABF3 [13]. Besides miR393, two other miRNA families, miR160 and miR167, are upregulated following PDC3000.
The influence of epigenetic regulation in controlling the adaptive responses of living organisms to changes in the environment is becoming a common theme in biology. RNA-directed DNA methylation (RdDM) is an epigenetic control mechanism driven by a subset of noncoding small interfering RNAs (siRNAs) that influence gene function without changing DNA sequence by inducing de novo methylation of cytosines, or by modification of histones, at their target genomic regions. The implication and roles of the RdDM mechanism in the orchestration of plant immune responses still remains to be characterized. A recent study in the model plant Arabidopsis showed that ARGONAUTE4, one of the characteristic components of the RdDM pathway, was required for plant immunity against bacterial pathogens. Here, in a genetic screen aiming to identify cellular factors integral in regulating immunity in Arabidopsis, we further identified that the RNA polymerases V, another crucial component of the RdDM pathway, is pivotal for plant immunity against fungal pathogens. Similarly, we identified that additional components of the RdDM pathway, but surprisingly not RNA polymerase IV, are similarly required for plant immunity. Based on genetic evidence, our results highlight the importance of RdDM as an additional layer of complexity in the regulation of plant immune responses.

Results/Discussion

Characterization of ocp1 Plants

The Arabidopsis ocp mutants were identified previously in a genetic screen [22] designed to isolate negative regulators of pathogen-induced defense responses. The H2O2-responsive and defense-related Ep5C gene promoter fused to GUS was used as reporter [24]. Here we described the characterization of the ocp1 mutant. Figure 1A shows the constitutive Ep5C::GUS expression in rosette leaves from ocp1 plants compared with its parental Col-0 line (line 5.2). ocp1 plants exhibited similar plant architecture and growth habit to the wild-type plants (Figure 1B). F1 hybrids from a backcross between parental and ocp1 plants showed the absence of GUS activity, and GUS activity segregated in the F2 progeny as a single recessive Mendelian locus [OCP1:ocp1, 111:33 (P<0.05, χ2 test)].

We hypothesize that the constitutive expression of Ep5C::GUS observed in ocp1 plants might be accompanied by an altered disease resistance response to pathogens as previously revealed in ocp3 and ocp11 plants [22,23,25,26]. Therefore, we inoculated ocp1 plants with the virulent necrotrophic fungal pathogen Botrytis cinerea and monitored the disease response in leaves in comparison with the parental line. Disease was scored by recording the extent of necrosis. Wild-type plants exhibited normal susceptibility to B. cinerea (Figure 1C), with inoculated leaves showing necrosis accompanied by extensive proliferation of the fungal mycelia. In contrast, ocp1 plants showed increased susceptibility to B. cinerea distinguished by moderate but statistical significant enlargement of necrotic areas at inoculation sites (Figure 1C).

Susceptibility of ocp1 plants to pathogens was also investigated with the bacterial pathogen Pseudomonas syringae pv. syringae (a wild-type strain of Pseudomonas syringae pv. maculicola and the oomycetes Hyaloperonospora arabidopsidis, constituting a third example for siRNA-mediated resistance responses [21]. However, it remains unclear how RdDM participates in this type of processes.

The understanding of the overall contribution and requirement of the different components that conform the RdDM pathway, and how important they are in the regulation of the RdDM-mediated processes, particularly in relation to plant immunity, is an issue that still remains to be fully understood. Previously we described a genetic screen in Arabidopsis design to identify mutants (ocp mutants) with altered immune responses [22]. This allowed identifying AGO4, through the characterization of its mutant allele ago4-2/ocp11, as an important component of the RdDM pathway in mediating plant immune responses towards Pseudomonas syringae pv. tomato (Pst DC3000) [23]. Towards characterizing the contribution of other components of the RdDM pathway in plant immunity, we report here on the isolation and characterization of ocp1, a recessive mutant allele of NRPD2. Our results support that RdDM, through the action of RNA Pol V, is pivotal in modulating immune responses towards pathogens.
plants at different times following inoculation with expression analysis of PR-1 cinerea (wild-type plant carrying the histochemical analysis of GUS activity in rosette leaves from a parental Figure 1. Characterization of ocp1 lesions). Representative leaves from wild-type and (D–E) Growth rates of virulent Ps plants to virulent B. cinerea and ocp1 plant (right). (B) Macroscopic comparison of 3-week-old wild-type (left) and avirulent Ps DC3000 (D) and avirulent Ps DC3000 (AvrRpm1) (E) in Col-0, ocp1 and npr1 or rpm1 plants. (F–G) RT-qPCR expression analysis of PR-1 (F) and PDF1.2a (G) in wild-type and ocp1 plants at different times following inoculation with PsDC3000 (F) and B. cinerea (G). Data represent the mean ± 5D; n = 3 biological replicates. doi:10.1371/journal.pgen.1002434.g001

OCP1 Is At3g23780 and Encodes NRPD2, the Second Largest Subunit of the RNA Pol IV and Pol V

The genetic lesion carried by ocp1 plants was identified by positional cloning (Figure S1). A single nucleotide deletion was detected on locus At3g23780, particularly in the third exon of the transcribed gene encoding NRPD2, the second largest subunit of the RNA Pol IV and Pol V protein complexes (Figure 2A and Figure S1C). The loss of a nucleotide residue created a change in the NRPD2 open reading frame that leads to a frame shift starting at residue 595 (Figure 2A) followed by an incorrect 22 amino acid C-terminal tail sequence before an in-frame stop codon (Figure S2). The mutation renders a protein of 616 amino acid residues, instead of the 1172 contained in NRPD2, that thus has lost almost half of the protein sequence, including the amino acids that contribute to the active site of RNA polymerases [28].

