Descendants of Primed Arabidopsis Plants Exhibit Resistance to Biotic Stress[1][W][OA]

Ana Slaughter, Xavier Daniel[2], Victor Flors, Estrella Luna, Barbara Hohn, and Brigitte Mauch-Mani*

Laboratoire de Biologie Moléculaire et Cellulaire, Institut de Biologie, Université de Neuchâtel, 2009 Neuchâtel, Switzerland (A.S., X.D., B.M.-M.); Plant Physiology Section, Universitat Jaume I, 12071 Castellón, Spain (V.F.); Centre for Sustainable Pest and Disease Management, Rothamsted Research, Harpenden, Hertfordshire, AL5 2JQ United Kingdom (E.L.); and Friedrich Miescher Institute, 4000 Basel, Switzerland (B.H.)

An attack of plants by pathogens or treatment with certain resistance-inducing compounds can lead to the establishment of a unique primed state of defense. Primed plants show enhanced defense reactions upon further challenge with biotic or abiotic stress. Here, we report that the primed state in Arabidopsis (*Arabidopsis thaliana*) is still functional in the next generation without additional treatment. We compared the reactions of Arabidopsis plants that had been either primed with β-aminobutyric acid (BABA) or with an avirulent isolate of the bacteria *Pseudomonas syringae* pv *tomato* (*PstavrRpt2*). The descendants of primed plants showed a faster and higher accumulation of transcripts of defense-related genes in the salicylic acid signaling pathway and enhanced disease resistance upon challenge inoculation with a virulent isolate of *P. syringae*. In addition, the progeny of primed plants was also more resistant against the oomycete pathogen *Hyaloperonospora arabidopsidis*. When transgenerationally primed plants were subjected to an additional priming treatment, their descendants displayed an even stronger primed phenotype, suggesting that plants can inherit a sensitization for the priming phenomenon. Interestingly, this primed to be primed phenotype was much reduced in the Arabidopsis β-amino-butyric acid priming mutant *ibs1* (induced BABA sterility1). Our results demonstrate that the primed state of plants is transferred to their progeny and confers improved protection from pathogen attack as compared to the descendants of unprimed plants.

Plants have developed sophisticated mechanisms to protect themselves against biotic and abiotic stresses (Jones and Dangl, 2006; Hirayama and Shinozaki, 2010). An impressive amount of information is available on the genes and signaling pathways involved in the plants' reaction following exposure to a given stress situation (Feys and Parker, 2000; Thomma et al., 2001; Kunkel and Brooks, 2002; Turner et al., 2002; Fujita et al., 2006; Hirayama and Shinozaki, 2007). Triggering of the defensive state of a plant can lead to acquired resistance in the case of biotic stresses or to acclimation for abiotic stresses (van Loon, 1997).

In numerous plant species acquired resistance functions against a wide array of pathogens (Kuc, 2001).

Interestingly, acquired resistance can spread systemically throughout the plant. When the induction of resistance is due to nonpathogenic rhizobacteria in the rhizosphere it is referred to as induced systemic resistance (van Loon, 1997). Induced resistance resulting from a natural induction with a pathogen or the induction with chemical inducers is referred to as systemic acquired resistance (SAR; Ryals et al., 1996; Sticher et al., 1997). The manifestation of SAR is tightly correlated with the local and systemic activation of defense-related genes such as genes coding for pathogenesis-related (PR) proteins (Ryals et al., 1996). As during pathogen stress, defense pathways can also be up-regulated by applied chemical stimuli. Resistance induced by the nonprotein amino acid β-amino-butyric acid (BABA) leads to a substantial induction of *PR* genes only upon challenge inoculation (Zimmerli et al., 2000). Based on northern blots it was originally described that BABA does not induce the expression of *PR* genes by itself (Zimmerli et al., 2000; Ton et al., 2005) but the availability of more sensitive technologies such as microarrays and PCR revealed that BABA treatment itself leads to an induction of *PR* genes, although to much lower levels than observed after pathogen attack (Zimmerli et al., 2008).

When a treatment puts a plant in a state of increased alertness with no or only minimal gene induction it is called sensitizing or priming (Jakab et al., 2001; Conrath et al., 2002; Prime-A-Plant Group et al., 2006).

