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

Ana López1, Vicente Ramírez1, Javier García-Andrade1, Victor Flors2, Pablo Vera1*

1 Instituto de Biología Molecular y Celular de Plantas (IBMCp), CSIC-UPV, Valencia, Spain, 2 Department of Experimental Sciences, Universidad Jaume I, Castellón, Spain

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 NRPD2 as the OVEREXPRESSOR OF CATIONIC PEROXIDASE 1 (OCP1). NRPD2 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, rdr1, rdr2, and drm1 drm2 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.


Editor: Craig S. Pikaard, Indiana University, United States of America

Received May 13, 2011; Accepted November 8, 2011; Published December 29, 2011

Copyright: © 2011 López et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: We acknowledge the financial support of the Spanish MICINN (grants BFU2009-09771 and Consolider-TRANSPLANTA to PV) and Generalitat Valenciana (Prometeo2010/020 to PV). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: vera@ibmc.upv.es

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, 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 hetrochromatic 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, ABI2 and ABI3 [13]. Besides miR393, two other miRNA families, miR160 and miR166, are upregulated following PDC3000...
Author Summary

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.

inoculation and target members of auxin-response factors (ARF) [14]. Thus, in response to bacterial infection, plants suppress multiple components of the auxin signaling pathway. In turn, bacteria have developed type III secretion effectors that repress transcription of miRNA genes, the host RNA silencing machinery is suppressed and therefore disease susceptibility increase [15]. Similarly, Lu et al. [16] identified a series of 10 miRNAs families in loblolly pine whose expression were suppressed, and the expression of the parental line. Disease was scored by recording the extent of necrotic areas at inoculation sites (Figure 1C).

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).

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 PDC3000. The npr1 mutant, which is compromised in resistance towards this pathogen [27] was used as a control. Resulting bacterial growth in inoculated leaves is shown in Figure 1D and indicates the wild-type and ocp1 mutant susceptibility was unchanged towards virulent PDC3000. In addition, plants were inoculated with an avirulent strain of PDC3000 carrying the avrRpm1 gene that triggers a hypersensitive cell death response in the plant that stops bacterial growth. The rpm1 mutant, compromised in the hypersensitive response and consequently hypersusceptible to the pathogen, was used as a control. Results showed the growth of PDC3000 (avrRpm1) in ocp1 plants was not significantly different to that observed in wild-type plants (Figure 1E). These results were consistent with normal accumulation of transcripts of the salicylic acid (SA)-responsive gene PR-1 at 48 h following inoculation with PDC3000 (Figure 1F), however induction occurs earlier in ocp1 plants. Interestingly, induction of the jasmonic acid (JA)-responsive gene PDF1.2a, a characteristic molecular response of plants to fungal attack, was compromised in ocp1 plants following inoculation with B. cinerea (Figure 1G). This later observation is congruent with the observation that ocp1 plants showed enhanced disease susceptibility to this pathogen (Figure 1C).
plants at different times following inoculation with *B. cinerea*. Data represent the mean 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* inoculation (dpi). Data points represent average lesion size (right). (B) Macroscopic comparison of 3-week-old wild-type (left) and *ocp1* plants (right). (C) Resistance response of wild-type and *ocp1* plants. (F–G) RT-qPCR experiments revealed that induction of the JA-responsive *PDF1.2a* gene 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 1C). 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] [5]. 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/nrpd2-23*.

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

**Figure 1. Characterization of *ocp1* plants.** (A) Comparative histochemical analysis of GUS activity in rosette leaves from a parental wild-type plant carrying the P<sub>Ep5C::GUS</sub> transgene (left), and *ocp1* mutant plant (right). (B) Macroscopic comparison of 3-week-old wild-type (left) and *ocp1* plants (right). (C) Resistance response of wild-type and *ocp1* plants to *virulent* *B. cinerea* plants 4 dpi. (D–E) Growth rates of virulent *PsDC3000* (D) and avirulent *PsDC3000* (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 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] [5]. 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/nrpd2-23*.

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, ocp1

(D) nrpd2 cucumberina

represent average lesion size inoculation with B. cinerea doi:10.1371/journal.pgen.1002434.g002 plants. The expression level by RT-PCR in mRNAs derived from Col-0, frameshift of the OCP1 protein starts is shown in blue. (B) nucleotide triplet, and the first amino acid change (S to T) where the allele is indicated in red bold uppercase letters in the wild-type significant differences at the P

At2g27040 encoding NRPD2. The G nucleotide residue deleted in the

nrpd2-2 mutant plants due to inoculation by B. cinerea [31]. We used methyltransfer tests employing the methylation-sensitive restriction endonuclease HauIII (where HauIII will not cut DNA if methylated), with subsequent amplification by PCR [32]. Initial experiments revealed that ocp1, as well as ago1-2/ocp11 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 retrotransposon AtSN1 [31]. We used methyltransfer analysis with these enzymes to check the methylation status of the

NRPD2 and Plant Immunity

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.