The result obtained in our mapping strategy was corroborated with a test of allelism between ocp1 plants and plants carrying a null allele of NRPD2, in particular with nrpd2-2 plants which carry a T-DNA insertion [SALK_046208] [3]. Analysis of GUS expression driven by the Ep5C gene promoter in 20 F1 plants derived from a cross between homozygous ocp1 plants with homozygous nrpd2-2 plants or, alternatively, from a reversed cross between nrpd2-2 plants with ocp1 plants, revealed that all F1 plants showed constitutive GUS expression (Figure S3). Conversely, control crosses between the parental Col-0 plants carrying the Ep5C::GUS gene construct (line 5.2) with either ocp1 plants or nrpd2-2 plants revealed no GUS expression in any of the F1 22 plants analyzed (Figure S3). The result indicates that the ocp1 and nrpd2-2 are mutant alleles of the same NRPD2 gene and supported the conclusion that the ocp1 mutation represents a loss of function allele. Hence, the ocp1 mutation will be referred also as ocp1/nrpd2-53.

From the type of mutation found, we cannot exclude the possibility that ocp1 plants are still able to produce a truncated version of the NRPD2 protein with a residual ability to interact with other components of the RNA polymerase complexes. Since Pol IV and Pol V complexes are comprised of a variety of interacting subunits, some being polymerase-specific while other subunits shared [i.e., NRPD2] [5,29,30], and with some cross-talk described for some of their subunits [i.e., between NRPD2 and NRPE1; [4]], we can not discard the possibility that the relationships between the different components of the two RNA polymerase complexes may become differentially altered in the ocp1 mutant. In this respect, the availability of the ocp1 allele may represent a valuable experimental tool to approach the biochemical regulation of the RdDM mechanism.

Interestingly, RT-PCR analyses of NRPD2 transcript levels in ocp1 plants revealed the absence of notable changes in gene expression compared with Col-0 plants (Figure 2B). This is in marked contrast with the expression observed in nrpd2-2 null mutant plants where no transcript amplification products can be obtained (Figure 2B). A comparison of the disease resistance response between ocp1 and nrpd2-2 plants revealed that while the ocp1 plants showed a moderate increase in susceptibility to B. cinerea, the nrpd2-2 null mutant responded to B. cinerea infection with a remarkable enhancement in susceptibility (Figure 2C). The enhanced susceptibility phenotype of nrpd2-2 plants was further corroborated by recording the susceptibility towards Plectosphaerella cucumerina, a different fungal necrotroph (Figure 2D). Consistent with the observed increase in disease susceptibility to P. cucumerina, RT-qPCR experiments revealed that induction of the JA-responsive PDF1.2a gene was disabled in nrpd2-2 plants compared to Col-0 (Figure 2E). These results mirror what occurs in ocp1 plants following B. cinerea infection (Figure 1F). Of importance for
Lesion size was measured in Col-0, ocp1plants. The expression level by RT-PCR in mRNAs derived from Col-0, nickelotide triplet, and the first amino acid change (S to T) where the sequence. Deduced amino acid sequences are indicated below each plants show an enhanced disease susceptibility of bigger magnitude than that observed in ocp1/nrpd2-53 plants, subsequently, the experiments related to disease resistance/susceptibility will be carried out employing the nrpd2-2 allele.