Priming of defense responses confers a fitness advantage over direct induction of resistance as observed in traditional SAR responses that infer high costs in absence of pathogens (van Hulten et al., 2006). An overview on molecular mechanism(s) possibly underlying priming for defense was recently presented by Conrath (2011). There is increasing evidence that plants have a memory of encountered stress situations that allow them to better adapt to changing conditions. In addition, several studies have shown the relevance of epigenetic mechanisms underlying plant defense responses (Alvarez-Venegas et al., 2006; March-Díaz et al., 2008; Walley et al., 2008; Jaskiewicz et al., 2011). Furthermore, the relation between *PR* gene expression and functionality of the SUPPRESSOR OF PR1-1 INDUCIBLE1 (SNI1) SNI1 protein has also been linked to epigenetic regulation of somatic DNA recombination. SNI1 represses *PR* gene transcription by modifying levels of histone H3 acetylation and histone H3K4 dimethylation (Mosher et al., 2006). Better understanding of how and in what situations plants can use this memory is of great scientific as well as applied interest.

Here, we analyze inheritance of priming. We compare the resistance of same-generation-primed plants and their descendants toward virulent *Pseudomonas syringae* (*Pst*) DC3000 and virulent isolates of the oomycete *Hyaloperonospora arabidopsidis*. To this end, Arabidopsis (*Arabidopsis thaliana*) plants either mock treated, treated with BABA, or inoculated with avirulent *Pst* and the progeny of these plants was analyzed for primed expression of defense-related *PR* genes and disease resistance. We found that when challenged with a pathogen, the progeny of primed plants showed strongly enhanced expression of defense-related genes and enhanced disease resistance compared to the progeny of nonprimed plants.

RESULTS

To study a possible transgenerational inheritance of priming and induced resistance, Arabidopsis lines that had been subjected to specific treatments with the goal of inducing resistance and/or priming were generated (Fig. 1). The role of BABA was tested in Arabidopsis accession Wassilewskija-0 (Ws-0) and in the priming

mutant *ibs1* (in the Ws-0 background; Ton et al., 2005). The parental lines Ws-0 and *ibs1* were treated once with water as a control or with BABA solution in water. The plants were grown to seed set and the resulting selfed progeny (S_1 progeny) was called WsH and *ibs1*H, respectively, in the case of water (H)-treated plants, and WsB and *ibs1*B, respectively, for BABA (B)-treated plants. Plants from the S_1 progeny (WsB and *ibs1*B) were again either treated with water or with BABA, respectively. They were grown to seed set thus giving rise to the S_2 progeny. S_2 plants derived from S_1 WsB and S_1 *ibs1*B plants and treated with water were called WsBH and *ibs1*BH, respectively. S_2 plants derived from S_1 WsB and S_1 *ibs1*B plants and treated with BABA were called WsBB and *ibs1*BB, respectively.
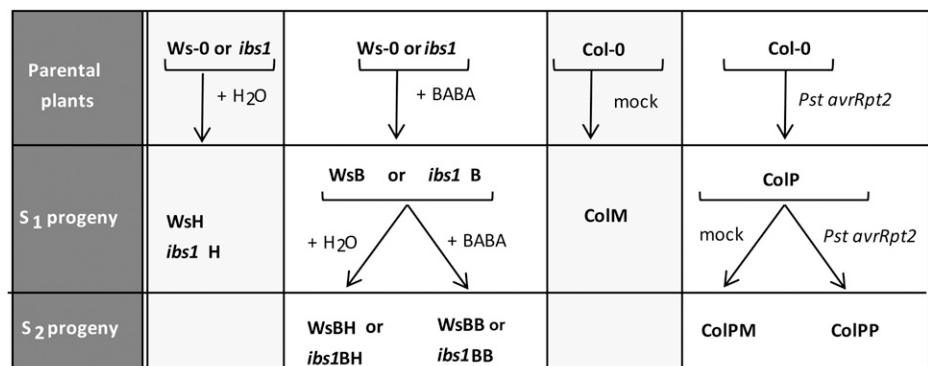
Arabidopsis plants accession Columbia-0 (Col-0) were inoculated with avirulent bacteria carrying the avirulence gene *avrRpt2* and were tested for a possible transgenerational effect (Fig. 1). Col-0 parental plants were either mock inoculated, giving rise to a S_1 progeny called ColM or inoculated with a suspension of avirulent *Pst* in buffer, yielding S_1 progeny seeds called ColP (P standing for *Pseudomonas*). ColP was sown and again either mock inoculated with buffer or inoculated with a suspension of avirulent *Pst* in buffer. The seeds harvested from these plants (S_2 progeny) were called ColPM and ColPP, respectively.