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 methyltransfer tests employing the methylation-sensitive restriction endonuclease HauIII (where HauIII will not cut DNA if methylated), with subsequent amplification by PCR [32]. Initial experiments revealed that ocp1, as well as ago1-2/ocp11 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 HauIII, 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 FspEI, MspJI and AvaII (160 target sequences in the PR-1 gene and 298 targets in the PDF1.2a gene), and where FspEI and MspJI 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 concuring 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. cinerea 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 ago4 mutant plants due to inoculation by B. cinerea was performed in relationship to nrpd2. Figure 4A shows an increase in

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. cinerea (C) or P. cucumerina (D). Data points represent average lesion size ± SE (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
nrpd1 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* *nrpd1* double mutant that would be defective in both Pol IV and Pol V activities was incorporated in this experiment for comparison. *nrpd1* *nrpd1* plants showed an enhanced disease susceptibility of a magnitude similar to that attained in *nrpd2* or *nrpd2* 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 *nrpd1* mutants, as well as the double *nrpd1* *nrpd1* 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 expression patterns of the *AtSN1* (B), *ABI5* contains no target sequences for *HaeIII* and was used as a control. doi:10.1371/journal.pgen.1002434.g003

**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 SS genes and the retrotransposon *AtSN1* (B). 

**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 SS genes and the retrotransposon *AtSN1* (B). *ABI5* contains no target sequences for *HaeIII* and was used as a control.

**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 SS genes and the retrotransposon *AtSN1* (B). *ABI5* contains no target sequences for *HaeIII* and was used as a control.

**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 SS genes and the retrotransposon *AtSN1* (B). *ABI5* contains no target sequences for *HaeIII* and was used as a control.

**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 SS genes and the retrotransposon *AtSN1* (B). *ABI5* contains no target sequences for *HaeIII* and was used as a control.

**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 SS genes and the retrotransposon *AtSN1* (B). *ABI5* contains no target sequences for *HaeIII* and was used as a control.

**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 SS genes and the retrotransposon *AtSN1* (B). *ABI5* contains no target sequences for *HaeIII* and was used as a control.

**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 SS genes and the retrotransposon *AtSN1* (B). *ABI5* contains no target sequences for *HaeIII* and was used as a control.

**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 SS genes and the retrotransposon *AtSN1* (B). *ABI5* contains no target sequences for *HaeIII* and was used as a control.

**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 SS genes and the retrotransposon *AtSN1* (B). *ABI5* contains no target sequences for *HaeIII* and was used as a control.
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 S5 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 entertain 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 acidobenzolar 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 WRKY6 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 WRKY6 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 nrd2 and ntpel 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 nrd1 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 nrd2 and ntpel plants, and much less reduced in nrd1 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.](image-url)

**Figure 4.** Comparative immune responses of RdDM mutants to inoculation with *B. cinerea* and *P. cucumerina*. (A) Disease susceptibility of Col-0, nrdp1, nrdp2, npe1, agd4-2/opp11 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 and PR-1 (D) transcript levels following inoculation with *P. cucumerina* in Col-0, nrdp1, npe1 and nrdp2 plants. Data represent the mean ± SD; n = 3 biological replicates. (E) Comparative disease susceptibility of rdr2, rdr1, dml1dm2 and nrdp2 mutants to *P. cucumerina*. ANOVA detected significant differences at the P < 0.05 level. doi:10.1371/journal.pgen.1002434.g004
Data are standardized for Col-0 histone modification levels. (C–D) Histone H3 K9 acetylation (H3K9ac) on the indicated gene promoters. Lys4 trimethylation (H3K4me3) on the indicated gene promoters. (B) leaf samples from Col-0, modifications of PR-1, PDF1.2a and nrpd1, nrpe1, Actin2 and gene promoters as present in nrpd2 plants. (A) Histone H3 H3K4me3 (C) and H3K9ac (D) modifications on PR-1 and PDF1.2a gene promoters in Col-0 and nrpd2 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 ± 5D; n = 3 biological replicates. doi:10.1371/journal.pgen.1002434.g005

decrease in repressive marks in the promoter of the PR-1 gene, as observed in nrpd2 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 WRK76 and WRK73 genes in healthy nrpd2 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 nrpd2 plants, in which these chromatin marks are already set in PR-1, P. cucumerina inoculation further increase 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 nrpd2 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 WRK76 and WRK73 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, nrpd2 plants constitutively carry increased H3K4me3 mark setting in WRK76 and WRK73 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].