**SUPERMAN, 5S Genes, and the AtSN1 Retroelement Are Hypomethylated in ocp1 Plants**

To further substantiate the molecular phenotype of ocp1 plants in relation to RdDM, we checked if the methylation status of different RdDM target sequences could be similarly affected in ocp1 and nrpd2-2 plants. We analyzed the methylation status in ocp1 plants of the RdDM pathway DNA target sequences SUPERMAN, ribosomal 5S genes and the retrotransposon AtSN1 [31]. We used methylation tests employing the methylation-sensitive restriction endonuclease HaeIII (where HaeIII will not cut DNA if methylated), with subsequent amplification by PCR [32]. Initial experiments revealed that ocp1, as well as ago1-2/ocp1I plants used as controls, exhibit a higher degree of hypomethylation in SUPERMAN gene compared to Col-0 plants (Figure 3A). Analyses were extended to the ribosomal 5S genes and the AtSN1 retrotransposon and we incorporated nrpd2-2, nrpd1-3 and nrpe1-1 mutants for comparison. Figure 3B shows mutants demonstrated higher degrees of hypomethylation in the sequences analyzed. DNA samples derived from ocp1 plants exhibited decreased amplification for the 5S and AtSN1 loci, confirming a clear DNA methylation deficiency in this mutant. The ABI5 gene, whose sequence contains no restriction sites for HaeIII, was used as a control. Methylation tests were also used to ascertain whether or not the enhanced induction observed for the PR-1 gene, or the repression of PDF1.2a, in the nrpd2 mutant following fungal infection correlated with defects in the DNA methylation of their promoter regions. Since both genes contain a large number of recognition sites for the methylation-sensitive restriction enzymes FspE1, MspJ1 and HaeII (160 target sequences in the PR-1 gene and 296 targets in the PDF1.2a gene), and where both sites must be methylated for the enzymes to cleave the DNA, we used restriction analysis with these enzymes with subsequent amplification by PCR to check the methylation status of the PR-1 and PDF1.2a genes. The results shown in Figure S4 and Figure S5 revealed that none of the promoters appear methylated, not even in Col-0 plants. Conversely, the sensitivity of the methylated 5S ribosomal DNA (Figure S4) to the aforementioned enzymes revealed the appropriateness of the method used to identify methylation of cytosine residues. The lack of a methylation footprint in the DNA of the defense-related PR-1 and PDF1.2a genes might suggest that the abnormal expression patterns concurring in nrpd2 mutant plants must obey not to a direct modification of cytosine residues but to other type of chromatin modification or mechanism similarly controlled either directly or indirectly by the RdDM pathway.

The Pol V Complex, But Not Pol IV, Is Required for the Correct Immune Response against *B. cinereae* and *P. cucumerina*

As for NRPD2, we addressed if other RdDM pathway components are similarly engaged in plant immunity. A comparative analysis of the disease resistance response of nrpd1, nrpe1, and ago1 mutant plants due to inoculation by *B. cinereae* was performed in relationship to nrpd2. Figure 4A shows an increase in understanding the immune-related phenotype of nrpd2-2 plants is the observation that expression of the SA-responsive *PR-1* gene was clearly enhanced following fungal inoculation in the mutant when compared to wild-type plants (Figure 2F). Since nrpd2-2 plants show an enhanced disease susceptibility of bigger magnitude than that observed in ocp1/nrpd2-53 plants, subsequently, the experiments related to disease resistance/susceptibility will be carried out employing the nrpd2-2 allele.

**Figure 2. ocp1 is a mutant allele of NRPD2.** (A) OCP1 corresponds to At2g27040 encoding NRPD2. The G nucleotide residue deleted in the ocp1 allele is indicated in red bold uppercase letters in the wild-type sequence. Deduced amino acid sequences are indicated below each nucleotide triplet, and the first amino acid change (S to T) where the frameshift of the OCP1 protein starts is shown in blue. (B) NRPD2 expression level by RT-PCR in mRNAs derived from Col-0, nrpd2-2 and ocp1 plants. The eEF1a house-keeping gene was used as a control. (C–D) nrpd2 plants show enhanced susceptibility to fungal pathogens. Lesion size was measured in Col-0, ocp1 and nrpd2-2 plants after inoculation with *B. cinereae* (C) or *P. cucumerina* (D). Data points represent average lesion size ± SD (n=30 lesions). ANOVA detected significant differences at the P<0.05 level. (E–F) RT-qPCR determination of PDF1.2a (E) and PR-1 (F) transcript levels following inoculation with *P. cucumerina*. Data represent the mean ± SD; n = 3 biological replicates. doi:10.1371/journal.pgen.1002434.g002
nrpe1 disease susceptibility to B. cinerea; the susceptibility being of a magnitude similar to that attained in nrpd2 plants. This enhancement in susceptibility was comparatively greater than that observed in ocp1 plants but less than in ago4-2/ocp11 plants. Conversely, nrpd1 plants did not exhibit a significant deviation from the normal disease response observed in Col-0 plants. This differential behavior was further corroborated in the Pol IV and Pol V defective mutants by challenging with P. cucumerina (Figure 4B). The nrpd1 nrpe1 double mutant that would be defective in both Pol IV and Pol V activities was incorporated in this experiment for comparison. nrpd1 nrpe1 plants showed an enhanced disease susceptibility of a magnitude similar to that attained in nrpd2 or nrpe1 plants. Furthermore, fungal biomass determination in leaves inoculated with P. cucumerina, as an alternative method for recording disease resistance, also revealed that the single nrpd2 and nrpe1 mutants, as well as the double nrpd1 nrpe1 mutant support significantly more fungal growth than Col-0 and the nrpd1 mutant (Figure S6). Therefore, the Pol V complex participates in the regulation of the immune response to necrotrophs while the Pol IV complex appears at least partially dispensable. This is sustained also by the observation that the single nrpe1 and nrpd2 plants, either in the course of infection with P. cucumerina (Figure 4C–4D) or upon chemical induction by treating plants with a solution of either 0.5 mM SA (Figure S7A) or 0.1 mM JA (Figure S7B). Notorious is the higher JA-triggered PDF1.2a gene induction in nrpd1 plants in comparison to Col-0 (Figure S7B). Conversely, this JA-triggered PDF1.2a gene induction is notably repressed in the Pol V defective mutants (Figure 4C and Figure S7B). This is in marked contrast with the altered expression pattern observed in nrpd2 plants where induction of PR-1 gene expression showed enhancement following inoculation with P. cucumerina (Figure 4D) or upon external application of SA (Figure S7A). Importantly, this pattern of gene expression was reproduced in nrpe1 plants (Figure 4D and Figure S7A). Moreover, the transcription factors WRKY6 and WRKY53 that bind W-box and transcriptionally regulate gene expression of SA-related genes, including PR-1 [33], and are themselves induced by pathogen infection [34], show similar enhanced level of induction following SA application in nrpd2 and nrpe1 plants when compared to Col-0 or nrpd1 plants (Figure S7C and S7D).

