# Next-Generation Systemic Acquired Resistance[1][W][OA]

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Systemic acquired resistance (SAR) is a plant immune response to pathogen attack. Recent evidence suggests that plant immunity involves regulation by chromatin remodeling and DNA methylation. We investigated whether SAR can be inherited epigenetically following disease pressure by *Pseudomonas syringae* pv *tomato* DC3000 (*Pst*DC3000). Compared to progeny from control-treated Arabidopsis (*Arabidopsis thaliana*; $C_1$), progeny from *Pst*DC3000-inoculated Arabidopsis ($P_1$) were primed to activate salicylic acid (SA)-inducible defense genes and were more resistant to the (hemi)biotrophic pathogens *Hyaloperonospora arabidopsidis* and *Pst*DC3000. This transgenerational SAR was sustained over one stress-free generation, indicating an epigenetic basis of the phenomenon. Furthermore, $P_1$ progeny displayed reduced responsiveness of jasmonic acid (JA)-inducible genes and enhanced susceptibility to the necrotrophic fungus *Alternaria brassicicola*. This shift in SA- and JA-dependent gene responsiveness was not associated with changes in corresponding hormone levels. Instead, chromatin immunoprecipitation analyses revealed that SA-inducible promoters of *PATHOGENESIS-RELATED GENE1*, *WRKY6*, and *WRKY53* in $P_1$ plants are enriched with acetylated histone H3 at lysine 9, a chromatin mark associated with a permissive state of transcription. Conversely, the JA-inducible promoter of *PLANT DEFENSIN1.2* showed increased H3 triple methylation at lysine 27, a mark related to repressed gene transcription. $P_1$ progeny from the defense regulatory mutant *non expressor of PR1* (*npr1*)*-1* failed to develop transgenerational defense phenotypes, demonstrating a critical role for NPR1 in expression of transgenerational SAR. Furthermore, the *drm1drm2cmt3* mutant that is affected in non-CpG DNA methylation mimicked the transgenerational SAR phenotype. Since *Pst*DC3000 induces DNA hypomethylation in Arabidopsis, our results suggest that transgenerational SAR is transmitted by hypomethylated genes that direct priming of SA-dependent defenses in the following generations.

To survive in hostile environments, plants have evolved the ability to prime their immune system against microbial pathogens. This priming results in a faster and stronger induction of defense mechanisms after pathogen attack (Conrath et al., 2006; Conrath, 2011). Although inducible defenses are often too weak to protect the host plant against disease by virulent pathogens, an augmented induction of these defenses can be highly effective, particularly when their expression precedes the delivery of susceptibility-inducing effectors by the invading pathogen (Ahmad et al., 2010).

A variety of environmental signals can trigger priming of plant defense, many of which indicate upcoming stress (Conrath et al., 2006). For example, localized pathogen attack causes systemic acquired resistance (SAR), which is associated with priming of defense (Kohler et al., 2002; Jung et al., 2009). The first systematic study of this phenomenon in tobacco (*Nicotiana tabacum*) revealed that SAR persists for at least 20 d (Ross, 1961). Studies over subsequent decades have mostly focused on the signaling pathways mediating SAR induction, which require endogenous accumulation of the plant hormone salicylic acid (SA) and the downstream signaling protein NON EXPRESSOR OF PR1 (NPR1; Durrant and Dong, 2004). NPR1 has also been implicated in the cross talk between SA- and jasmonic acid (JA)-dependent defense pathways, which enables plants to mount an appropriate defense reaction, depending on the nature of the attacker and the stage of infection (Spoel et al., 2003; Koornneef and Pieterse, 2008).

Recent studies have revealed that systemic accumulation of SA during the onset of SAR is preceded by a variety of metabolic signals, such jasmonates (Truman et al., 2007) and indole-derived compounds (Truman et al., 2010). The exact nature of the systemic SAR

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signal in Arabidopsis (*Arabidopsis thaliana*) after localized infection by avirulent *Pseudomonas syringae* remains complex and has been a matter of debate (Attaran et al., 2009). Apart from methyl salicylate (MeSA; Vlot et al., 2008a, 2008b), glycerolipids (Chaturvedi et al., 2008), azeleic acid (Jung et al., 2009), and glycerol-3-P (Chanda et al., 2011) have been implicated. As a plausible explanation, Liu et al. (2011b) recently proposed that SAR is controlled by an interaction between at least two mobile signals, MeSA and a complex formed between the lipid transfer protein DIR1 and glycerolipid or lipid derivatives. Liu et al. (2011a) also reported that the dependency of SAR on MeSA is determined by the light regime. When SAR was induced late in the day and plants received little light in subsequent hours, MeSA and its metabolizing enzymes were found to be essential for SAR. By contrast, when induction was performed in the morning and was followed by an extended light period, SAR developed in the absence of MeSA. Together, these studies illustrate that the onset of SAR in Arabidopsis is mediated by a multitude of transiently expressed signaling networks that can vary according to the environmental conditions. The mechanisms of long-lasting maintenance of SAR, on the other hand, have remained less intensely studied. Recent studies have begun to analyze how epigenetic regulatory mechanisms, such as DNA methylation and chromatin remodeling, can have long-lasting impacts on gene expression and plant immunity (Bruce et al., 2007; van den Burg and Takken, 2009; Alvarez et al., 2010).

Previously, we demonstrated that the costs of priming in Arabidopsis are outweighed by its benefits under relatively high disease pressure (van Hulten et al., 2006). This suggests that priming is a beneficial defense strategy in hostile environments. Whether priming can be inherited epigenetically from disease-exposed Arabidopsis remains unknown, even though it can be expected that transgenerational defense priming would provide benefits for short-generation plant species with limited ability to outlive disease outbreaks. The objective of this study was to examine whether disease-exposed Arabidopsis produces progeny that are primed for defense. We provide evidence for transgenerational SAR and have explored the mechanistic basis of this epigenetic immune response.