**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, nrpd1, nrpe1 and nrpd2 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 Col-0 histone modification levels. (C–D) nrpd2 and nrpe1 Plants Show Enhanced Resistance to PsDC3000

Enhanced activation of SA-mediated defenses is characteristic of plants resistant to biotrophic pathogens, like PsDC3000, 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 nrpd2 and nrpe1 plants suggest these mutants may be altered in the resistance to PsDC3000. Consequently, we addressed Pol IV and Pol V defective mutants in search for defects in the immune response to PsDC3000. We used ago4-2/ocp11 and nrpl plants as controls, both exhibiting heightened PsDC3000 disease susceptibility [25,27]. Interestingly, a significant enhanced disease resistance to PsDC3000 was observed in nrpd2, nrpe1, and in nrpd1 nrpe1 plants, when compared to Col-0 plants (Figure 6). In contrast, statistically significant effects were not observed in nrpd1 plants relative to Col-0 in response to PsDC3000, giving further support to the idea that RNA Pol IV seems not engaged in plant immunity. The observed heightened resistance towards PsDC3000 in nrpd2 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 PsDC3000 is severely compromised. Although there
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 P. DC3000, 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 ndp2 and ndp1 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 ndp2 and ndp1 mutants, despress the priming mechanism for SA-mediated resistance through pertinent chromatin modifications, and renders enhanced resistance to P. DC3000. As a tradeoff, presumably mediated through endogenous antioxidant 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 ndp2 and ndp1 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 research 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 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/ocp11, ndp1, rpm1-1, ndr2, drm1/2 and drm1/drm2 plants were previously described [23]. ndp2-2 (SALK_046208); ndp1-11 (SALK_029919) and ndp1-3 (SALK_128428) were obtained from the Salk Institute Genomic Analysis Laboratory (http://signal.salk.edu/). ndp1 ndp1 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 *ocp1* mutant was backcrossed twice to the *P*_{DC3000}:*GUS line to confirm its recessive inheritance. *ocp1* 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*El, *Aan*I and *Msp*I were performed as described [32]. The relative DNA fragment amounts corresponding to *SUPERMAN*, *5S* and *ASII* were obtained after 30, 25 and 35 respective PCR cycles. For *ABI3*, 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 RT-qPCR 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 antiH3K4m3 (#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 pb located between markers CER45355 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 npd2. The result obtained in our cloning strategy was corroborated with a test of allelism between ocp1 plants and plants carrying the npd2-2 allele. Analysis of GUS expression driven by the Ehp3C gene promoter in 20 F1 plants derived from a cross between homozygous ocp1 plants with homozygous npd2-2 plants or, alternatively, from a reversed cross of npd2-2 plants with ocp1 plants, revealed that all F1 plants showed constitutive GUS expression. Conversely, control crosses between the parental Col-0 plants carrying the Ehp3C::GUS gene construct (line 5.2) with either ocp1 plants or npd2-2 plants revealed no GUS expression in any of the 22 F1 plants analyzed. These complementation analyses indicate that the ocp1 and npd2 are mutant alleles of the same NRPD2 gene. Hence, the ocp1 mutation will be referred also as ocp1/npd2-53.

(TIF)

Figure S4 PR-1 and PDF1.2a genes appear not to be methylated in their DNA sequences. Genomic DNA isolated from Col-0, npd2, npd1 and npd1 plants were digested (+) or not (−) with FidEII, MspII or AvaII 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.

(TIF)

Figure S5 Nucleotide sequence of PR-1 and PDF1.2a 5′ promoter regions. Restriction sites for FidEII (green) and MspII (blue) endonucleases are indicated by color sequences. Red circle marks 46II 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, npd1, npd1, npd1 and npd2 plants quantified by qPCR. Plants were inoculated with P. cucumerina by spraying full expanded leaves with a solution containing 5×10⁶ 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.

(TIF)

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, npd1, npd1 and npd2 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, npd1, npd1 and npd2 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.

(TIF)

Figure S8 Histone modifications on control genes and effect of the priming agent BTH. (A-B) Histone H3K4me3 (A) and H3K9ac (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, npd1 and npd2 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, npd1, npd1 and npd2 plants. Comparative levels of histone H2K9m2 (A) and H3K27me3 (B) modifications on Actin2, PR-1 and PDF1.2a gene promoters in Col-0, npd1, npd1 and npd2 plants. (C) Comparative levels of H2K9m2 and H3K27me3 modifications in Actin2, PR-1 and PDF1.2a gene promoters in Col-0 plants before and after inoculation with P. cucumerina. Data are standarized for non-treated 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 npd2 plants following inoculation with P. cucumerina. Comparative levels of induced modifications in histone H3K4me3 marks on the promoters of WRKY6 and WRKY53 following inoculation of Col-0 and npd2 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)

References

Acknowledgments
We acknowledge E. Luna and J. Ton for their technical assistance and fruitful discussion in relation to ChIP experiments. We also thank J. L. Carrasco for helpful discussions and D. Pascual for technical assistance.

Author Contributions
Conceived and designed the experiments: AL, PV. Performed the experiments: AL VR, JG-AVF. Analyzed the data: AL VR, JG-AVF PV. Wrote the paper: PV.