**Figure 3.** *ocp1* plants show hypomethylation in RdDM target DNA sequences. Genomic DNA isolated from Col-0, ocp1 and ago4-2/ocp11 plants (A) and nrpd2, nrpd1 and nrpe1 (B) was digested (+) or not (−) with HaeIII and amplified by PCR for SUPERMAN promoter (A), the ribosomal S5 genes and the retrotransposon AtSN1 (B). ABI5 contains no target sequences for HaeIII and was used as a control.

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**Table 1.** Pol V, RDR2, AGO4, DRM1 and DRM2 mutants show compromised immune responses to *P. cucumerina*

| Mutant | Immune Response |
|--------|-----------------|
| Col-0  | Susceptible     |
| nrpd1  | Susceptible     |
| nrpe1  | Susceptible     |
| nrpd2  | Susceptible     |
| nrpd1 nrpe1 | Susceptible |
| drm1 drm2 | Susceptible |
| drm1 drm2 rdr2 | Susceptible |
| drm1 drm2 rdr2 ago4 | Susceptible |

**Table 2.** SA–Mediated Defense Genes Are Poised for Enhanced Activation by Histone Modifications in Pol V Defective Mutants

The previous results suggest that in Pol V defective mutants SA-related defense genes are poised for enhanced activation following perception of pathogenic cues and concurrently JA-related defenses appear impeded for induction. This will be congruent with a solution of either 0.5 mM SA (Figure S7A) or 0.1 mM JA (Figure S7B). Notorious is the higher JA-triggered PDF1.2a gene induction in nrpd1 plants in comparison to Col-0 (Figure S7B). Conversely, this JA-triggered PDF1.2a gene induction is notably repressed in the Pol V defective mutants (Figure 4C and Figure S7B). This is in marked contrast with the altered expression pattern observed in nrpd2 plants where induction of PR-1 gene expression showed enhancement following inoculation with *P. cucumerina* (Figure 4D) or upon external application of SA (Figure S7A). Importantly, this pattern of gene expression was reproduced in nrpe1 plants (Figure 4D and Figure S7A). Moreover, the transcription factors WRKY6 and WRKY53 that bind W-box and transcriptionally regulate gene expression of SA-related genes, including PR-1 [33], and are themselves induced by pathogen infection [34], show similar enhanced level of induction following SA application in nrpd2 and nrpe1 plants when compared to Col-0 or nrpd1 plants (Figure S7C and S7D).

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| nrpd1  | Susceptible     |
| nrpe1  | Susceptible     |
| nrpd2  | Susceptible     |
| nrpd1 nrpe1 | Susceptible |
| drm1 drm2 | Susceptible |
| drm1 drm2 rdr2 | Susceptible |
| drm1 drm2 rdr2 ago4 | Susceptible |

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with a notion where Pol V may regulate a priming phenomenon for SA-mediated defense responses that ultimately would modulate the speed and extent of gene activation. However, the lack of a methylation footprint in the DNA of the defense-related PR-1 and PDF1.2a genes (Figure S3 and Figure S6) suggest that the observed abnormal gene expression patterns concurring in the Pol V defective mutants is not to be due to an altered DNA methylation pattern resulting from a defective RdDM pathway. However, one could still entertained the possibility that changes in chromatin structure such as those obeying to covalent modification of histones, which are also under the control of the RdDM pathway, may be on the basis for the enhanced expression observed for PR-1 and, therefore, for the altered resistance phenotypes in the mutant plants. This would be congruent with the recent identification of a mechanism linking chromatin modification in wild type plants, through the differential modification of histones in several genes encoding WRKY transcription factors (i.e. WRKY6, WRKY29 or WRKY53), with priming of a defense response following pharmacological treatment with the SA analogue acidobenzoar S-methyl (BTH) which functions as a priming agent in plants [39]. Thus, we hypothesized that in Pol V defective mutants PR-1 could be poised for enhanced activation of gene expression by a differential modification of histones.