The Priming State Induced by BABA Is Transferred to the Next Generation

A transient accumulation of transcripts of salicylic acid (SA)-dependent marker genes is a hallmark of BABA-IR and priming (Zimmerli et al., 2000; Ton et al., 2005). Therefore, we studied *PR1* expression, over a time course of 48 h, in 5-week-old plants of WsH, *ibs1*H, WsB, *ibs1*B, WsBH, *ibs1*BH, WsBB, and *ibs1*BB that were soil drenched with 25 ppm BABA or with water as a control, respectively (see Supplemental Fig. S1A for experimental design).

BABA treatment induced a transient enhancement of *PR1* transcript levels in WsH plants (Fig. 2). Interestingly, transcript levels of *PR1* were 3-fold higher in WsB and 4.6-fold higher in WsBB compared to WsH. In contrast, WsBH plants contained similar transcript levels as WsH (Fig. 2A), indicating that priming for

Figure 1. Experimental design. Parent plants: Ws-0 (Ws wild-type plants), *ibs1* (a priming mutant in Ws-0 background), and Col-0 (Col wild-type plants). S_1 progeny: descendants from the selfed parent plants; S_2 progeny: descendants from the selfed S_1 plants. The plants were treated with: water = H; BABA = B; mock treated with buffer = M; or inoculated with avirulent *Pst* pv *tomato* containing *avrRpt2* = P.

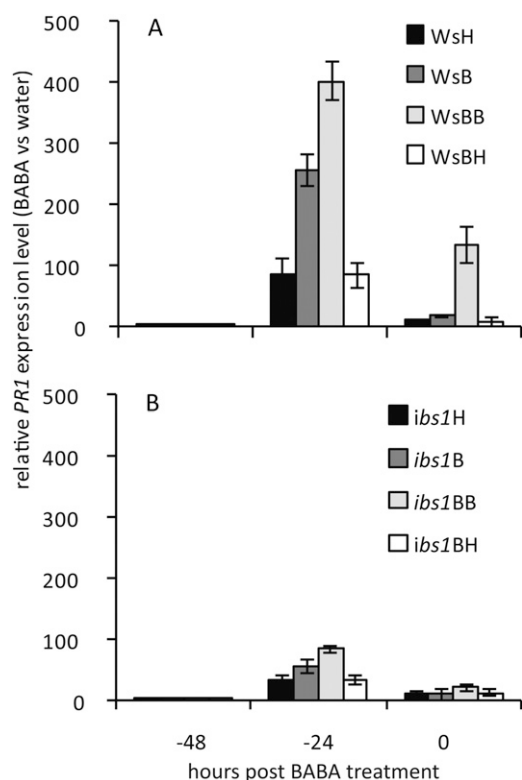| Parental plants	Ws-0 or *ibs1* + H₂O	Ws-0 or *ibs1* + BABA	Col-0 mock	Col-0 *Pst avrRpt2*
S_1 progeny	WsH *ibs1* H	WsB or *ibs1* B + H₂O / + BABA	ColM	ColP mock / *Pst avrRpt2*
S_2 progeny		WsBH or *ibs1*BH WsBB or *ibs1*BB		ColPM ColPP

Figure 2. Basal *PR1* expression levels in the progeny of Ws-0 plants and *ibs1* plants (see Fig. 1). Four-week-old plants were soil drenched with water or a solution of BABA to a final concentration of 25 ppm in the soil. Transcript levels were analyzed with qRT-PCR. Expression was normalized to the sample treated with water at 0 h. *PR1* expression in the different Ws-0 lines (A) and in the different *ibs1* lines (B) is shown. The values represent means ± SD of three replicates. Similar results were obtained in three independent experiments.