## RESULTS

### Plants Exposed to Fitness-Reducing Levels of Disease Produce Resistant Offspring

Six plants were inoculated five times over a period of 3 weeks with increasing doses of the bacterial pathogen *P. syringae* pv *tomato* DC3000 (*Pst*DC3000), while six control plants were subjected to mock inoculations. All parental plants were allowed to set seed under similar growth conditions (Fig. 1A). Pathogen-infected parental plants ($P_0$; P = pathogen) suffered severe fitness costs, as was evidenced by dramatically reduced growth and seed production in comparison to control-treated parental plants ($C_0$; C = control; Fig. 1, A and 1B). Nevertheless, $P_1$ progeny from pathogen-infected plants and $C_1$ progeny from control-treated plants did not differ statistically in seed size, germination efficiency, or plant growth (Supplemental Fig. S1). We subsequently compared basal levels of resistance in $P_1$ and $C_1$ progenies against the oomycete pathogen *Hyaloperonospora arabidopsidis* using lactophenol trypan blue staining of infected leaves. Pathogen colonization was reduced in $P_1$ plants compared to $C_1$ plants (Fig. 1C), which was statistically significant for all six $P_1$ lines from individual $P_0$ plants (Supplemental Fig. S2A). In an independent experiment, three $P_1$ progeny lines from the SA-insensitive *npr1-1* mutant failed to develop transgenerational resistance in comparison to three corresponding $C_1$ lines from this mutant, while three $P_1$ wild-type lines again displayed enhanced resistance compared to the corresponding $C_1$ wild-type lines (Fig. 1C; Supplemental Fig. S2B). Similar results were obtained after inoculation with a bioluminescent strain of *Pst*DC3000 (*Pst*DC3000-lux; Fan et al., 2008): Whereas $P_1$ wild-type plants developed less bioluminescence and fewer disease symptoms than $C_1$ wild-type plants, no such differences were observed between $P_1$ and $C_1$ progeny from the *npr1-1* mutant (Supplemental Fig. S4). Hence, transgenerational resistance is dependent on an intact NPR1 protein and is effective against different (hemi) biotrophic pathogens.

To examine the durability of the transgenerational resistance, four individual plants from different $C_1$ or $P_1$ progeny lines were allowed to set seed under stress-free conditions, providing $C_1C_2$ and $P_1C_2$ progeny lines, respectively. In addition, six individuals from different $P_1$ lines were exposed to fitness-reducing levels of *Pst*DC3000 disease to provide $P_1P_2$ progeny lines (Fig. 1, A and B). Compared to $C_1C_2$ plants, $P_1P_2$ and $P_1C_2$ plants were more resistant to *H. arabidopsidis* (Fig. 1C), which was statistically significant for independent $P_1P_2$ and $P_1C_2$ progeny lines (Supplemental Fig. S3). It can thus be concluded that transgenerational resistance is sustained over one stress-free generation.

### Transgenerational Resistance Is Associated with Priming of SA-Dependent Genes

The involvement of NPR1 in transgenerational resistance resembles pathogen-induced SAR, which is based on NPR1-dependent priming of SA-inducible defense (Kohler et al., 2002; Jung et al., 2009). To examine whether transgenerational resistance is associated with similar defense priming, we quantified responsiveness of the SA-inducible *PATHOGENESIS-RELATED GENE-1 (PR-1)* upon treatment with SA. As is shown in Figure 2A, $P_1$ plants displayed a faster and stronger induction of *PR-1* than $C_1$ plants, indicating that $P_1$ progeny are primed for SA-inducible defenses. This augmented responsiveness of the *PR-1* gene was

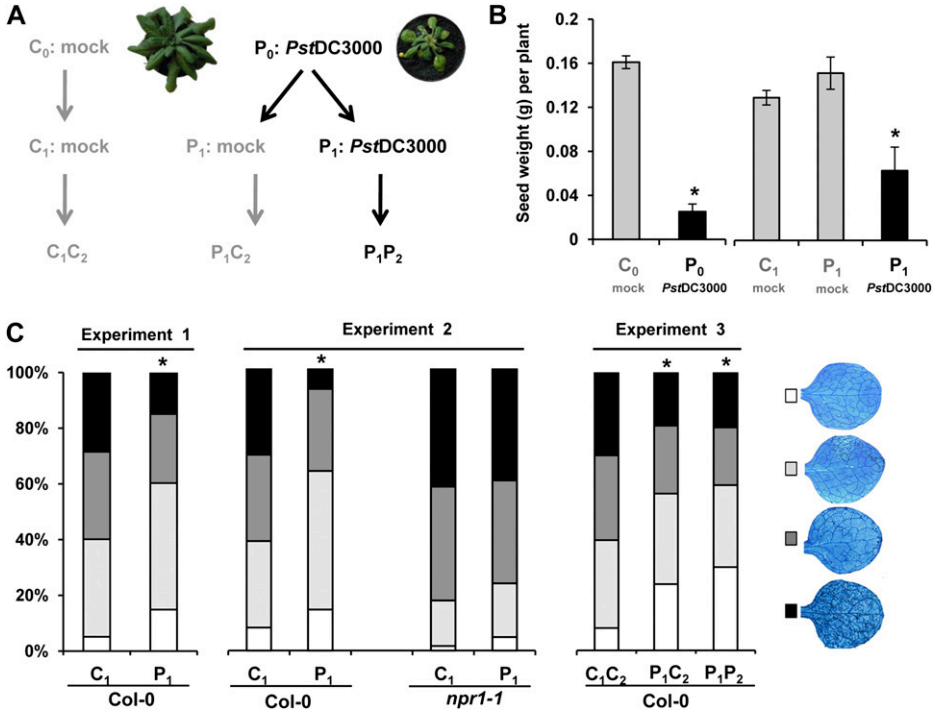


**Figure 1**. Transgenerational SAR in progeny from healthy and diseased Arabidopsis. A, Experimental design for the generation of progeny lines. Plants were inoculated five times at intervals of 3 to 4 d by dipping the leaves in a control solution ($C_0$) or a solution containing *Pst*DC3000 ($P_0$), after which plants were allowed to set seed to provide and $P_1$ progenies, respectively. Insets show representative growth phenotypes of $C_0$ and $P_0$ after mock and *Pst*DC3000 inoculations. $C_1$ and $P_1$ plants were allowed to set seed under stress-free conditions, providing $C_1C_2$ and $P_1C_2$ progeny, respectively. A separate batch of $P_1$ plants was exposed to similar *Pst*DC3000 disease pressure as $P_0$ plants to provide $P_1P_2$ progeny. B, Seed production by mock- and *Pst*DC3000-inoculated parental plants. Data represent mean values (±SE; $n = 4–6$) of grams of seed weight per plant. Asterisks indicate statistically significant differences compared to mock-inoculated $C_0$ or $C_1$ plants (Student's *t* test; $\alpha = 0.05$) C, Basal resistance against *H. arabidopsidis* WACO9 in $C_1$ and $P_1$ progenies of wild-type plants (Col-0; Experiment 1), $C_1$ and $P_1$ progenies of Col-0 and *npr1-1* (Experiment 2), and $C_1C_2$, $C_1P_2$, and $P_1P_2$ progenies of Col-0 (Experiment 3). At 6 d after conidiospore inoculation, stained leaves were microscopically examined and assigned to different classes. Asterisks indicate statistically significant differences in class distributions in comparison to $C_1$ or $C_1C_2$ plants ($\chi^2$ test; $\alpha = 0.05$).

