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1 # The role of DNA (de)methylation in immune responsiveness of Arabidopsis

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31 # Summary

32 DNA methylation is antagonistically controlled by DNA-methyltransferases and DNA-
33 demethylases. The level of DNA methylation controls plant gene expression on a global
34 level. We have examined impacts of global changes in DNA methylation on the Arabidopsis
35 immune system. A range of hypo-methylated mutants displayed enhanced resistance to the
36 biotrophic pathogen *Hyaloperonospora arabidopsidis* (*Hpa*), whereas two hyper-methylated
37 mutants were more susceptible to this pathogen. Subsequent characterization of the hypo-
38 methylated *nrpe1* mutant, which is impaired in RNA-directed DNA methylation, and the
39 hyper-methylated *ros1* mutant, which is affected in DNA demethylation, revealed that their
40 opposite resistance phenotypes are associated with changes in cell wall defence and salicylic
41 acid (SA)-dependent gene expression. Against infection by the necrotrophic pathogen
42 *Plectosphaerella cucumerina*, *nrpe1* showed enhanced susceptibility, which was associated
43 with repressed sensitivity of jasmonic acid (JA)-inducible gene expression. Conversely, *ros1*
44 displayed enhanced resistance to necrotrophic pathogens, which was not associated with
45 increased responsiveness of JA-inducible gene expression. Although *nrpe1* and *ros1* were
46 unaffected in systemic acquired resistance to *Hpa*, they failed to develop transgenerational
47 acquired resistance against this pathogen. Global transcriptome analysis of *nrpe1* and *ros1*
48 at multiple time-points after *Hpa* infection revealed that 49% of the pathogenesis-related
49 transcriptome is influenced by NRPE1- and ROS1-controlled DNA methylation. Of the 166
50 defence-related genes displaying augmented induction in *nrpe1* and repressed induction in
51 *ros1*, only 25 genes were associated with a nearby transposable element and NRPE1- and/or
52 ROS1-controlled DNA methylation. Accordingly, we propose that the majority of NRPE1- and
53 ROS1-dependent defence genes are regulated *in trans* by DNA methylation.

Significance Statement

The recent interest in epigenetic regulation of plant environmental responses prompted us to further explore the regulatory function of DNA (de)methylation in the Arabidopsis immune system. We demonstrate that DNA (de)methylation processes control components of both innate and acquired immunity, and show that half of the pathogenesis-related transcriptome of Arabidopsis is controlled by DNA (de)methylation, of which the majority of defence-associated genes are regulated *in trans*.

Introduction

Plants activate defence mechanisms in response to microbial attack. This innate immune response operates through conserved signalling mechanisms, such as the recognition of microbe- or damage-associated molecular patterns (MAMPs and DAMPs), production of reactive oxygen and nitrogen species, and induction of plant defence hormones, such as salicylic acid (SA) and jasmonic acid (JA; Thomma *et al.*, 2001). Together, these signalling events lead to a coordinated transcriptional response that controls production of long-distance defence signals, pathogenesis-related proteins and antimicrobial metabolites. Expression of innate immunity is often transient, but can lead to a form of acquired immunity that manifests itself as a 'priming' of inducible defences (Prime-A-Plant Group *et al.*, 2006).

Primed plants respond faster and stronger to a secondary defence stimulus, such as pathogen attack, wounding, or treatment with chemical defence elicitors (Conrath, 2006; Frost *et al.*, 2008; Ahmad *et al.*, 2010). Plants can develop different types of defence priming, which are controlled by partially different signalling mechanisms. Some priming responses are triggered by plant-microbe interactions, such as pathogen-induced systemic acquired resistance (SAR; Durrant and Dong, 2004) or root microbe-induced systemic resistance (ISR; Van Wees *et al.*, 2008), whereas others can be induced by application of specific chemicals, such as beta-amino butyric acid (BABA; Luna *et al.*, 2014a). On a temporal scale, there are types of defence priming that are relatively short-lived and disappear over days (Luna *et al.*, 2014b), whereas priming of SA- and JA-dependent

defences are long-lasting (Luna *et al.*, 2014b; Worrall *et al.*, 2012), and can even be transmitted to the next generation, resulting in transgenerational acquired resistance (TAR; Luna *et al.*, 2012; Rasmann *et al.*, 2012; Slaughter *et al.*, 2012). The durable and heritable character of priming of SA-dependent immunity have suggested involvement of epigenetic regulatory mechanisms, such as chromatin remodelling and DNA (de)methylation, which can account for long-lasting changes in defence gene responsiveness (Jaskiewicz *et al.*, 2011; Pastor *et al.*, 2013; Conrath *et al.*, 2015).

DNA methylation is critical for diverse biological processes including gene expression and genome stability. The pattern of DNA methylation is controlled by an equilibrium between methylation and de-methylation activities (Law and Jacobsen, 2010). In plants, cytosine-specific DNA methyltransferases (MTases) are responsible for DNA methylation, which add a methyl group to the fifth carbon of cytosines (Pavlopoulou and Kossida, 2007). *De novo* DNA methylation is controlled by small interfering RNAs (siRNAs). This RNA-directed DNA methylation (RdDM) is mediated by two overlapping pathways, controlling initiation and establishment of DNA methylation in every sequence context (CG, CHG and CHH; H = any nucleotide but G; Matzke and Mosher, 2014). Initiation of *de novo* DNA methylation involves transcription of target sequences by DNA-DEPENDENT RNA POLYMERASE II (Pol II). Some Pol II transcripts can be amplified by RNA-DEPENDENT RNA POLYMERASE 6 (RDR6), which are processed by DICER-LIKE (DCL) 2 and 4 into 21-22 nucleotide (nt) siRNAs. These siRNAs can induce low levels of DNA methylation via DNA-DEPENDENT RNA POLYMERASE V (Pol V) and the DNA methyltransferase DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2; Nuthikattu *et al.*, 2013). This initiation of DNA methylation activates the second RdDM pathway, in which DNA-DEPENDENT RNA POLYMERASE IV (Pol IV) generates single-stranded RNA molecules, which are copied and amplified into double-stranded RNAs by RNA-DEPENDENT RNA POLYMERASE 2 (RDR2), processed into 24 nt siRNAs by DCL3, and loaded onto ARGONAUTE 4 (AGO4). The latter protein enables base-pairing between the siRNA with Pol V-produced RNA transcripts, after which DRM2 is recruited for establishment of DNA methylation (Matzke and Mosher, 2014). DRM2-dependent CHH methylation cannot be maintained in the absence of siRNAs, and requires on-going activity by the Pol IV-RDR2-dependent RdDM pathway (Law and Jacobsen, 2010). However, once established, asymmetrical CHH methylation can spread into

113 symmetrical CG or CHG methylation that is stably preserved through DNA replication by
114 METHYLTRANSFERASE 1 (MET1) and CHROMOMETHYLASE 3 (CMT3), respectively. DNA de-
115 methylation in plants occurs either passively, during DNA replication, or can occur actively
116 through DNA glycosylase/lyase activity (Zhu, 2009). In Arabidopsis, three DNA
117 glycosylases/lyases have been identified: REPRESSOR OF SILENCING 1 (ROS1), DEMETER
118 (DME), DEMETER-LIKE 2 (DML2) and DEMETER-LIKE 3 (DML3), where ROS1 is predominantly
119 responsible for DNA de-methylation in vegetative tissues (Zhu, 2009; Gong and Zhu, 2011;
120 Penterman *et al.*, 2007).