gene expression was switched off after one generation. The progeny of the *ibs1* mutant showed a similar pattern although the amplitude of the reaction was much reduced (Fig. 2B). Our results show that the progeny of Ws-0 as well as of *ibs1* are sensitized to the priming of *PR1* triggered by BABA, thus displaying a memory of the treatments to which their parents had been subjected to. Furthermore, the facts that *ibs1* lines are severely affected in the induction of *PR1* upon infection and that Ws-0 or *ibs1* descendants do not show induction of *PR1* in the absence of BABA treatments (Supplemental Fig. S2, water-treated plants) strongly indicate that this phenomenon is related to priming of defense. The results also show that the memory had to be rescued, as in the absence of further priming treatments the level of *PR1* transcripts was not elevated. This is consistent with the transient nature of the priming activity.

Resistance against *Pst* DC3000 Is More Effective in the Progeny of BABA-Treated Plants

Parent Ws-0 and *ibs1* plants and descendants as shown in Figure 1 were either treated by soil drench

with 25 ppm BABA or water, respectively. Forty-eight hours after treatment (time 0; Supplemental Fig. S1a), plants were inoculated with virulent *Pst* DC3000. Bacterial growth was monitored by quantitative reverse transcription (qRT)-PCR using *Pst*-DC3000-specific primers over a time course of 72 h after inoculation.

Descendants of BABA-primed plants (WsB, WsBB, and WsBH) showed enhanced resistance against *Pst* DC3000 compared to WsH (Fig. 3; Supplemental Fig. S3). In addition, the level of resistance of the descendants was stronger according to the number of treatments accumulated through previous generations. Furthermore, a similar pattern of resistance was observed after BABA treatment prior to infection, resulting in a more pronounced resistance in the progeny of primed Arabidopsis (Fig. 3B). BABA-treated *ibs1* progeny also showed transgenerational enhanced resistance against *Pst* DC3000 although they lacked the improved capacity to express BABA-IR observed in wild-type progeny plants.

Priming efficiency, expressed as the quotient of bacterial growth between two generations varied from 1.6 to 1.9 in BABA-treated Ws-0 lines. This quotient was between 1.1 and 1.4 for BABA-treated *ibs1* lines, water-treated *ibs1* lines, and for water-treated Ws-0 lines (Supplemental Table S1).

Transgenerational Priming for Defense Is Supported by SA Marker Gene Sensitization

The priming state of the plants was reflected at the molecular level in the Ws-0 lines and *ibs1* lines upon inoculation with *Pst* DC3000. *PR1* (Fig. 3C) and *PR2* and *PR5* transcript accumulation (Supplemental Fig. S4, A and B) remained low in all inoculated water controls. In BABA-treated plants inoculated with *Pst* DC3000, *PR1* accumulation peaked at 24 h post inoculation (hpi) in WsH. WsB plants reacted earlier and stronger and the reaction was even more pronounced in WsBB (Fig. 3C). WsBH plants displayed similar kinetics and intensity of *PR1* transcript accumulation as the WsH plants.

In the *ibs1* lines the accumulation of *PR1* transcripts was delayed and attenuated compared to the corresponding Ws-0 line. *ibs1*H plants displayed a low relative expression level peaking at 24 hpi while in *ibs1*B the expression level was twice as high but only at 48 hpi. *ibs1*BB peaked at 24 hpi with 3-fold-higher level than *ibs1*H. As for WsBH, in *ibs1*BH *PR1* accumulation levels reverted to *ibs1*H level.