By using chromatin immunoprecipitation (ChIP) we analyzed trimethylation of histone H3 Lys4 (H3K4me3) and acetylation of histone H3 Lys9 (H3K9ac) on the promoter of the PR-1 gene. For comparison, the promoter of the JA-inducible PDF1.2a gene, that of the constitutively expressed Actin2 gene and also those of the WRK66 and WRKY53 genes were similarly studied. The specificity of the ChIP reaction was evaluated in advance by measuring histone modifications on these genes in Col-0 plants treated with BTH (Figure S8A and S8B). On the PR-1 promoter H3K4me3 and H3K9ac marks increased after BTH application while these marks did not change in the promoters of Actin2 or PDF1.2a (Figure S8A and S8B). As for PR-1, these chromatin marks were similarly increased in the promoters of WRK66 and WRKY53 upon treatment with BTH (Figure S8C). Thus chromatin marks normally associated with active genes [39,40] are set in the promoters of SA-related defense genes by the priming stimulus of BTH. Interestingly, determination of H3K4me3 (Figure 5A) and H3K9ac (Figure 5B) chromatin marks in the PR-1 promoter in ChIP samples derived from nrpd2 and nrpe1 plants, revealed that these marks are already set in these two mutants, although PR-1 gene activation does not take place. Thus, Pol V defective mutants mimic Col-0 plants treated with the priming agent BTH. This reconciles with the idea that the PR-1 gene is switch on for priming in the Pol V defective mutant and explains why this gene shows enhanced induction upon pathogenic attack in the same mutants (Figure 4D). In the nrpd1 mutant only a moderate increase in the setting of these chromatin marks in the promoter of PR-1 was detected (Figure 5A and 5B). No variation in similar activation marks was observed in the promoters of the Actin2 and PDF1.2a genes (Figure 5A and 5B). Other histone marks, such as H3K9me2 and H3K27me3, both of which repressive marks normally associated with heterochromatin and established through the RdDM pathway [41], appear notably reduced in the PR-1 promoter in ChIP samples derived from nrpd2 and nrpe1 plants, and much less reduced in nrpd1 plants, when compared to Col-0 plants (Figure S9A and S9B). Moreover, Col-0 plants respond to P. cucumerina infection with reduction in the setting of these two repressive histone marks in the PR-1 gene promoter but not in the promoters of the PDF1.2a or Actin2 genes (Figure S9C). The dismantling of histone repressive marks in infected plants, along with the concurring increase in histone activation marks and

Figure 4. Comparative immune responses of RdDM mutants to inoculation with B. cinerea and P. cucumerina. (A) Disease susceptibility of Col-0, nrpd1, nrpe1, nrpd2, ocp1 and ago4-2/ocp11 plants to B. cinerea. (B) Comparative disease susceptibility of the Pol IV and Pol V defective mutants to P. cucumerina. (C–D) RT-qPCR of PDF1.2a (C) and PR-1 (D) transcript levels following inoculation with P. cucumerina in Col-0, nrpd1, nrpe1 and nrpd2 plants. Data represent the mean ± SD; n = 3 biological replicates. (E) Comparative disease susceptibility of rdr2, ddr1, drm1/drm2 and nrpd2 mutants to P. cucumerina. ANOVA detected significant differences at the P<0.05 level. doi:10.1371/journal.pgen.1002434.g004
Figure 5. Histone H3 modifications. Comparative level of histone modifications of PR-1, PDF1.2a and Actin2 gene promoters as present in leaf samples from Col-0, nprd1, nrpe1 and nprd2 plants. (A) Histone H3 Lys4 trimethylation (H3K4me3) on the indicated gene promoters. (B) Histone H3 K9 acetylation (H3K9ac) on the indicated gene promoters. Data are standardized for mock inoculated Col-0 histone modification levels. Data represent the mean ± SD; n = 3 biological replicates.

HRK4me3 (C) and H3K9ac (D) modifications on PR-1 and PDF1.2a gene promoters in Col-0 and nprd2 plants 48 h after inoculation with P. cucumerina. (D) (−) mock inoculated plants, (+) P. cucumerina inoculated plants. Data are standardized for mock inoculated Col-0 histone modification levels. Data represent the mean ± SD; n = 3 biological replicates.

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decrease in repressive marks in the promoter of the PR-1 gene, as observed in nprd2 and nrpe1 plants, gives further support to the implication of Pol V in regulating defense gene activation.