121 Recently, DNA methylation and chromatin modifications have emerged as a potential
122 regulatory mechanism of defence priming. Arabidopsis mutants impeded in DNA
123 methylation have been reported to show increased basal resistance to (hemi)biotrophic
124 pathogens (López *et al.*, 2011; Luna *et al.*, 2012; Yu *et al.*, 2013; Dowen *et al.*, 2012).
125 Specifically, mutants in non-CG methylation, such as the Pol IV/Pol V mutant *nrpd2,* the pol
126 V mutant *nrpe1* and the MTase triple mutant *ddm1 ddm2 cmt3*, display constitutive priming
127 of SA-dependent *PR1* gene expression (López *et al.*, 2011; Luna *et al.*, 2012). Other studies
128 have shown that infection of Arabidopsis by the hemi-biotrophic pathogen *P. syringae* pv.
129 *tomato* DC3000 (*Pst*DC3000) reduces DNA methylation (Dowen *et al.*, 2012; Yu *et al.*, 2013;
130 Pavet *et al.*, 2006), offering a plausible explanation for long-term and transgenerational
131 defence gene priming upon enduring disease stress. However, despite evidence for *cis*-
132 regulation of defence gene priming by histone modifications (Jaskiewicz *et al.*, 2011; López
133 *et al.*, 2011; Luna *et al.*, 2012), the relationship between DNA de-methylation and defence
134 gene priming is less well documented. In a pioneering study, Dowen *et al.* (2012) reported a
135 correlation between pathogen-induced DNA hypo-methylation and pathogen-induced
136 transcription of proximal genes, suggesting that reduced DNA methylation contributes to
137 regulation of pathogen-induced gene expression. However, it remained unclear in how far
138 pathogen-induced DNA hypo-methylation contributes to transcriptional priming of defence
139 genes. Mutants defective in DNA methylation show constitutive priming of *PR1* gene
140 expression (López *et al.*, 2011; Luna *et al.*, 2012), demonstrating that DNA hypo-methylation
141 primes *PR1* gene induction. Interestingly, however, the promoter of *PR1* is normally not
142 methylated. Furthermore, Slaughter *et al.* (2012) found that transgenerational priming of
143 the *PR1* gene in isogenic progeny from BABA-treated plants is not associated with changes

144 in DNA methylation of *PR1*. Together, these results suggest that regulation of defence gene
145 priming by DNA methylation is not solely based on *cis*-acting mechanisms.

146 To date, the exact mechanisms by which DNA methylation controls plant immunity
147 remains unclear. Further investigation is required to establish what types of plant immunity
148 are influenced by DNA methylation, which regulatory mechanisms of DNA (de)methylation
149 control plant immunity, and how DNA methylation regulates defence gene priming on a
150 genome-wide scale. Here, we have addressed these questions through comprehensive
151 phenotypic and transcriptomic analysis of Arabidopsis mutants that are oppositely affected
152 in DNA methylation, but that do not express developmental growth phenotypes. Our study
153 reveals that DNA (de)methylation processes play critical roles in certain types of innate and
154 acquired immunity. We furthermore show that DNA (de)methylation exerts a global
155 influence on the responsiveness of the defence-related transcriptome via predominantly
156 *trans*-regulatory mechanisms.

157 RESULTS

158 Opposite effects of DNA methylation and DNA de-methylation on basal resistance to
159 *Hyaloperonospora arabidopsidis*.

160 To determine impacts of DNA (de)methylation on resistance against biotrophic
161 pathogens, we evaluated a range of Arabidopsis mutants in DNA (de)methylation
162 mechanisms for basal resistance to the obligate biotrophic oomycete *Hyaloperonospora*
163 *arabidopsidis* (*Hpa*). To prevent pleiotropic effects of developmental phenotypes, we only
164 selected mutants with normal (wild-type) growth phenotypes under the conditions of our
165 patho-assays (Fig. 1a). T-DNA insertions in *ros1* (SALK_135293), *ros3* (SALK_022363C) and
166 *cmt3* (SALK_148381) were confirmed by PCR of genomic DNA (Fig. S1a), while
167 transcriptional knock-down of *ROS1* and *NRPE1* gene expression was confirmed by reverse-
168 transcriptase quantitative PCR (RT-qPCR) analysis in *ros1* and *nrpe1*, respectively (Fig. S1b).
169 Three-week-old seedlings were spray-inoculated with *Hpa* conidiospores and collected six
170 days later for trypan blue staining. Microscopic examination of *Hpa* colonization revealed
171 that two mutants defective in RdDM, *nrpe1* (Pontier *et al.*, 2005) and *drd1* (Kanno *et al.*,
172 2004), showed a statistically significant reduction in the number of leaves producing

173 conidiospores and oospores (class III and IV; Fig. 1b). The *cmt3* mutant, which is defective in
174 maintenance of CHG methylation (Lindroth *et al.*, 2001), also showed enhanced resistance
175 in comparison to Col-0, although to a lesser extent than *nrpe1* and *drd1* (Fig. 1b). The *ddm1*
176 mutant, which is affected DNA methylation at all sequence contexts in intergenic regions
177 (Vongs *et al.*, 1993; Jeddeloh *et al.*, 1998; Zemach *et al.*, 2013), was tested in the fourth
178 generation of homozygosity and showed the strongest level of resistance amongst all
179 genotypes tested (Fig. 1b). In contrast to the hypo-methylated mutants, the DNA glycosylase
180 mutant *ros1*, which is hyper-methylated at all DNA sequence contexts (Zhu *et al.*, 2007;
181 Gong *et al.*, 2002), was significantly more susceptible to *Hpa* than Col-0 plants (Fig. 1b). This
182 enhanced susceptibility was similar to that of SA-insensitive *npr1* plants (Cao *et al.*, 1994;
183 Fig. S2a). The *ros3* mutant, which is affected in an RNA-binding protein that interacts with
184 ROS1 (Zheng *et al.*, 2008), also showed enhanced susceptibility to *Hpa* (Fig. 1b), although
185 this phenotype was not consistent over multiple experiments (Fig. S2a). Conversely, all
186 other mutants tested showed similar resistance phenotypes between independent
187 experiments (Fig. S2a). Together, these results point to opposite roles of DNA methylation
188 and DNA de-methylation in basal resistance to *Hpa*. Subsequent experiments focused on the
189 hypo-methylated *nrpe1* mutant and hyper-methylated *ros1* mutant, whose *Hpa* resistance
190 phenotypes were confirmed by qPCR quantification of oomycete biomass (Fig. S2b).

191 **DNA methylation regulates effectiveness of callose deposition and SA-dependent *PR1***
192 **gene induction upon *Hpa* infection.**

193 Reinforcement of the cell wall by deposition of callose-rich papillae contributes to
194 slowing down pathogen colonization at relatively early stages of infection (Voigt, 2014;
195 Ellinger *et al.*, 2013; Luna *et al.*, 2011). To determine the role of DNA (de)methylation in this
196 induced defence layer against *Hpa*, we compared the effectiveness of callose deposition in
197 relation to *Hpa* colonization between the wild-type Col-0, hypo-methylated *nrpe1*, and
198 hyper-methylated *ros1*. To this end, leaves were collected at 48 hours post inoculation (hpi)
199 for calcofluor/analine blue double staining and analysed by epifluorescence microscopy. To
200 assess the defence-contributing activity of callose, all germinating spores were assigned to
201 two mutually exclusive classes: i) spores that were effectively arrested by callose and ii)
202 spores that were not arrested by callose. Using this classification, the *ros1* mutant showed a
203 statistically significant reduction in callose effectiveness in comparison to Col-0 plants (χ^2; *p*

204 < 0.001; Fig. 2a). This indicates that the enhanced DNA methylation in this mutant represses
205 the effectiveness of callose deposition.