also observed in plants from $P_1C_2$ and $P_1P_2$ progenies (Fig. 2A), demonstrating that the priming is maintained over one stress-free generation. We then examined whether this transgenerational priming targets regulatory genes of SA-induced defense. To this end, we profiled transcription of *WRKY6*, *WRKY53*, and *WRKY70*, which had previously been found to be active during priming of NPR1-dependent defense by β-amino-butyric acid (Van der Ent et al., 2009), acibenzolar *S*-methyl (BTH; Jaskiewicz et al., 2011), or *P. syringae* pv *maculicola* (Jaskiewicz et al., 2011). At time points preceding augmented *PR-1* induction, $P_1$ plants showed enhanced expression of *WRKY6*, *WRKY53*, and *WRKY70* in comparison to $C_1$ plants (Fig. 2B). It can thus be concluded that transgenerational priming targets multiple regulatory steps in NPR1-dependent resistance.

### Transgenerational Cross-Effects on JA-Dependent Resistance

Infection with *Pst*DC3000 activates SA-dependent defense but suppresses JA-dependent resistance against the necrotrophic fungus *Alternaria brassicicola* (Spoel et al., 2003, 2007). To examine whether transgenerational resistance is associated with a similar suppression of JA-dependent defense, we assessed basal resistance against the necrotrophic fungus *A. brassicicola* in $C_1$ and $P_1$ wild-type plants. At 5 d after inoculation, $P_1$ plants developed larger chlorotic lesions and allowed increased hyphal colonization by the fungus (Fig. 3A). We subsequently investigated whether responsiveness of the JA-inducible genes *PLANT DEFENSIN1.2* (*PDF1.2*) and *VEGETATIVE STORAGE PROTEIN2* (*VSP2*) is affected in $P_1$ wild-type plants. At 4 and 8 h after exogenous JA application, both marker genes showed reduced expression in $P_1$ wild-type plants (Fig. 3B), which was absent in $P_1$ progeny from the *npr1-1* mutant (Supplemental Fig. S5). Despite these cross talk effects on JA-dependent resistance, ultraperformance liquid chromatography coupled to mass spectrometry (UPLC-MS/MS) analysis of $P_1$ and $C_1$ plants revealed no significant differences in endogenous levels of JA, JA-Ile, the JA precursor 12-oxo-phytodienoic acid (OPDA), SA, or

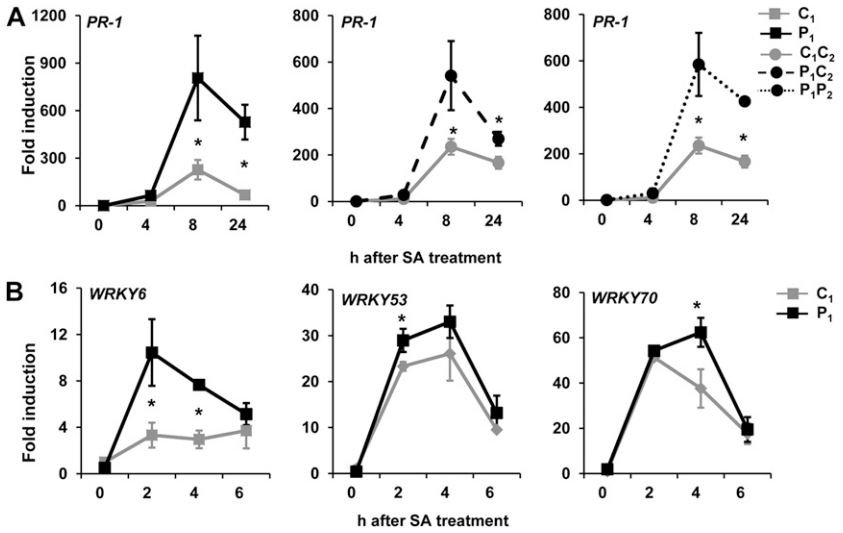


**Figure 2**. Transgenerational priming of SA-induced defense gene expression. A, Quantitative reverse transcription PCR (qRT-PCR) analysis of SA-induced *PR-1* transcription in $C_1$ and $P_1$ progenies (left), $C_1C_2$ and $C_1P_2$ progenies (center), and $C_1C_2$ and $P_1P_2$ progenies (right) at 4, 8, and 24 h after treatment with 0.5 mM SA. B, qRT-PCR analysis of SA-induced transcription of *WRKY6*, *WRKY53*, and *WRKY70* in $C_1$ and $P_1$ progenies at 2, 4, and 8 h after treatment with 0.5 mM SA. Gene expression analyses were performed in 2-week-old plants. Data represent average fold induction values (±SE; $n = 3$), relative to transcription levels before hormone treatment in $C_1$ plants ($2^{\Delta Ct}_{PR1} = 0.00042$; $2^{\Delta Ct}_{WRKY6} = 0.0076$; $2^{\Delta Ct}_{WRKY53} = 0.00092$; $2^{\Delta Ct}_{WRKY70} = 0.0078$) or in $C_1C_2$ plants ($2^{\Delta C}_{PR1} = 0.00030$). Asterisks indicate statistically significant differences in gene induction values (Student's *t* test; $\alpha = 0.05$).

SA-glucoside (SAG) between $P_1$ and $C_1$ plants (Fig. 3C). Hence, the shifted balance between SA- and JA-dependent defenses in $P_1$ plants is not caused by changes in hormone levels, but rather by adjustments in the downstream response pathways.