As for PR-1, H3K4me3 activation marks are also constitutively set in the promoters of the WRKY6 and WRKY53 genes in healthy nprd2 and nrpe1 plants (Figure S8C), again mirroring the effect carried out by BTH on Col-0 for these promoters (Figure S8C). Further analysis demonstrated that Col-0 plants respond to P. cucumerina infection with a drastic increase in the setting of H3K4me3 and H3K9ac activation marks in the promoters of PR-1 (Figure 5C and 5D). In nprd2 plants, in which these chromatin marks are already set in PR-1, P. cucumerina inoculation further increases H3K4me3 marks on the PR-1 promoter to levels that are even higher than those attained in Col-0 (Figure 5C). However, for H3K9ac marks no further increase was observed in nprd2 plants, suggesting that this type of mark is completely set in the mutant. In contrast, no variation in the setting of these chromatin marks was detected in the PDF1.2a promoter upon fungal infection (Figure 5C and 5D). For WRKY6 and WRKY53 gene promoters, Col-0 plants respond to P. cucumerina infection by similarly increasing H3K4me3 mark setting in both promoters (Figure S10). Compared to Col-0, nprd2 plants constitutively carry increased H3K4me3 mark setting in WRKY6 and WRKY53 gene promoters and do not show further increases upon inoculation, but instead slightly decrease (Figure S9). Together, these data imply that Pol V, either directly or indirectly, regulates the extent of chromatin modifications on SA defense-related gene promoters, and may be the underlying mechanism controlling priming marks facilitating the more rapid activation of gene expression observed upon perception of pathogenic cues. As reported for other genes, the observed covalent modifications in chromatin might provoke increases in the accessibility of DNA or perhaps in the provision of docking sites for gene activators [42,43].

nprd2 and nrpe1 Plants Show Enhanced Resistance to P. o. DC3000

Enhanced activation of SA-mediated defenses is characteristic of plants resistant to biotrophic pathogens, like P. o. DC3000, and is on the basis for a systemic type of immunity known as systemic acquired resistance (SAR) [44]. Our results on a priming effect for enhanced expression of SA defense-related genes in nprd2 and nrpe1 plants suggest these mutants may be altered in the resistance to P. o. DC3000. Consequently, we addressed Pol IV and Pol V defective mutants in search for defects in the immune response to P. o. DC3000. We used ago4-2/ocp11 and nrpe1 plants as controls, both exhibiting heightened P. o. DC3000 disease susceptibility [23,27]. Interestingly, a significant enhanced disease resistance to P. o. DC3000 was observed in nprd2, nrpe1, and in nprd1 nrpe1 plants, when compared to Col-0 plants (Figure 6). In contrast, statistically significant effects were not observed in nprd1 plants relative to Col-0 in response to P. o. DC3000, giving further support to the idea that RNA Pol IV seems not engaged in plant immunity. The observed heightened resistance towards P. o. DC3000 in nprd2 and nrpe1 plants indicated that in wild-type plants Pol V is required for susceptibility to this pathogen. However, in ago4-2/ocp11 plants resistance to P. o. DC3000 is severely compromised. Although there
The function, such as those observed in observations is that in wild type plants Pol V negatively regulates arsenal controlled by SA. A simpler explanation for these which suggests these plants are more prone to mobilize the defense SA defense-related genes and respond to pathogen attack with a specific type of pathogenic insult [45]. Our results demonstrated equilibrium that occasionally culminates with the partial inhibition underlying complexity in the control of disease resistance by necrotrophic fungi in Pol V defective mutants. This reveals an function, and while required for an effective defense response it stated [23] one can speculate that AGO4 can serve a novel is no obvious explanation for this contrasting effect, as previously stated [23] one can speculate that AGO4 can serve a novel function, and while required for an effective defense response it may operate independently of the RdDM pathway.

An important observation derived from the results presented is the co-existence of an enhanced disease resistance to a biotrophic bacteria, like PsDC3000, with an enhanced susceptibility to necrotrophic fungi in Pol V defective mutants. This reveals an underlying complexity in the control of disease resistance by RdDM. The SA and JA signal pathways are under an antagonistic equilibrium that occasionally culminates with the partial inhibition of one pathway when the other is facilitated. Consequently the interaction between pathways serves to optimize responses to a specific type of pathogenic insult [45]. Our results demonstrated that npd2 and npe1 plants are poised for enhanced activation of SA defense-related genes and respond to pathogen attack with a marked enhancement in the induced expression of marker genes, which suggests these plants are more prone to mobilize the defense arsenal controlled by SA. A simpler explanation for these observations is that in wild type plants Pol V negatively regulates a priming mechanism for SA-mediated disease resistance while keeping intact a JA-mediated disease resistance. Defects in Pol V function, such as those observed in npd2 and npe1 mutants, de-repress the priming mechanism for SA-mediated resistance through pertinent chromatin modifications, and renders enhanced resistance to PsDC3000. As a tradeoff, presumably mediated through endogenous antagonistic cross talk mechanisms, mis-regulation of the JA-mediated disease resistance occurs. This thus explaining the repressed expression of JA-marker gene and the heightened susceptibility of npd2 and npe1 plants to fungal pathogens. However, although this mechanism seems very likely, we still cannot disregard the possibility that RdDM may be similarly required for normal expression of one or more unknown genes involved in JA signaling. Disruption of RdDM could thus lead to a disruption of JA signaling which would in turn result in hyper-activation of SA signaling. In fact, mutant plants with JA-mediated signaling pathway defects and hypersensitivity to fungal necrotrophs concurrently present a less repressed SA-mediated signaling pathway, resulting in a more efficient defense response when challenged with biotrophic pathogens [45,46]. Experiments directed towards identification of an epigenetic footprint associated to the JA pathway merits future reach and will help clarify the complexity of the antagonistic cross-talk mechanism between the SA and the JA signal transduction pathways.