206 In addition to cell wall defence, resistance to *Hpa* relies on post-invasive SA-
207 dependent defences (Lawton *et al.*, 1995; Thomma *et al.*, 1998; Ton *et al.*, 2002). To
208 examine whether DNA (de)methylation affects SA-dependent defences, we quantified
209 relative transcript accumulation of the SA-inducible *PR1* marker gene at 48 and 72 hpi with
210 *Hpa*, using RT-qPCR (Fig. 2b). Consistent with previous results (López *et al.*, 2011), the more
211 resistant *nrpe1* mutant displayed a stronger induction of the *PR1* gene, which was
212 statistically significant at 48 hpi with *Hpa* ($p = 0.026$). Conversely, the more susceptible *ros1*
213 mutant showed repressed *PR1* induction at 48 hpi compared to Col-0 ($p = 0.028$). As the
214 *nrpe1* mutant does not show constitutive expression of *PR1* gene, we conclude that the DNA
215 hypo-methylation in *nrpe1* primes SA-dependent defence against *Hpa*, whereas DNA hyper-
216 methylation in *ros1* represses this type of defence.

217 **Role of NRPE1- and ROS1-dependent DNA methylation in basal resistance against**
218 **necrotrophic fungi.**

219 López *et al.* (2011) demonstrated that mutants in RNA-directed DNA methylation
220 display enhanced susceptibility to the necrotrophic fungus *Plectosphaerella cucumerina*,
221 which is associated with repressed responsiveness of JA-dependent defence genes. To
222 examine whether the increased level of DNA methylation in *ros1* has an opposite effect on
223 basal resistance to necrotrophic fungi, we compared 4.5-week Col-0, *nrpe1* and *ros1* for
224 basal resistance against the Ascomycete fungus *P. cucumerina*. Basal resistance was
225 quantified by necrotic lesion diameter, which is a reliable parameter to assess necrotrophic
226 colonization by this fungus after droplet inoculation (Ton and Mauch-Mani, 2004; Pétriacq
227 *et al*. 2016). At six days post inoculation, the *nrpe1* mutant developed larger lesions than
228 Col-0 (Fig. 3a and S3a), confirming previous results by López *et al.* (2011). Conversely, *ros1*
229 plants displayed significantly smaller necrotic lesions than Col-0 (Fig. 3a and S3a), indicating
230 enhanced basal resistance to *P. cucumerina*. The disease phenotypes of *nrpe1* and *ros1*
231 were validated by qPCR quantification of fungal DNA (Fig. S3b), confirming that both
232 mutants are oppositely affected in disease resistance to *P. cucumerina*. Furthermore, similar
233 results were obtained by quantifying microscopic colonization by a different necrotrophic

234 fungus, *A. brassicicola* (Fig. S3c). It can thus be concluded that DNA hyper-methylation in the
235 *ros1* mutant boosts basal disease resistance to necrotrophic fungi.

236 Basal resistance against *P. cucumerina* and *A. brassicicola* partially relies on JA-
237 dependent defences (Thomma *et al.*, 1998; Thomma *et al.*, 1999; Ton and Mauch-Mani,
238 2004). To investigate whether the enhanced resistance of *ros1* is based on increased
239 sensitivity of JA-inducible defence gene expression, we analysed plants for *PDF1.2* and *VSP2*
240 expression at 4, 8 and 24 hours after spraying of the leaves with 50 mM JA. Consistent with
241 the earlier notion that mutations in RdDM repress defence gene responsiveness to JA (López
242 *et al.*, 2011), the *nrpe1* mutant showed significantly lower and/or delayed JA induction of
243 both genes in comparison to wild-type plants (Fig. 3b). Surprisingly, despite the fact that the
244 *ros1* mutant was more resistant to both *P. cucumerina* and *A. brassicicola* (Fig. 3a and S3), it
245 also showed repressed induction of *PDF1.2* and *VSP2* by JA, which was statistically
246 significant at 4 hours post treatment with JA (Fig. 3b). Thus, increased resistance of *ros1* to
247 necrotrophic fungi is not based on primed responsiveness of JA-inducible gene expression.

248 **ROS1-dependent de-methylation does not play a role in within-generation systemic**
249 **acquired resistance (SAR), but is required for transgenerational acquired resistance (TAR).**

250 SAR is a pathogen-inducible form of acquired immunity that is expressed systemically
251 (Durrant and Dong, 2004). Recently, it was shown that pathogen-induced acquired
252 immunity can be transmitted to following generations in Arabidopsis (TAR; Slaughter *et al.*,
253 2012; Luna *et al.*, 2012). This resistance could be mimicked by genetic mutations in the DNA
254 methylation machinery (Luna *et al.*, 2012; Luna and Ton, 2012), suggesting that DNA de-
255 methylation is responsible for the generation and/or transmission of the response. To
256 investigate the role of NRPE1- and ROS1-dependent DNA (de)methylation during within-
257 generation SAR, 3 lower leaves of 4.5-week-old plants were infiltrated with avirulent
258 *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) carrying the avirulence gene *avrRpm1*.
259 Three days after SAR induction, systemic leaves were challenged with *Hpa*. As expected,
260 SAR-treated Col-0 plants displayed a statistically significant reduction in *Hpa* colonization
261 compared to control-treated plants (Fig. 4a). SAR in *Pst avrRpm1*-infected *nrpe1* plants was
262 borderline statistically significant (p = 0.072), probably due to the masking effect of this
263 mutant’s elevated basal resistance (Fig. 1a). Notably, the *ros1* mutant was fully capable of

264 mounting a statistically significant SAR response against *Hpa* infection, indicating that ROS1-
265 dependent DNA de-methylation does not play a role in within-generation SAR.

266 We then investigated the role of NRPE1- and ROS1-dependent DNA (de)methylation in
267 TAR. To this end, Col-0, *nrpe1* and *ros1* were inoculated three times with increasing doses of
268 virulent *Pst* and allowed to set seed. Three-week-old F1 seedlings from *Pst*- (P1) and mock-
269 treated (C1) parent plants were tested for resistance against *Hpa* (Fig. 4b). P1 progeny from
270 *Pst*-infected Col-0 showed increased basal resistance in comparison to C1 progeny from
271 mock-treated Col-0 (p = 0.017). By contrast, there was no statistically significant difference
272 in *Hpa* resistance between P1 and C1 progenies of *nrpe1* (p = 0.538). Levels of resistance in
273 C1 progeny from *nrpe1* were statistically similar to that of P1 progeny from Col-0 (p =
274 0.148), which is consistent with the notion that reduced DNA methylation mimics TAR (Luna
275 *et al.*, 2012; Luna and Ton, 2012). Like the *nrpe1* mutant, P1 and C1 progenies from *ros1* did
276 not show a difference in *Hpa* resistance (p = 0.697). However, C1 progeny from *ros1*
277 displayed enhanced susceptibility in comparison to both P1 and C1 progeny of Col-0 (p <
278 0.001), indicating that the lack of TAR in *ros1* is due to this mutant's inability to transmit
279 and/or express transgenerational acquired immunity.