### Transgenerational Chromatin Modifications at Defense Gene Promoters

Posttranslational modifications at the N-terminal tail of histone H3 can influence defense-related gene expression (van den Burg and Takken, 2009; Alvarez et al., 2010). Because these chromatin modifications can have long-lasting impacts on plant gene expression (Vaillant and Paszkowski, 2007), we investigated whether the altered responsiveness of *PR-1* and *PDF1.2* in $P_1$ plants is associated with changes in chromatin structure at the promoter regions of these genes. For this purpose, we performed chromatin immunoprecipitation (ChIP) analysis of *PR-1* and *PDF1-2* promoter DNA using antibodies against acetylated histone H3 at Lys-9 (H3K9ac) and triple-methylated H3 at Lys-27 (H3K27me3). Compared with $C_1$ plants, *PR-1* promoter DNA of $P_1$ plants was associated with enhanced levels of H3K9ac (Fig. 4A), which was apparent using different primer pairs against separate regions of the promoter (Supplemental Fig. S6). By contrast, $P_1$ progeny from the *npr1-1* mutant failed to show increased levels of H3K9ac at the *PR1* promoter (Figs. 4B; Supplemental Fig. S6). Acetylation of H3K9 marks an increased transcriptional capacity (Eberharter and Becker, 2002) and could therefore contribute to priming of the *PR-1* gene in $P_1$ plants. Furthermore, the *PDF1-2* promoter from $P_1$ wild-type plants was not altered in H3K9ac levels but displayed statistically significant enrichment with H3K27me3 (Fig. 4A), which was apparent with different primer pairs against this promoter (Supplemental Fig. S6). Conversely, $P_1$ progeny from the *npr1-1* mutant failed to show H3K27me3 enrichment at the *PDF1.2* promoter (Fig. 4B; Supplemental Fig. S6). Since H3K27me3 is associated with transcriptional silencing (Zhang et al., 2007), this histone modification could contribute to the suppressed responsiveness of *PDF1.2* in $P_1$ plants (Fig. 3B). To further investigate the role of H3K9ac in priming of SA-dependent defense, we analyzed the promoter regions of *WRKY6* and *WRKY53*. Both regulatory genes can be primed by pretreatment with BTH or *P. syringae* pv *maculicola* (Jaskiewicz et al., 2011) and showed augmented responsiveness to SA in $P_1$ plants (Fig. 2B). As observed for *PR-1*, the promoters of *WRKY6* and *WRKY53* in $P_1$ wild-type plants were enriched with H3K9ac, whereas this response was absent in $P_1$ *npr1-1* plants. Hence, transgenerational acetylation of H3K9 requires an intact NPR1 protein and targets multiple SA-inducible gene promoters.

### Role of DNA Methylation in Transgenerational Resistance

Although histone modifications can have long-lasting impacts on gene expression (Vaillant and Paszkowski, 2007), there is no convincing evidence that they can be transmitted through meiosis. By contrast, there is ample evidence for transmission of DNA methylation to following generations. It is also known that *PstDC3000* triggers DNA hypomethylation in Arabidopsis (Pavet et al., 2006). To examine the role of DNA methylation in *PstDC3000*-induced transgenerational resistance, we compared transgenerational resistance phenotypes between wild-type plants and the *drm1drm2cmt3* triple mutant (*ddc*), which is reduced in non-CpG DNA methylation (Chan et al., 2006). Unlike other DNA methylation mutants, the *ddc* mutant expressed normal growth phenotypes under our growth conditions until the onset of flowering, which would otherwise complicate the interpretation of our

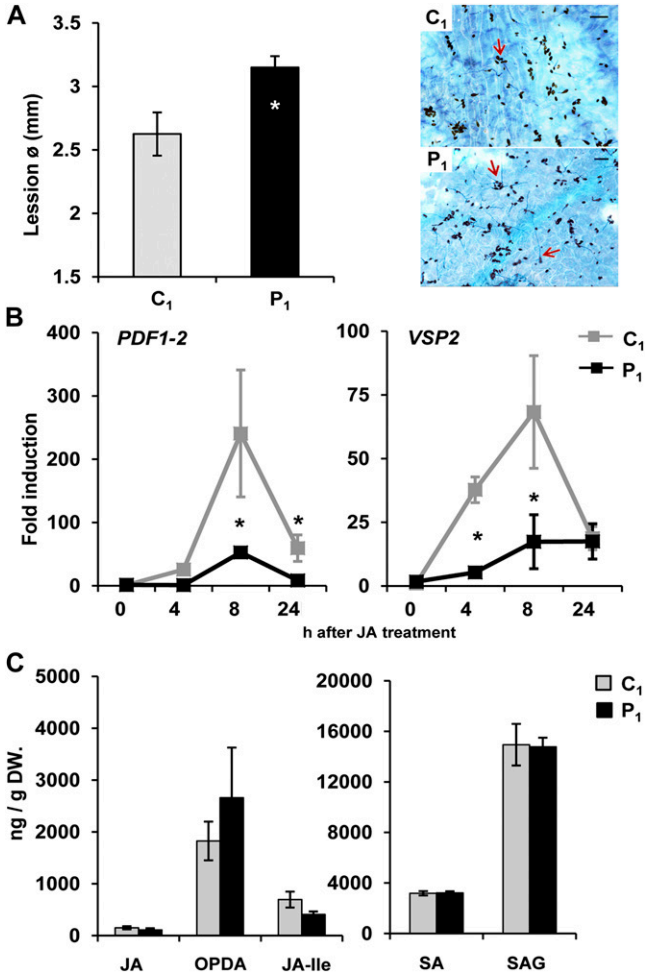


**Figure 3**. Transgenerational cross-effects on JA-dependent resistance. A, Resistance against the necrotrophic fungus *A. brassicicola* in 5-week-old $C_1$ and $P_1$ plants. Left: Average lesion diameters (±SE; $n = 15$) at 5 d after spore inoculation. Asterisk indicates a statistically significant difference in lesion diameter between lines (Student's $t$ test; $\alpha = 0.05$). Right: Representative photographs of fungal colonization, visualized by trypan blue staining at 4 d after inoculation. Bars = 100 $\mu$m. Red arrows indicate germinated spores. B, qRT-PCR analysis of JA-induced transcription of *PDF1.2* and *VSP2* in $C_1$ and $P_1$ progenies at 4, 8, and 24 h after treatment of the leaves with 0.1 mM JA. Gene expression analyses were based on 2-week-old plants from $C_1$ and $P_1$ progenies. Data represent average fold induction values (±SE; $n = 3$) relative to transcription levels before hormone treatment in $C_1$ plants ($2^{\Delta Ct}_{PDF1\text{-}2} = 0.0011$; $2^{\Delta Ct}_{VSP2} = 0.0060$). Asterisks indicate statistically significant differences in gene induction (Student's $t$ test; $\alpha = 0.05$). C, UPLC-MS/MS quantification of JA, JA-Ile, OPDA, SA, and SAG in mature leaves from 5-week-old plants. Shown are average values in nanograms per gram of dry weight (DW; ±SE; $n = 3$). No statistically significant differences were detected between $C_1$ and $P_1$ plants (Student's $t$ test; $\alpha = 0.05$).