A deeper understanding on how the RdDM and associated chromatin modification acts as a mechanism controlling gene priming and induced immune responses in plants, and how pathogens may counteract this epigenetic regulation for their own benefit will open new avenues for the a better knowledge on how plant immunity is orchestrated.

**Materials and Methods**

**Plant Material and Growth Conditions**

*Arabidopsis* were grown in a growth chamber (19 to 23°C, 85% relative humidity, 100 μE m⁻² s⁻¹ fluorescent illumination) under a 10/14 h light/dark photoperiod. All mutants are in Col-0 background. ago4-2/ago11, npr1, tpm1-1, ntr2, ddr1-6 and dm1/dm2 plants were previously described [23]. npd2-2 (SALK_046208); npe1-11 (SALK_029919) and npd1-3 (SALK_128428) were obtained from the Salk Institute Genomic Analysis Laboratory (http://signal.salk.edu/). npd1 npe1 double mutant was obtained from T. Lagrange.

**GUS Staining**

Plant leaves were incubated overnight at 37°C in GUS staining buffer as previously described [22]. The oep1 mutant was backcrossed twice to the plants were crossed to Ler, and F1 plants were allowed to self. F2 plants were scored for co-segregation of high constitutive GUS activity with simple sequence length polymorphisms (SSLP) [40]. Molecular markers were derived from the polymorphism database between the Ler and Col-0 ecotypes (http://www.arabidopsis.org).

**PCR-Based Methylation Assays**

Methylation tests using the methylation-sensitive endonuclease *Hae*III, *Fpy*EI, *Aau*II and *Msp*I/*I* were performed as described [32]. The relative DNA fragment amounts corresponding to *SUPER-MAN*, 35 and *AtSN1* were obtained after 30, 25 and 35 respective PCR cycles. For *ABI2*, 30 (A) or 26 (B) PCR cycles were used. **PR-1 and PDF1.2a** methylation assays are provided in a supplemental file.

**Expression Analysis**

Gene expression analysis, by either RT-PCR or qRT-PCR was performed as described previously [23]. The primers used to amplify the different genes and DNA regions, and the PCR conditions employed for genotyping T-DNA insertions, and RT-PCR and qRT-PCR experiments are provided in the supporting information file Text S1.

**Bacterial and Fungal Bioassays**

Bacterial strains were grown overnight and used to infect 5-week-old *Arabidopsis* leaves by infiltration and bacterial growth determined as described [23]. Twelve samples were used for each data point and represented as the mean ± SEM of log c.f.u./cm². *B. cinerea* and *P. cucumerina* bioassays were performed as previously described [24]. Fungal disease symptoms were evaluated by determining the lesion diameter (in mm) of a minimum of 30 lesions. All experiments were repeated at least three times with similar results.
Chromatin Immunoprecipitation

Chromatin isolation and immunoprecipitation were performed as described [47]. Chip samples, derived from three biological replicates, were amplified in triplicate and measured by quantitative PCR using primers for PR-1, WRKY6, WRKY53 and Actin2 as reported [39]. The rest of primers are described in Text S1. All ChIP experiments were performed in three independent biological replicates. The antibodies used for immunoprecipitation of modified histones from 2 g of leaf material were antiH3K4me3 (#07-473 Millipore), antiH3K4ac (#07-352 Millipore), antiH3K9me2 (ab1772 Abcam) and anti-H3K27me3 (ab6002 Abcam).

Supporting Information

Figure S1 ocp1 is At3g23780 and Encodes NRPD2. To identify the genetic lesion carried by ocp1 plants, we performed positional cloning of the mutation. To map the position of ocp1 in the genome, we crossed ocp1 plants to Landsberg erecta (Ler) plants, and F2 plants were scored for co-segregation of high constitutive GUS activity with simple sequence length polymorphisms (SSLP) [48]. An initial analysis of 40 ocp1 individuals allocated the ocp1 mutation in chromosome III, between markers nga162 and AthGAPAB which define an interval of 22.2 cM. Further analysis of 472 plants with 12 new polymorphic markers allowed narrowing the position of ocp1 to an interval of 246,291 bp located between markers CER455355 and CER454777 and comprising 6 BAC clones (A). Four new SSLP markers and one dCAPF (Derived Cleaved Amplified Polymorphic Sequences) marker were analyzed for this mapping interval, and we deduced that the ocp1 lesion was located between markers CER457821 and CER457824, delimiting an interval of 36 kb that comprised a region of 10 ORFs (B). DNA sequencing of this 36 kb interval allowed us to find a guanosine residue deleted in the third exon of the NRPD2 gene (C).

(TIF)

Figure S2 Comparative amino acid sequences of NRPD2 and OCP1. In blue is indicated the 22 extra amino acid residues preceding the premature stop codon arising due to the nucleotide deletion identified in the ocp1 mutant. In red is indicated the S to T transition due to the change in the open reading frame as a consequence of the deleted nucleotide.