280 **NRPE1- and ROS1-dependent DNA (de-)methylation influences nearly half of the**
281 **pathogenesis-related transcriptome**

282 DNA methylation patterns are known to affect gene expression (Law and Jacobsen,
283 2010). Since *nrpe1* and *ros1* are antagonistically affected in both DNA methylation and
284 responsiveness of *PR1* expression during *Hpa* infection (Fig. 2b), we further explored global
285 impacts of both mutations on the pathogenesis-related transcriptome of *Hpa*-infected
286 Arabidopsis, using Affymetrix Gene 1.0 ST arrays. To account for transcriptomic responses
287 during expression of penetration defence (48 hpi) and post-invasive defence during hyphal
288 colonization (72 hpi), we isolated RNA from Col-0, *nrpe1* and *ros1* at 48 and 72 hpi,
289 respectively. First, we assessed the global impacts of mutations in *NRPE1* and *ROS1* by
290 determining the number of differentially expressed genes between each mutant and Col-0
291 at any time-point and condition ($q \leq 0.01$). This analysis revealed that 1975 and 1150 genes
292 are differentially expressed in the *ros1* and *nrpe1*, respectively. By comparing these gene
293 sets with the 967 genes that are differentially expressed in Col-0 between mock and *Hpa*-
294 inoculated leaf samples (i.e. the *Hpa*-responsive genes), we found that 49% of all *Hpa*-

295 responsive genes are affected by mutations in *NRPE1* and/or *ROS1* (477/967 = 49%; Fig. 5a).
296 Hence, nearly half of the pathogenesis-related transcriptome of Arabidopsis is controlled
297 directly or indirectly by NRPE1- and ROS1-dependent DNA (de-)methylation.

298 **Defence-related genes that are primed by DNA hypo-methylation and/or repressed by**
299 **DNA hyper-methylation are strongly enriched with SA-dependent defence genes.**

300 The resistance phenotypes of *nrpe1* and *ros1* to *Hpa* can be caused by constant
301 changes in defence gene expression, changes in defence gene responsiveness to pathogen
302 attack, or a combination of both. Comparison of mock-inoculated *nrpe1* and *ros1* relative to
303 Col-0 identified 1215 genes with enhanced expression in *nrpe1* and/or repressed expression
304 in *ros1* at 48 and/or 72 hpi (Fig. 5b). Of these, 256 genes were also *Hpa*-inducible in Col-0
305 plants (Fig. 5b). We then searched for defence-related genes with increased *Hpa*
306 responsiveness in the more resistant *nrpe1* mutant (i.e. 'primed') and/or repressed
307 responsiveness in the more susceptible *ros1* mutant. To this end, the group of 700 *Hpa*-
308 inducible genes (shown in green; Fig. 5b) were filtered ***i***) for a statistically significant
309 difference between *Hpa*-inoculated *nrpe1* and *ros1* (48 and/or 72 hpi; $q \leq 0.01$) and ***ii***) for a
310 statistically significant difference between at least one of the *Hpa*-inoculated mutants and
311 *Hpa*-inoculated Col-0 (48 and/or 72 hpi; $q \leq 0.01$). As evidenced by a heat map projection of
312 the gene expression profiles (Fig. 5c, Fig. S4), this filter identified 166 defence-related genes
313 with primed *Hpa* responsiveness in *nrpe1* and/or repressed *Hpa* responsiveness in *ros1*
314 (supplemental data file 1). Of these 166 genes, 46 were altered in *Hpa* responsiveness only,
315 whereas 120 showed a combination of differential expression between mock-treated plants
316 and differential responsiveness to *Hpa* (Fig. 5b). Interestingly, in comparison to all other
317 gene sets, the genes displaying differential *Hpa* responsiveness showed the highest
318 proportion of gene ontology (GO) terms 'Systemic Acquired Resistance' and 'Salicylic Acid
319 Biosynthetic Process' (Fig. 5b). This outcome supports our notion that the resistance
320 phenotypes of *nrpe1* and *ros1* are predominantly based on changes in defence gene
321 responsiveness, rather than changes in constitutive gene expression.

322 **The majority of ROS1- and/or NRPE1-controlled defence genes is not associated with**
323 **ROS1- and/or NRPE1-dependent DNA methylation in their promoter regions.**

324 In subsequent analyses, we focused on the selection of 166 defence-related genes
325 that are primed by DNA hypo-methylation and/or repressed by DNA hyper-methylation.
326 First, we determined reproducibility of these microarray results by profiling transcript
327 accumulation of 4 randomly selected genes in an independent experiment, using RT-qPCR.
328 As is shown in Figure S5, all 4 genes showed reproducible expression profiles to the
329 microarray experiment. Next, we examined whether the selection of 166 defence-related
330 genes are regulated directly (*in cis*) or indirectly (*in trans*) by NRPE1 and ROS1-dependent
331 DNA (de-)methylation. Because NRPE1 and ROS1 are known to control DNA methylation at
332 or around transposable elements (TEs; Law and Jacobsen, 2010), we investigated whether
333 the selection of 166 genes are enriched with nearby TEs. Using the TAIR10 annotation for
334 known TEs, the 166 genes showed a weak enrichment of TEs within 2 kb upstream of their
335 transcriptional start, relative to a background of all other Arabidopsis genes on the
336 microarray (Fig. 6a). By contrast, no TE enrichment was found for genic or 2 kb-downstream
337 regions of the 166 genes (Fig. 6a). We then examined whether the TE-enriched promoter
338 regions are subject to NRPE1- or ROS1-dependent DNA (de-)methylation. To this end, we
339 used publically available C-methylomes of *nrpe1* and *ros1* (Qian *et al.*, 2012; Stroud *et al.*,
340 2013) to create a combined C-methylome of sufficient sequence coverage (≥5 reads,
341 8363349 positions), before determining which of these positions are hypo-methylated in
342 *nrpe1* and/or hyper-methylated in *ros1*. From this list, we selected genes with at least 3
343 differentially methylated cytosines at the same context (CG, CHG or CHH) within their 2kb
344 promoter region. Although the promoters of 166 defence-related genes were marginally
345 enriched for NRPE1-dependent CHG and/or CHH methylation (Fig. 6b), this enrichment was
346 not statistically significant in comparison to all other genes on the microarray (χ^2 tests; p =
347 0.3150 and 0.2837, respectively). Furthermore, the 166 gene promoters were not enriched
348 for ROS1-dependent hypo-methylation. Together, this indicates that the majority of 166
349 defence genes are indirectly (*trans*-)regulated by NRPE1- and/or ROS1-dependent DNA
350 (de)methylation.