bioassays. Upon inoculation with *H. arabidopsidis*, three independent $C_1$ and $P_1$ progeny lines from the *ddc* mutant expressed similar levels of resistance, whereas the corresponding $P_1$ lines of the wild-type displayed enhanced resistance in comparison to $C_1$ wild-type lines. Interestingly, however, all *ddc* lines expressed significantly higher levels of resistance in comparison to $C_1$ wild-type lines (Fig. 5A; Supplemental Fig. S7). The *ddc* mutant also expressed constitutively higher levels of resistance against *Pst*DC3000-lux but was enhanced susceptible to the necrotrophic pathogen *A. brassicicola* (Supplemental Fig. S8). These results indicate that the hypomethylated DNA status in the *ddc* mutant mimics the transgenerational resistance phenotype of $P_1$ wild-type plants.

To examine whether the constitutive resistance against hemibiotrophic pathogens in *ddc* is based on a priming of the SA response, we compared levels of *PR-1* gene induction between $C_1$ and $P_1$ plants of wild-type and *ddc* plants. At 4 and 8 h after SA application, *ddc* plants showed significantly enhanced *PR-1* transcription compared to wild-type plants ($P = 0.031$), which was similar in $C_1$ and $P_1$ progenies of the mutant (Fig. 5B). Hence, DNA hypomethylation in the *ddc* mutant mimics transgenerational priming of SA-dependent defense. Since infection by *Pst*DC3000 induces DNA hypomethylation in Arabidopsis (Pavet et al., 2006), our results indicate that transgenerational resistance from *Pst*DC3000-infected plants is transmitted by hypomethylated DNA.

## DISCUSSION

Our study demonstrates that disease resistance can be carried forward to the next generation from plants exposed to fitness-reducing disease pressure. It indicates an epigenetic mechanism of disease protection, which could function as a plant memory of disease stress encountered in previous generations. A recent study by Kathiria et al. (2010) demonstrated increased homologous recombination in progeny from tobacco mosaic virus-infected tobacco, which was associated with enhanced disease resistance. Increased homologous recombination has also been reported in progeny from Arabidopsis exposed to short-wavelength radiation (ultraviolet-C) or flagellin, which persisted in subsequent, untreated generations (Molinier et al., 2006). Hence, transgenerational responses to stress are emerging as a widespread defense phenomenon in plants. Our study reveals an epigenetic mechanism for transgenerational resistance against biotic stress. Like SAR, transgenerational resistance protects against (hemi)biotrophic pathogens, requires an intact NPR1 protein, and is associated with priming of SA-dependent defense (Kohler et al., 2002; Ton et al., 2002; Durrant and Dong, 2004; Jung et al., 2009; Figs. 1 and 2). Therefore, we propose to define this phenomenon as transgenerational SAR or "next-generation SAR."

Independently from our findings, two independent studies by Slaughter et al. (2012) and Rasmann et al. (2012) demonstrate similar transgenerational resistance phenomena in response to priming-inducing stimuli. Slaughter et al. (2012) discovered that progeny of Arabidopsis that had been treated with $\beta$-aminobutyric acid or an avirulent isolate of *Pst*DC3000 (*PstavrRpt2*) are primed for SA-dependent resistance

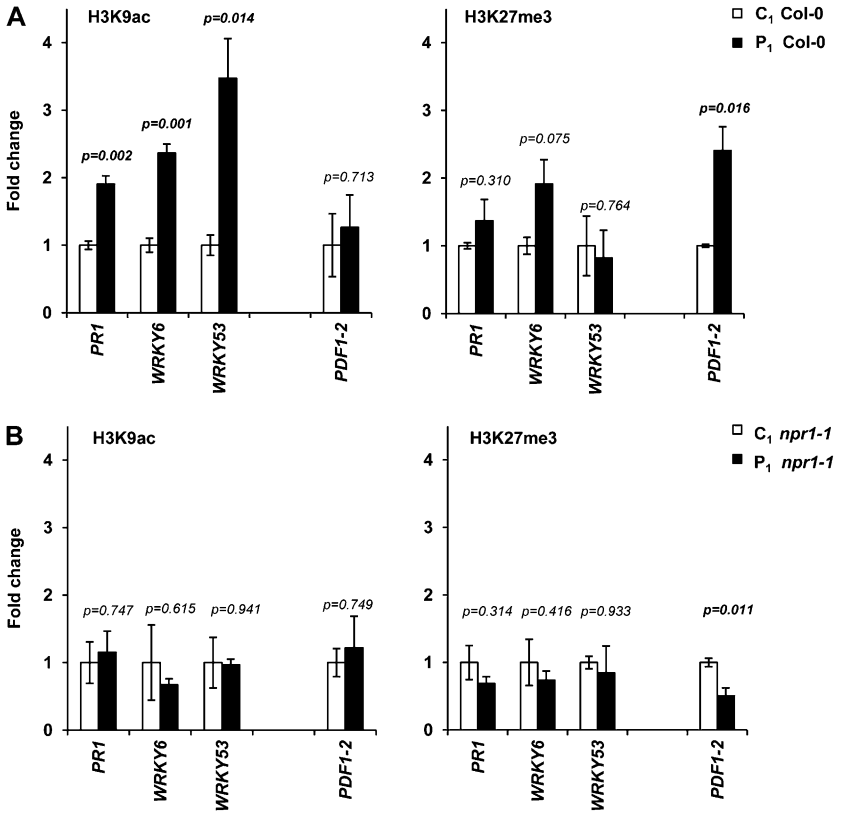


**Figure 4**. Transgenerational modifications of histone H3 at defense gene promoters in Col-0 (A) but not in *npr1-1* (B). After ChIP, promoter DNA of SA-inducible *PR-1*, *WRKY6*, *WRKY53*, and JA-inducible *PDF1.2* was quantified by qPCR relative to DNA amounts in chromatin extracts before immunoprecipitation (input) using antibodies against H3K9ac or H3K27me3. Data represent average fold change values (±SE; $n = 3$) in $P_1$ plants compared to $C_1$ plants. *P* values indicate statistical differences between $C_1$ and $P_1$ progenies (Student's *t* test).