(TIF)

Figure S3 ocp1 is allelic to nrpd2. The result obtained in our cloning strategy was corroborated with a test of allelism between ocp1 plants and plants carrying the nrpd2-2 allele. Analysis of GUS expression driven by the Ep3SC gene promoter in 20 F1 plants derived from a cross between homozygous ocp1 plants with homozygous nrpd2-2 plants or, alternatively, from a reverse crossed between nrpd2-2 plants with ocp1 plants, revealed that all F1 plants showed constitutive GUS expression. Conversely, control crosses between the parental Col-0 plants carrying the Ep3SC::GUS gene construct (line 5.2) with either ocp1 plants or nrpd2-2 plants revealed no GUS expression in any of the 22 F1 plants analyzed. These complementation analyses indicate that the ocp1 and nrpd2 are mutant alleles of the same NRPD2 gene. Hence, the ocp1 mutation will be referred also as ocp1/nrpd2-3.

(TIF)

Figure S4 PR-1 and PDF1.2a genes appear not to be methylated in their DNA sequences. Genomic DNA isolated from Col-0, nrpd2, nrpd1 and nrpe1 plants were digested (+) or not (−) with FspE1, MspJ or AvaI and amplified by PCR using specific primers for the indicated promoter regions. The ribosomal 5S DNA sequences, which are methylated, were used as a control.

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Figure S5 Nucleotide sequence of PR-1 and PDF1.2a 5′ promoter regions. Restriction sites for FspE1 (green) and MspJ (blue) endonucleases are indicated by color sequences. Red circle marks AvaII restriction site. Arrows denote position of primers used to amplify the respective promoter regions as indicated in supplemental Methods. The ATG translation initiation codon for the transcribed genes is shown in bold.

(TIF)

Figure S6 Growth of P. cucumerina on leaves from Col-0, nrpd1, nrpd2-1, nrpd2-2 and nrpd2 plants quantified by qPCR. Plants were inoculated with P. cucumerina by spraying full expanded leaves with a solution containing 5×10^6 spores/ml. Five days after inoculation DNA was extracted from leaves and the amount of the P. cucumerina β-tubulin gene quantified by qPCR. Data are standarized for the presence of the P. cucumerina β-tubulin gene in Col-0. Data represent the mean ± SD; n = 3 biological replicates.

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Figure S7 Transcript abundance by RT-qPCR on control genes following spray treatment with SA and JA. Abundance of PR-1 (A), WRKY6 (C) and WRKY53 (D) transcripts in Col-0, nrpd1, nrpd2 plants 48 h after spraying with a solution containing (+) or not containing (−) 0.5 mM SA. (B) Abundance of PDF1.2a transcripts in Col-0, nrpd1, nrpd2 plants 48 h after spraying with a solution containing (+) or not containing (−) 0.1 mM JA. Data represent the mean ± SD; n = 3 biological replicates.

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Figure S8 Histone modifications on control genes and effect of the priming agent BTH. (A–B) Histone H3K4me3 mark on the promoters of PR-1, PDF1.2a and Actin2 genes in Col-0, nrpd1, nrpd2 plants 48 h after spraying with a solution containing (+) or not containing (−) 0.1 mM BTH (+) or a wettable powder (−) as a control. (C) Comparative level of histone H3K4me3 modification on WRKY6 and WRKY53 promoters in Col-0, nrpd2 plants and after treatment for gene priming of Col-0 plants with 0.1 mM BTH. Data are standarized for Col-0 histone modification levels. Data represent the mean ± SD; n = 3 biological replicates.

(TIF)

Figure S9 Histone H3K9me2 and H3K27me3 modifications in PR-1, PDF1.2a and Actin2 genes in Col-0, nrpd1, nrpd2 plants. Comparative levels of histone H2K9m2 (A) and H3K27me3 (B) modifications on Actin2, PR-1 and PDF1.2a gene promoters after treatment of Col-0 plants for priming with 0.1 mM BTH (+) or a wettable powder (−) as a control. (C) Comparative level of histone H3K4me3 modification on WRKY6 and WRKY53 promoters in Col-0, nrpd1, nrpd2 plants and after treatment for gene priming of Col-0 plants with 0.1 mM BTH. Data are standarized for Col-0 histone modification levels. Data represent the mean ± SD; n = 3 biological replicates.

(TIF)

Figure S10 Histone H3K4me3 modification on WRKY6 and WRKY53 gene promoters in Col-0 and nrpd2 plants following inoculation with P. cucumerina. Comparative levels of induced histone modifications in histone H3K4me3 marks on the promoters of WRKY6 and WRKY53 following inoculation of Col-0 and nrpd2 plants with P. cucumerina. BTH-induced H3K4me3 modifications in Col-0 plants are included for comparison of the magnitude of the induced modifications in the two genes. Data are standarized.
for non-treated Col-0 histone modification levels. Data represent the mean ± SD; n = 3 biological replicates. (TH)

Text S1  Primer sequences. (DOCX)

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