351 **Selection of 25 defence-regulatory genes that are *cis*-regulated by NRPE1- and/or ROS1-**
352 **dependent DNA (de-)methylation.**

353 To search for defence regulatory genes that are *cis*-regulated by NRPE1-/ROS1-
354 dependent DNA (de)methylation, we analysed the 2 kb gene promoter regions from the 166
355 NRPE1-/ROS1-controlled defence genes for *i*) TE presence and *ii*) occurrence of > 3 hypo-
356 methylated cytosines in *nrpe1* and/or hyper-methylated cytosines in *ros1*. A total of 25 gene
357 promoters met these criteria (Fig. 6c). To illustrate the DNA (de)methylation activities in
358 these promoters, Figure S6 plots the positions of TEs and differentially methylated cytosines
359 in *nrpe1* and *ros1*. Furthermore, using data from a recent ChIP-sequencing study with a
360 polyclonal antibody against native NRPE1 protein (Zhong *et al.*, 2015), we show that physical
361 binding of NRPE1 largely coincides with hypo-methylated regions in the *nrpe1* mutant,
362 thereby confirming localised activity by the Pol V complex. The group of 25 *cis*-regulated
363 genes includes genes with annotated defence regulatory activity, such as genes encoding for
364 pattern recognition receptors (PRRs), leucine-rich repeat (LRR) resistance proteins, CYP81D1
365 and DOWNY MILDEW RESISTANT 6 (Table S1), each of which has the potential to control a
366 larger set of defence genes.

367 ## DISCUSSION

368 **Role of DNA (de)methylation processes in basal resistance.**

369 Our study has shown that DNA methylation and de-methylation activities
370 antagonistically regulate basal resistance of Arabidopsis. While previous studies reported
371 similar effects by mutations in DNA methylation (Dowen *et al.*, 2012; Yu *et al.*, 2013; López
372 *et al.*, 2011; Luna *et al.*, 2012; Le *et al.*, 2014), we provide a comprehensive comparison of
373 the effects of hypo- and hyper-methylated DNA on basal resistance against both biotrophic
374 (*H. arabidopsidis*) and necrotrophic pathogens (*P. cucumerina* and *A. brassicicola*).
375 Furthermore, we show that the enhanced resistance in the hypo-methylated *nrpe1* mutant
376 and the enhanced susceptibility in the hyper-methylated *ros1* mutant were linked to
377 opposite changes in the effectiveness of callose deposition and the speed and intensity of
378 SA-dependent *PR1* gene induction. Hence, DNA (de)methylation determines the
379 effectiveness of multiple layers of basal defence against biotrophic pathogens. Conversely,

380 the enhanced susceptibility of *nrpe1* to necrotrophic *P. cucumerina* was associated with
381 reduced responsiveness of JA-induced *PDF1.2* and *VSP2* expression, confirming the earlier
382 notion that NRPE1-dependent RdDM suppresses JA-dependent resistance via the
383 antagonistic action of SA on JA responses (López *et al.*, 2011). Surprisingly, *ros1* also
384 displayed reduced responsiveness of JA-induced *PDF1.2* and *VSP2* expression, despite the
385 fact that this mutant was more resistant to both *P. cucumerina* and *A. brassicicola*. This
386 suggests that DNA hyper-methylation in *ros1* boosts basal resistance against necrotrophic
387 pathogens independently of JA-dependent defences. The unexpected finding that *nrpe1* and
388 *ros1* are both affected in JA responsiveness might be explained by the recent discovery that
389 RdDM regulates *ROS1* expression positively through DNA methylation of a target sequence
390 between the TE-containing promoter and 5' UTR of *ROS1* (Williams *et al.*, 2015; Lei *et al.*,
391 2015). As a consequence, *ROS1* is scarcely expressed in RdDM mutant backgrounds (Li *et al.*,
392 2012), explaining why mutations in both RdDM and *ROS1* can cause similar phenotypes. For
393 instance, (Le *et al.*, 2014) recently discovered that both *nrpe1* and the *rdd* (*ros1 dml2 dml3*)
394 triple demethylase mutant have enhanced susceptible to *Fusarium oxysporum* due to lack of
395 RdDM-induced DNA de-methylation at corresponding defence genes. By contrast, our
396 experiments show that *nrpe1* and *ros1* display opposite resistance phenotypes to *H.*
397 *arabidopsidis* and *P. cucumerina* (Figs. 1, 3a and S3). Hence, basal resistance against *H.*
398 *arabidopsidis* and *P. cucumerina* is not controlled by RdDM-induced ROS1 activity, but
399 rather by antagonistic activities of RdDM and ROS1-dependent DNA de-methylation on
400 corresponding defence genes.

401 **Role of DNA methylation in acquired resistance.**

402 Transgenerational acquired resistance (TAR) in progeny from *Pst*-infected Arabidopsis
403 manifests itself as priming of SA-dependent defences, which can be mimicked by mutations
404 in the DNA methylation machinery (Luna *et al.*, 2012). Our current study has expanded
405 these initial observations by exploring the function of DNA (de)methylation in both SAR and
406 TAR. The *nrpe1* mutant showed weakened within-generation SAR against *Hpa*. However,
407 since *nrpe1* expresses enhanced basal resistance to *Hpa* (Fig. 1a), we propose that this
408 mutant's SAR response was partially masked by its elevated level of basal resistance. The
409 *ros1* mutant, on the other hand, was fully capable of expressing SAR (Fig. 4a). Hence, DNA
410 (de)methylation does not play a major role in within-generation SAR. By contrast, P1

411 progenies from *Pst*-infected mutant plants failed to show increased *Hpa* resistance in
412 comparison to corresponding C1 progenies, indicating that TAR requires regulation by intact
413 *NRPE1* and *ROS1* genes. The resistance in C1 progeny from *nrpe1* was statistically similar to
414 that of P1 progeny from wild-type plants (Fig. 4b), thereby confirming our previous
415 conclusion that hypo-methylation mimics TAR (Luna *et al.*, 2012; Luna and Ton, 2012).
416 Conversely, levels of susceptibility in P1 and C1 progenies of the *ros1* mutant were
417 significantly higher than that of C1 progeny from the wild-type. Since *ros1* is not impaired in
418 within-generation SAR, we propose that Arabidopsis employs ROS1-dependent de-
419 methylation for the imprinting of TAR in the parental generation.

420 The exact mechanisms by which acquired immunity is transmitted from infected
421 parental plants to P1 progeny remains unknown. Yu *et al.* (2013) showed that *Pst* infection
422 of Arabidopsis represses RdDM genes, such as *AGO4*, *AGO6*, *NRPD2*, and *RDR1*, which offers
423 a plausible explanation as to why *Pst* induces DNA hypo-methylation in Arabidopsis (Pavet
424 *et al.*, 2006; Dowen *et al.*, 2012). It is tempting to speculate that *Pst*-induced repression of
425 RdDM acts in concert with ROS1, in order to mediate heritable hypo-methylation of DNA.
426 Comprehensive bisulfite-sequence analysis of both vegetative tissues and reproductive
427 tissues from healthy and *Pst* DC3000-infected plants, as well as their resulting progenies,
428 will be necessary to resolve the exact role of DNA (de)methylation during the imprinting,
429 meiotic transmission and expression of TAR.