against *H. arabidopsidis* and *Pst*DC3000. Furthermore, Rasmann et al. (2012) show that Arabidopsis and tomato (*Solanum lycopersicum*) subjected to herbivory or mechanical damage produce progeny that are primed to express JA-dependent resistance against herbivores. Together, our three studies demonstrate that transgeneration priming of defense is a robust and broadly distributed mechanism of phenotypic plasticity in plants to environmental stress. Moreover, the triplicate demonstration that the priming of defense can be transmitted to following generations indicates an epigenetic basis of the phenomenon.

Interestingly, Slaughter et al. (2012) reported that induction of transgenerational resistance induced one single inoculation with an avirulent *PstDC3000* strain disappears after one stress-free generation. By contrast, our study revealed that transgenerational resistance upon repeated inoculations with plant fitness-reducing doses of the virulent *PstDC3000* can be sustained over one disease-free generation (Figs. 1C and 2A). This difference indicates that the intensity of disease-related stress is proportional to the durability of transgenerational resistance in progeny. Hence, plants are capable of adjusting the stability of transgenerational defense priming according to the severity of disease pressure in their environment. Future experiments are necessary to establish the exact relationship between disease severity and epigenetic stability of transgenerational priming.

Spoel et al. (2007) reported that virulent *Pst*DC3000 renders plants more susceptible to the necrotrophic pathogen *A. brassicicola* through suppression of the JA pathway. Our results suggest that this NPR1-dependent signaling cross talk can be transmitted to the following generation (Fig. 3). Interestingly, $P_1$ plants did not accumulate different levels of endogenous SA or JA (Fig. 3C), indicating that the altered balance between SA- and JA-dependent defense is based on changes in the sensitivity of the downstream response pathways. WKRY70 has been implicated as a downstream regulator of negative cross talk between SA- and JA-dependent signaling (Li et al., 2006). Indeed, $P_1$ plants expressed enhanced levels of *WRKY70* (Fig. 2B), supporting a regulatory role of this transcription factor in transgenerational cross talk. We furthermore found that the observed change in responsiveness of SA-inducible *PR-1* and JA-inducible *PDF1.2* is marked by NPR1-dependent modifications of histone H3 at their gene promoters (Fig. 4). Together, our results suggest that transgenerational SAR is based on a shifted balance between SA- and JA-dependent defense, which is maintained by NPR1-dependent modifications of chromatin structure at promoters of JA- and SA-responsive defense genes.

Evidence is emerging that plant defense is regulated by changes in chromatin structure (van den Burg and Takken, 2009). Arabidopsis mutants in chromatin remodeling enzymes are affected in JA- and SA-dependent

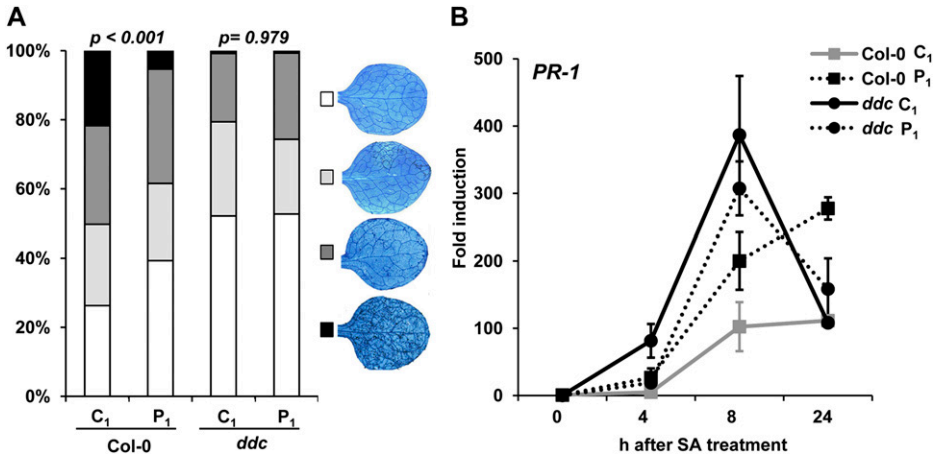


**Figure 5.** Role of non-CpG DNA methylation in transgenerational SAR. A, Level of resistance against *H. arabidopsidis* WACO9 in $C_1$ and $P_1$ progeny from Col-0 and the *dmr1dmr2ctm3* (*ddc*) mutant. At 6 d after conidiospore inoculation, stained leaves were microscopically examined and assigned to different severity classes. *P* values indicate statistical differences in class distributions between $P_1$ and $C_1$ progeny of each genotype ($\chi^2$ test). B, qRT-PCR analysis of SA-induced *PR-1* transcription at 4, 8, and 24 h after treatment of $C_1$ and $P_1$ progeny of Col-0 and *ddc* plants with 0.5 mM SA. Data represent average fold induction values (±SE; $n = 3$) relative to transcription levels before hormone treatment in $C_1$ Col-0 plants ($2^{\Delta Ct}_{PR1} = 0.0020$).

resistance (Devoto et al., 2002; March-Díaz et al., 2008; Wu et al., 2008). Furthermore, the SAR-inducing chemical BTH triggers posttranslational modifications of histone H3 in the *PR-1* promoter (Mosher et al., 2006), and Jaskiewicz et al. (2011) recently demonstrated that *P. syringae* pv *maculicola* primes stress-inducible expression of *WRKY* genes that is associated with NPR1-dependent H3 modifications at their promoters. Although the latter two studies describe relatively short-term responses within days after treatment, they are consistent with our finding that $P_1$ progeny are primed for NPR1-dependent defense. However, there is no evidence that histone modifications can be transmitted through meiosis. Conversely, various plant traits have been demonstrated to be transmitted by DNA methylation, which can remain stable over multiple generations (Kalisz and Purugganan, 2004). In particular DNA hypomethylation has been associated with plant defense. For instance, Arabidopsis responds to infection by *Pst*DC3000 by hypomethylation of genomic DNA (Pavet et al., 2006). Furthermore, inbreeding of the Arabidopsis *decrease in DNA methylation1* mutation, causing genome-wide DNA hypomethylation, gives rise to a heritable but metastable defense allele, called *bal1*, which confers NPR1-dependent resistance (Stokes et al., 2002; Yi and Richards, 2009). Hence, DNA hypomethylation can cause epigenetic inheritance of disease resistance. Our finding that non-CpG DNA hypomethylation by the *ddc* mutations mimics the phenotype of transgenerational SAR supports this notion (Fig. 5; Supplemental Figs. S7 and S8). Furthermore, evidence from both animal and plant systems suggests that (de)methylated DNA can direct posttranslational modifications of histone H3 (Vaillant and Paszkowski, 2007; Cedar and Bergman, 2009). Therefore, we propose that transgenerational SAR is inherited through hypomethylated regulatory genes, which direct NPR1-dependent histone H3 modifications in following generations to induce and maintain priming of SA-dependent defense genes.

Small interfering RNAs (siRNAs) can control gene transcription through changes in DNA methylation (Brodersen and Voinnet, 2006; Vaucheret, 2006). Interestingly, the complementary study by Rasmann et al. (2012) demonstrated that two Arabidopsis mutants impaired in the biogenesis of siRNAs (*dcl2 dcl3 dcl4* and *nrpd2a nrpd2b*) failed to produce progeny with transgenerational resistance against herbivores. Since the *ddc* mutant is affected in RNA-directed DNA methylation (Kurihara et al., 2008), our results support a similar role by siRNAs during the onset of transgenerational SAR. *Pst*DC3000 has recently been reported to trigger the accumulation of 27 RNA interference families in Arabidopsis (Zhang et al., 2011). Furthermore, micro-RNAs and siRNAs have both been implicated in the response of Arabidopsis to *P. syringae* pathogens (Li et al., 2011). However, the siRNA families eliciting transgenerational SAR and herbivore resistance must be different to prime SA- and JA-inducible defenses, respectively. Our additional observation that transgenerational SAR is associated with a suppression of JA-dependent resistance (Fig. 3) suggests that these siRNAs could act antagonistically on each other.

Priming of defense is a beneficial defense strategy in hostile environments with relatively minor costs (van Hulten et al., 2006; Ahmad et al., 2010). Epigenetic regulation of priming would allow plants to protect their progeny against recurring biotic stress without permanent genetic fixation of the trait and its associated costs. A better understanding of this phenomenon will create opportunities to improve disease resistance in agricultural crops. Food security is an

important challenge in the 21st century (Baulcombe et al., 2009), and there is a pressing need to develop sustainable ways of pest and disease control to safeguard yields while minimizing pesticide use. An efficient induction of epigenetically imprinted defense priming would allow us to generate seed stocks of crops with broad-spectrum resistance that would require fewer chemicals to control disease.

## CONCLUSION

Disease-exposed Arabidopsis produces progeny with enhanced disease resistance, which can be maintained over one stress-free generation. This transgenerational SAR is effective against (hemi)biotrophic pathogens, requires an intact NPR1 protein, and is associated with priming of SA-dependent genes. Furthermore, transgenerational SAR is associated with an NPR1-dependent repression of JA-dependent defense genes and enhanced susceptibility to the necrotrophic fungus *A. brassicicola*. This shift in the cross talk balance between SA- and JA-dependent defenses is associated with permissive and repressive histone H3 modifications at SA- and JA-inducible gene promoters, respectively. The hypomethylated *ddc* mutant of Arabidopsis mimics the transgenerational SAR phenotype, suggesting that SAR is inherited through pathogen-induced hypomethylation at non-CpG DNA sites.

## MATERIALS AND METHODS

### Plant Material

Arabidopsis (*Arabidopsis thaliana*) Columbia-0 (Col-0), *npr1-1* (Cao et al., 1994), and *drm1-2 drm2-2 cmt3-11* (Chan et al., 2006) were cultivated under standard conditions (8.5:15.5 h light:dark; 20°C photophase, 18°C scotophase; 65% relative humidity) in a sand:compost mixture (1:3). Seeds (approximately 50 per pot) were stratified at 4°C in the dark for 2 d. For experiments lasting longer than 3 weeks, plants were individually transferred at the seedling stage (approximately 10 d old) to 60-mL pots.

### Imprinting of Transgenerational Defense Priming

Inoculation with *Pseudomonas syringae* pv *tomato* DC3000 (*Pst*DC3000) was performed five times by dipping rosettes in a bacterial suspension containing 10 mM $MgSO_4$ and 0.01% (v/v) Silwet L-77 at intervals of 3 to 5 d between treatments. To ensure constant disease pressure, the first three inoculations were performed at $10^8$ cells/mL and the last two at $10^9$ cells/mL. Plants were maintained at 100% relative humidity from the 1st inoculation until the 1 week after the 5th inoculation. Flowering was induced by transferring plants to long-light conditions (16:8 h light:dark) between the 3rd and 4th inoculation. Mock-inoculated plants ($C_0$ and $C_1$) were treated with 10 mM $MgSO_4$ (0.01% Silwet L-77) without bacteria and maintained under similar conditions. $C_1$ and $P_1$ progeny lines were collected from individual $C_0$ and $P_0$ parental plants. To assess durability of transgenerational SAR, four individuals from different $C_1$ and $P_1$ lines were allowed to set seed under stress-free conditions, providing $C_1C_2$ and $P_1C_2$ progeny lines, respectively. A separate batch of four individuals from different $P_1$ lines was exposed to fitness-reducing levels of *Pst*DC3000 disease to provide $P_1P_2$ progeny lines.

### Fitness Assays

Growth rates and seed production were determined as described previously (van Hulten et al., 2006). Seed size was estimated on the basis of seed area (Herridge et al., 2011). Germination efficiency was determined after 3 d of cultivation under standard growth conditions following 2 d of stratification.