430 **Global regulation of defence gene expression by DNA (de)methylation.**

431 The combination of post-translational histone modifications, histone variants and DNA
432 methylation determines the level of compaction of chromatin (Saze *et al.*, 2012; Richards,
433 2006). This epigenetic regulation is especially important in genomic regions that are
434 enriched with repetitive sequences and transposable elements (TE) to ensure genome
435 stability. The chromatin state can also influence basal and pathogen-inducible expression of
436 defence genes by determining accessibility of the transcriptional machinery, such as
437 transcription factors and DNA dependent RNA polymerase II (Pol II). To establish global
438 impacts of DNA (de)methylation on defence gene expression, we performed whole-genome
439 transcriptome analysis of the DNA (de)methylation mutants at different time-points after
440 *Hpa* inoculation. Comparison between differentially expressed genes in *Hpa*-inoculated
441 wild-type plants against all differentially expressed in *nrpe1* and/or *ros1* revealed that nearly

442 half of all *Hpa*-responsive genes (49%) are under direct or indirect control by DNA
443 (de)methylation processes (Fig. 5a). This outcome shows that the pathogenesis-related
444 transcriptome of Arabidopsis is under substantial and global regulation by DNA
445 (de)methylation. Next, we focused on the patterns of gene expression that could explain the
446 resistance phenotypes of *nrpe1* and *ros1* to *Hpa*. We reported that the 166 genes with
447 increased *Hpa* responsiveness in the more resistant *nrpe1* mutant and/or decreased *Hpa*
448 responsiveness in the more susceptible *ros1* mutant were more strongly enriched with GO
449 terms 'Systemic Acquired Resistance' and 'Salicylic Acid Biosynthetic Process' than the 136
450 *Hpa*-inducible genes, whose expression was only altered in mock-treated *nrpe1* and *ros1*
451 (Fig. 5b). This indicates that the resistance phenotypes of *nrpe1* and *ros1* are predominantly
452 caused by changes in responsiveness of defence genes. We therefore conclude that DNA
453 (de)methylation regulates transcriptional responsiveness of SA-dependent defence genes on
454 a genome-wide scale.

455 DNA (de)methylation could regulate defence gene responsiveness via *cis*- and *trans*-
456 regulatory mechanisms (Fig. 7). To explore a possible *cis*-regulatory role of NRPE1/ROS1-
457 dependent DNA (de)methylation, we examined TE occurrence and NRPE1-binding
458 sequences in the selection of 166 defence-related gene promoters that are antagonistically
459 controlled by *NRPE1* and *ROS1*. Surprisingly, we only detected relatively weak over-
460 representation of TEs in the 166 gene promoters compared to the genomic background
461 average (Fig. 6a), even though RdDM and ROS1 are both known to act on TE-containing
462 intergenic sequences (Chan *et al.*, 2005). Moreover, the 166 gene promoters were not
463 statistically enriched with sequences that are de-methylated in *nrpe1* and/or hyper-
464 methylated in *ros1* (Fig. 6b). We therefore conclude that the influence of NRPE1/ROS1-
465 dependent (de)methylation on defence gene responsiveness is predominantly enacted by
466 *trans* regulatory mechanisms.

467 There are different mechanisms by which DNA (de)methylation can regulate defence
468 gene induction *in trans* (Fig. 7). For instance a small number of signalling genes that are
469 directly *cis*-regulated by DNA (de)methylation can control induction of a much larger group
470 of defence genes. In fact, of the 166 genes with altered *Hpa* responsiveness, we identified
471 only 25 genes whose promoters contain a TE and show evidence for NRPE1-/ROS1-
472 dependent DNA (de)methylation and/or binding to the NRPE1 unit of Pol V (Figs. 6c and S6).

473 Since their responsiveness to *Hpa* is influenced by mutations in *NRPE1* and *ROS1* (Fig. 5c), it
474 is plausible that these 25 genes are *cis*-regulated by NRPE1-/ROS1-dependent DNA
475 (de)methylation. This group includes genes with annotated regulatory activity in plant
476 defence (Fig. S6; Table S1), such as PRR and R proteins, which can initiate downstream
477 defence pathways and activate a wider range of defence genes. An alternative mechanism
478 by which DNA (de)methylation can *trans*-regulate defence genes is through influencing
479 chromatin density at distant genome loci. Like DNA methylation, chromatin density has
480 been reported to have a long-lasting impacts on gene expression and responsiveness
481 (Vaillant and Paszkowski, 2007). Furthermore, both mechanisms are highly co-regulated,
482 since Arabidopsis mutants affecting in DNA methylation are also altered in post-
483 translational modifications of histones that mark chromatin density (Law and Jacobsen,
484 2010). Previous studies have shown that priming of defence genes is associated with post-
485 translational modifications of histone proteins in their promoter regions, such as triple-
486 methylation of lysine 4 and acetylation of lysine 9 in the tail of histone H3 (Jaskiewicz *et al.*,
487 2011; López *et al.*, 2011; Luna *et al.*, 2012). Hence, chromatin structure can act as a *cis*-
488 regulatory mechanism of defence gene priming. Interestingly, however, some defence gene
489 promoters are subject to histone modifications in primed plants, even when these regions
490 are not methylated at the DNA level (Slaughter *et al.*, 2012; López *et al.*, 2011). Under these
491 premises, it is tempting to speculate that the Pol V-associated chromatin-remodelling
492 complex (Zhong *et al.*, 2012; Liu *et al.*, 2014; Zhu *et al.*, 2013) can increase chromatin
493 density at multiple chromosomal positions via cross-linking distant loci (Fig. 7). In this
494 scenario, it is possible that Pol V-dependent DNA methylation at specific TEs influences
495 chromatin structure at genomically distant defence genes. This mechanism would enable
496 *trans*-regulation of defence genes by RdDM, and explain earlier reports that TAR is
497 associated with histone modifications at defence genes that are not associated with nearby
498 DNA methylation (Luna *et al.*, 2012; Slaughter *et al.*, 2012). Chromatin immuno-precipitation
499 of NRPE1 followed by chromosome conformation capture analysis ('ChIP-loop') and next
500 generation sequencing is one future approach which could resolve whether the Pol V
501 complex indeed cross-links *cis*-methylated DNA regions with *trans*-regulated defence genes
502 during pathogen attack.

503 # EXPERIMENTAL PROCEDURES

504 **Plant material**

505 Seeds of *ros1-4* (SALK_135293), *ros3* (SALK_022363C) and *cmt3-11* (SALK_148381)
506 were obtained from the Col-0 Salk T-DNA collection (Alonso *et al.*, 2003) and verified to be
507 homozygous for the T-DNA insertion (Fig. S1a); *nrpe1-11* (SALK_029919) and *drd1-6* (Kanno
508 *et al.*, 2004) were kindly provided by P. Vera and D. C. Baulcombe respectively. Knock-down
509 of ROS1 and NRPE1 gene expression was confirmed by RT-qPCR (Fig. S1b). Seeds of the F4 of
510 *ddm1-2* (Vongs *et al.*, 1993) were kindly provided by V. Colot. Growth conditions are
511 detailed in the Supplemental Methods.

512 **Basal resistance assays**

513 To quantify basal resistance against *H. arabidopsidis* (isolate WACO9), seedlings were
514 grown for three weeks before spray inoculation with a suspension containing 10^5
515 conidiospores ml^{-1}, as described in the Supplemental Methods. For basal resistance assays
516 to *P. cucumerina* and *A. brassicicola*, fungi was grown in darkness at room temperature on
517 full-strength PDA plates and half-strength PDA agar plates containing 20 g l^{-1} sucrose and
518 30 g l^{-1} $CaCO_3$, respectively. Fungal spores were collected by scraping water-flooded plates.
519 Plants (4.5 week-old) were inoculated by applying 6 µl-droplets (10^6 spores ml^{-1}) onto four
520 leaves of similar physiological age per plant. Inoculated plants were kept at 100% humidity
521 until scoring disease or sample collection (as described in the Supplemental Methods). To
522 investigate defence responsiveness to JA, 4.5-week-old Arabidopsis plants were sprayed
523 with 0.016% v.v ethanol and 0.01% v.v Silwet L-77 (Vac-In-Stuff; catalogue number VIS-30) in
524 dH_2O with (treatment) or without (mock) 0.1 mM (±)-jasmonic acid (JA; Sigma; catalogue
525 number J2500).

526 **SAR assays**

527 SAR was induced in 4.5-week old plants, using avirulent *Pseudomonas syringae* pv.
528 *tomato* DC3000, carrying *avrRpm1*. Four lower leaves per plant were pressure infiltrated
529 using with 10 mM $MgSO_4$ with or without (mock) 10^7 cfu ml^{-1} *Pst*DC3000(*avrRpm1*), using a
530 needleless syringe. Plants were challenged three days later by spray inoculation with *H.*
531 *arabidopsidis* (10^5 conidiospores ml^{-1}). At 5 dpi, distal leaves from infiltrated leaves were

532 collected for trypan blue staining. For TAR assays, plants were grown under long day
533 conditions (16h light/8h dark, 21°C, 80% relative humidity, light intensity 100-
534 140 µmol s^{-1} m^{-2}) and spray-inoculated at 21 days, 28 days and 35 days after germination
535 with 10 mM $MgSO_4$ containing 10^8 cfu ml^{-1} *Pst* DC3000 (P0; diseased) or 10 mM $MgSO_4$ (C0;
536 mock). Progeny from P0 and C0 plants (P1 and C1) were grown for three weeks and
537 challenged by spray-inoculating *H. arabidopsidis* (10^5 conidiospores ml^{-1}). At 6 dpi, leaves
538 were collected for trypan blue staining. All staining procedures are detailed in the
539 Supplemental Methods. Bacteria were grown overnight at 28°C in liquid KB or LB medium
540 containing 50 mg l^{-1} rifampicin and, for *Pst*DC3000(*avrRpm1*), 50 mg l^{-1} kanamycin.

541 **RNA extraction and RT-PCR**

542 Samples were snap-frozen in liquid nitrogen and ground to a fine powder. RNA was
543 extracted using modified guanidinium thiocyanate-phenol-chloroform extraction methods,
544 as detailed in the Supplemental Methods. To remove residual DNA, samples were treated
545 with DNAse I (Promega) for 30 min at 37°C. First strand cDNA synthesis and RT-PCR analysis
546 were performed as described in the Supplemental Methods.

547 **Microarray analysis.**

548 Col-0, *nrpe1* and *ros1* plants were grown as described for *Hpa* basal resistance assays.
549 Samples were taken at 48 and 72 hpi by pooling leaves from 10 to 12 seedlings per
550 treatment from the same pot. Four biologically replicated samples were used to represent
551 each treatment/genotype combination. RNA was extracted, as described above, and
552 analysed using Affymetrix Arabidopsis Gene 1.0 ST arrays, according to manufacturer's
553 instructions. Details of array processing and statistical analysis using R-packages oligo
554 (Carvalho and Irizarry, 2010) and Limma (Smyth, 2004; Ritchie *et al.*, 2015) are included in
555 the Supplemental Methods. Data have been deposited at EMBL (E-MTAB-3963). GO-term
556 overrepresentation analysis was performed using Gorilla (Eden *et al.*, 2009).

557 **Analysis of sequencing data.**

558 Bisulfite sequencing reads from two previous studies (Qian *et al.*, 2012; Stroud *et al.*,
559 2013) were downloaded from NCBI's SRA (accession numbers SRR353936-SRR353939,
560 SRR534177, SRR534182 and SRR534193). Processing of raw sequence data is detailed in the

561 Supplemental Methods. ChIP-seq data from (Zhong *et al.*, 2015) were downloaded from
562 NCBI's GEO (series number GSE61192).

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570 ## SHORT LEGENDS FOR SUPPORTING INFORMATION

571 **Figure S1: Genetic characterization of selected mutants.**

572 **Figure S2: Repeats of pathogenicity assays to determine basal resistance in DNA**
573 **(de)methylation mutants against *H. arabidopsidis*.**

574 **Figure S3: Basal resistance phenotypes of Col-0, *nrpe1* and *ros1* to the necrotrophic fungi**
575 ***Plectosphaerella cucumerina* and *Alternaria brassicicola*.**

576 **Figure S4: Transcript levels of 166 *Hpa*-inducible genes with augmented induction in *nrpe1***
577 **and/or repressed induction in *ros1*.**

578 **Figure S5: Micro-array validation of transcriptional profiles from an independent *Hpa***
579 **experiment.**

580 **Figure S6: Schematic overview of the 2 Kb promoter regions of 25 defence-related genes**
581 **that are *cis*-regulated by DNA (de)methylation.**

582 **Table S1: Annotations of 25 candidate defence-regulatory genes that are *cis*-regulated by**
583 **NRPE1- and/or ROS1-dependent DNA (de-)methylation.**

584 **Supplemental data file 1: Gene transcripts showing statistically significant differences in**
585 **normalized hybridization signal (Affymetrix Arabidopsis Gene 1.0 ST arrays) between**
586 **Col-0, *nrpe1*, and *ros1* at 48 and 72 hours after mock or *Hpa* inoculation.**

587 **Supplemental methods file: Details about plant growth conditions, basal resistance**
588 **assays, staining procedures & resistance classifications, nucleic acid extractions & qPCR,**
589 **primer sequences, microarray analysis, and analysis of sequencing data.**

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770 ## FIGURE LEGENDS

771 **Figure 1: Basal resistance to *Hyaloperonospora arabidosidis* in *Arabidopsis thaliana***
772 **mutants that are affected in DNA (de)methylation.**

773 **(a)** Growth phenotypes of tested Arabidopsis genotypes before infection. Genotypes
774 correspond to those of the bars in (b) below each picture.

775 **(b)** Levels of basal resistance to *H. arabidopsidis* (*Hpa*) in DNA methylation mutants (*ddm1*
776 F4, *nrpe1*, *drd1*, and *cmt3*) and DNA de-methylation mutants (*ros3* and *ros1*). Six days after
777 spray inoculation of 3-week-old plants (10^5 conidiospores ml^{-1}), 200 leaves from 35 plants
778 per genotype were microscopically assigned to different *Hpa* colonization classes following
779 trypan blue staining. Shown are relative numbers of leaves assigned to different
780 colonization classes. Inserts show representative levels of classes. Asterisks indicate
781 statistically significant differences in class distributions compared to Col-0 ($\chi 2$ test; $p < 0.05$).

782 **Figure 2: Effectiveness and responsiveness of inducible defences against *H. arabidopsidis***
783 **in *nrpe1*, *ros1* and Col-0.**

784 **(a)** Effectiveness of callose deposition against *Hpa* infection at 48 hours after inoculation of
785 3-week-old plants (10^5 conidiospores ml^{-1}). Defence phenotypes were determined by epi-
786 fluorescence microscopy in at least 10 leaves per genotype, and assigned to 2 different
787 classes based on presence or absence of successful penetration into the mesophyll by *Hpa*.
788 Inserts on the right show an example of each class. Germinating *Hpa* spores appear in blue
789 (calcofluor white-stained) and callose deposition is indicated by the presence of yellow
790 staining (analine blue-stained). Asterisks indicate statistically significant differences in class
791 distributions compared to Col-0 ($\chi 2$ test; $p < 0.05$). Scale bars = 100 µm.