### Basal Resistance Assays

*Hyaloperonospora arabidopsidis* WACO9 bioassays were performed as described before (Van der Ent et al., 2009). Infected leaves (>75) from at least 15 randomly selected plants were collected at 6 d after spray inoculation with $10^5$ conidiospores/mL, stained with lactophenol–trypan blue (Koch and Slusarenko, 1990), and scored using light microscopy. Colonization levels were assigned to four classes: I, no pathogen growth; II, hyphal colonization without conidiophores; III, hyphal colonization with conidiophores and sporadic oospores; and IV, extensive colonization, conidiophores, and frequent oospores. Differences in class distributions between progenies were analyzed for statistical differences by $\chi^2$ contingency tests or $\chi^2$ goodness of fit tests using Genstat software (13th edition).

*Pst*DC3000 assays were performed with a bioluminescent luxCDABE-tagged strain (PstDC3000-lux; Fan et al., 2008). Five-week-old plants were inoculated by dipping the leaves in a bacterial suspension containing $10^8$ colony-forming units/mL in 10 mM $MgSO_4$ and 0.01% (v/v) Silwet L-77. Symptoms were scored at 3 d after inoculation as described before (Van der Ent et al., 2009). Bacterial colonization was estimated from the intensity of bioluminescence, using a liquid nitrogen cooled CCD detector (Princeton Instruments) at maximum sensitivity. Digital photographs were taken at 3 d after inoculation under bright light (exposure time of 0.5 s) and in darkness (exposure time of 300 s) using WinView/32 software at fixed black-and-white contrast settings. Bacterial luminescence showed no major variation within colonized leaf areas and was proportional to the number of pixels on digital photographs. Bacterial titers in each plant were expressed as ratios between numbers bioluminescent pixels and total leaf pixels from bright light pictures, as described previously for digital callose quantification (Luna et al., 2011).

*Alternaria brassicicola* inoculation was performed when plants were 5 weeks old by applying 5-$\mu$L drops of $1 \times 10^6$ spores/mL onto four leaves of approximately similar age, as described (Ton and Mauch-Mani, 2004). Disease symptoms and pathogen colonization were evaluated at 5 d after inoculation and based on mean lesion diameters from 15 to 20 plants and microscopy examination of lactophenol-trypan blue stained leaves, respectively.

### Gene Expression Assays

Hormone-induced gene expression assays were based on 2-week-old plants after spraying with water, 0.1 mM JA, or 0.5 mM SA, supplemented with 0.01% Silwet (L-77). Samples were collected at the indicated time points, consisting of pooled shoots from four to six plants per replicate. RNA extraction, cDNA synthesis, and quantitative PCR reactions were essentially performed as described before (Van der Ent et al., 2009). Briefly, two technical replicates of each sample were subjected to the quantitative PCR reaction. PCR efficiency (E) of primer pairs were estimated from multiple amplification plots using the Equation $(1+E) = 10^{slope}$ (Ramakers et al., 2003) and were confirmed to provide (1+E) values close to 2 (ranging between 1.9 and 2.0). Transcript levels were calculated relative to the reference gene *At1g13440* (Czechowski et al., 2005) using the $2^{\Delta Ct}$ (cycle threshold) method, where $\Delta Ct = Ct$ (reference gene) $-$ Ct (gene of interest). Primer sequences were designed against the 3′-end of the gene and have been published before (Czechowski et al., 2004; Van der Ent et al., 2009). Fold induction values of gene expression were normalized to average $2^{\Delta Ct}$ values relative to wild-type $C_1$ or $C_1C_2$ plants at 0 h before hormone treatments, which are indicated in the figure legends.

### UPLC-Quadruple MS/MS Analysis

Mature leaf samples were collected from five plants per replicate. Extraction and quantification of SA, SAG (2-*O*-$\beta$-D-glucoside), JA, JA-Ile, and OPDA were analyzed by UPLC-MS/MS as described (Flors et al., 2008), with modification from (Forcat et al., 2008). Samples were analyzed by a triple quadrupole tandem mass spectrometer (TQD; Waters). Liquid chromatography separation was performed using an Acquity UPLC BEH C18 analytical column (Waters) at a flow rate of 300 mL/min. Quantifications were carried out with MassLynx 4.1 software (Waters) using internal standards as a reference for extraction recovery and the standard curves as quantifiers.

## ChIP

Assays were performed according to the manufacturer's protocol (EpiQuik Plant ChIP kit; Epigentek) using mature leaves from 5-week-old plants. Each biologically replicated sample from independent experiments ($n$ = 3) consisted of pooled leaves from four to five plants. Chromatin samples were immunoprecipitated using antibodies against acetyl-histone H3K9 (Millipore 07-352) and trimethyl-H3K27 (Millipore 07-449). Abundance of DNA in chromatin extracts was analyzed by quantitative PCR from two independent precipitations using an ABI PRISM 7900 HT sequence detection system (Applied Biosystems). Reactions were performed in a final volume of 25 $\mu$L containing Jump Start SYBR Green (Sigma-S4438). Gene-specific primers were designed to cover gene promoter regions containing known cis-elements (Supplemental Table S1 and Supplemental Fig. S6; Koornneef et al., 2008; Jaskiewicz et al., 2011). Results were normalized to initial DNA amounts in the input control, as described (Haring et al., 2007), and standardized to levels in corresponding $C_1$ progeny.

## Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Fitness of $C_1$ and $P_1$ plants.

**Supplemental Figure S2.** Level of resistance against *H. arabidopsidis* WACO9 in independent $C_1$ and $P_1$ progeny lines from individual wild-type and *npr1-1*.

**Supplemental Figure S3.** Level of resistance against *H. arabidopsidis* WACO9 in independent $C_1C_2$, $P_1C_1$, and $P_1P_2$ progeny lines from individual wild-type $C_1$ or $P_1$ plants.

**Supplemental Figure S4.** Basal resistance against bioluminescent *Pst*DC3000-lux in $C_1$ and $P_1$ wild-type and *npr1-1* plants.

**Supplemental Figure S5.** Lack of transgenerational repression of the JA response in the *npr1-1* mutant.

**Supplemental Figure S6.** Transgenerational modifications of histone H3 at different regions of the *PR-1* and *PDF1-2* gene promoters.

**Supplemental Figure S7.** No difference in resistance between $C_1$ and $P_1$ progenies of the *dmr1dmr2ctm3* (*ddc*) mutant.

**Supplemental Figure S8.** Basal resistance of Col-0 and *ddc* plants against bioluminescent *Pst*DC3000-lux *Alternaria brassicicola*.

**Supplemental Table S1.** Primers used for quantification of immunoprecipitated promoter DNA.

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