792 **(b)** RT-qPCR quantification of *PR1* gene expression in Col-0, *nrpe1* and *ros1* at 48 and 72
793 hours after inoculation with *Hpa* or mock treatment. Data represent mean values of relative
794 expression (± SEM) from 4 biologically replicated samples. Asterisks indicate statistically
795 significant differences in comparison to Col-0 (Student's *t* test; $p < 0.05$).

796 **Figure 3: Basal resistance to *Plectosphaerella cucumerina* and JA-induced gene expression**
797 **in *nrpe1*, *ros1* and Col-0.**

798 **(a)** Levels of basal resistance to *P. cucumerina*. Shown are mean lesion diameters (± SEM; 27
799 plants) at six days after droplet inoculation of 4.5-week-old plants. Asterisks indicate
800 statistically significant differences between Col-0 and mutant plants (Student's *t* test; $p <$
801 0.05).

802 **(b)** RT-qPCR quantification of *PDF1.2* and *VSP2* gene expression in Col-0, *nrpe1* and *ros1* at
803 0, 4, 8 and 24 hours after spraying with 0.1 mM jasmonic acid (JA). Data represent mean
804 values of relative expression (± SEM; n= 3). Asterisks indicate statistically significant
805 differences in comparison to Col-0 samples (Student's *t* test; $p < 0.05$).

806 **Figure 4: Systemic acquired resistance (SAR) and transgenerational acquired resistance**
807 **(TAR) in Col-0, *nrpe1* and *ros1*.**

808 **(a)** Quantification of within-generation SAR against *Hpa*. Four leaves of 4.5-week-old plants
809 were infiltrated with either avirulent *Pseudomonas syringae* pv. *tomato* DC3000 *avrRpm1*
810 (*Pst* DC3000 *avrRpm1*) or 10 mM $MgSO_4$ (mock). Three days after SAR induction, plants
811 were spray-inoculated with *Hpa* (10^5 conidiospores ml^{-1}). At six days after inoculation, 4-6
812 leaves from 15 plants per genotype were stained with trypan blue and microscopically
813 assigned to different *Hpa* colonization classes (right panels). Asterisks indicate statistically
814 significant differences in class distributions between SAR- and mock-treated plants (χ^2 test;
815 $p < 0.05$).

816 **(b)** Quantification of TAR against *Hpa* in P1 and C1 progenies from *Pst* DC3000- and mock-
817 inoculated plants, respectively. Parental plants were spray-inoculated 3 consecutive times at
818 3-4 day intervals with *Pst* DC3000 or 10 mM $MgSO_4$ (mock), and allowed to set seed. Leaves
819 of 3-week-old progenies were inoculated with *Hpa* (10^5 conidiospores ml^{-1}) and examined
820 for pathogen colonization 6 days later, as detailed in the legend of Figure 1a. Asterisks
821 indicate statistically significant differences in class distributions between P1 and C1
822 progenies (χ^2 test; $p < 0.05$).

823 **Figure 5: The pathogenesis-related transcriptome of Col-0, *nrpe1* and *ros1* during infection**
824 **by *H. arabidopsidis*.**

825 **(a)** Venn diagram showing numbers of differentially expressed genes at 48 and/or 72 hours
826 post inoculation (hpi) between mock- (m) and *Hpa*-inoculated (h) Col-0 (*Hpa*; green),
827 between Col-0 and *nrpe1* for any time-point and condition (*nrpe1*; blue), and between Col-0
828 and *ros1* for any time-point and any condition (*ros1*; red). Each time-point (48 and 72 hpi)
829 was analysed separately; numbers represent the sum of differentially expressed genes at
830 one or both time-points. Genes were considered to be differentially expressed at LIMMA-
831 reported q-value ≤ 0.01 (global adjust, FDR).

832 **(b)** *Hpa*-inducible genes that show augmented induction in *nrpe1* and/or repressed
833 induction in *ros1* are enriched with gene ontology (GO) terms 'Systemic Acquired
834 Resistance' (GO:0009627) and 'Salicylic Acid Biosynthetic Process' (GO:0009697).

835 **(c)** Transcript levels of all 166 *Hpa*-inducible genes with augmented induction in *nrpe1*
836 and/or repressed induction in *ros1*. Genes were selected when differentially expressed
837 between *ros1* and *nrpe1*, as well as between Col-0 and *ros1,* and/or between Col-0 and
838 *nrpe1,* at either time-point after inoculation. Heat map projections represent z-scores of
839 transcript levels.

840 **Figure 6: Transposable element (TE) occurrence and DNA methylation features in 166**
841 **defence genes whose responsiveness is primed in *nrpe1* and/or repressed in *ros1*.**

842 **(a)** Relative TE occurrence in the selection of 166 genes compared to other genes
843 considered in the transcriptome analysis (genes on array). For the 2kb upstream regions (5';
844 relative to transcriptional start site) and the 2kb downstream regions (3'; relative to poly-
845 adenylation site), 100 windows of 20 bp were used; for gene body regions, 100 windows of
846 1% of the gene length were used.

847 **(b)** Relative occurrence of differentially methylated cytosines (DmCs) in 2 kb gene promoter
848 regions of nrpe1 and ros1. Dark shades: DmC frequencies within the selection of 166 *Hpa*-
849 responsive genes with augmented induction in *nrpe1* and/or repressed induction in *ros1*
850 during *Hpa* infection; Light shades: DmC frequencies in all other genes considered in the
851 transcriptome analysis. Shown are promoters with at least three differentially methylated

852 DmCs in *nrpe1* or *ros1*, relative to Col-0. Results are based on publically available bisulfite-
853 sequencing data of *nrpe1* and *ros1* (Qian et al., 2012; Stroud *et al*., 2013).

854 **(c)** Venn diagram representing a selection of the 166 gene promoters (2 kb) that contain one
855 or more TEs (green), have at least three hyper-methylated cytosines in the *ros1* mutant
856 (blue), and have at least three hypo-methylated cytosines in the *nrpe1* mutant (red)

857 **Figure 7: Model of *cis*- and *trans*-regulation of defence gene responsiveness by DNA**
858 **(de)methylation.**

859 Responsiveness of defence genes can be *cis*-regulated via RNA-directed DNA methylation
860 (RdDM; blue) and/or ROS1-mediated DNA de-methylation (red) of nearby DNA regions, such
861 as transposable elements (TEs; purple). *Trans*-regulation of defence genes that are not
862 associated with nearby DNA methylation can be achieved via different mechanisms. Apart
863 from indirect regulation by *cis*-controlled regulatory genes (top), chromatin remodellers in
864 the RdDM protein complex can cross-link with distant genomic regions and influence post-
865 translational histone modifications at distal genes that are not associated with DNA
866 methylation. Red arrows indicate stimulation of DNA methylation and/or post-translational
867 histone modifications (blue triangles and circles) by the RdDM complex. Green lines indicate
868 repression of DNA methylation by ROS1, or transcriptional repression by post-translational
869 histone modifications. The black arrow indicates stimulation of defence gene induction by
870 defence regulatory proteins.