The same trend was observed for *PR2* and *PR5* transcript accumulation (Supplemental Fig. S4, A and B). These results show that although all plants reacted to BABA treatment with an induction of *PR* gene transcripts following inoculation with *Pst* DC3000, the reaction was much more pronounced in the priming-competent Ws-0 lines than in the priming-impaired *ibs1* lines.
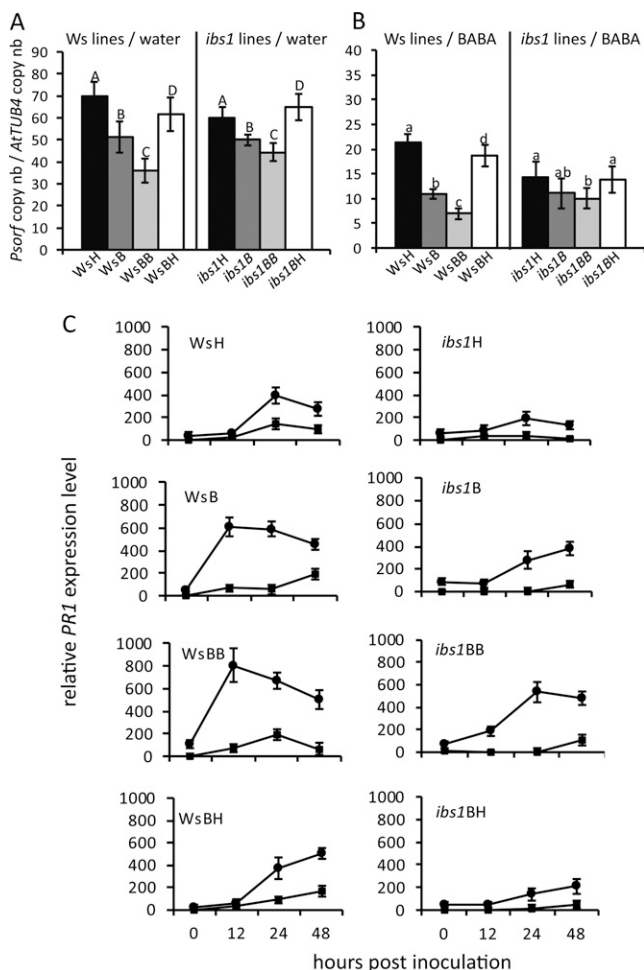
Figure 3. Descendants of BABA-treated Ws-0 plants are more resistant to virulent *Pst*. Three-week-old plants were treated with BABA (25 ppm final concentration in the soil) or water 2 d prior to inoculation with *Pst* DC3000 (OD$_{600}$ = 0.08). A, Growth of *Pst* DC3000 in the water-treated Ws-0 lines (WsH, WsB, WsBB, and WsBH) and *ibs1* lines (*ibs1*H, *ibs1*B, *ibs1*BB, and *ibs1*BH) at 72 hpi. B, Growth of *Pst* DC3000 in BABA-treated Ws-0 lines and in *ibs1* lines at 72 h postinoculation. Bacterial growth was quantified by qRT-PCR as transcript levels of *Psorf* normalized to the transcript level of the Arabidopsis gene *AtTUB4*. Capital letters indicate statistically significant bacterial growth within the water-treated Ws-0 and *ibs1* lines (ANOVA, Student-Newman-Keuls, *n* = 3, *P* < 0.05). Small letters indicate statistically significant bacterial growth within the BABA-treated Ws-0 or *ibs1* lines. C, qRT-PCR analysis of *PR1* gene expression in BABA-treated (circles) and water-treated (squares) Ws-0 and *ibs1* lines. Expression was normalized to the corresponding sample treated with water at 0 h. The values represent means ± sd of three replicates. Similar results were obtained in three independent experiments.

Transgenerational Inheritance of Priming Induced by Avirulent *Pst*

To demonstrate that transgenerational priming is not only observed following chemical induction, we performed a set of experiments using a natural priming phenomenon. Since preliminary tests had shown that S$_1$ descendants of both Ws-0 and Col-0, respectively, showed transgenerational priming (data not shown),

we decided to use Col-0 plants for this experiment to demonstrate that transgenerational priming was also not bound to a specific Arabidopsis accession. Leaves of accession Col-0 were infiltrated with avirulent *Pst*, leading to a localized hypersensitive reaction. An overview of treatments and generations is shown in Figure 1.

Challenge inoculation of the various Col-0 lines with virulent *Pst* DC3000 showed that transgeneration priming is also observed following an inducing inoculation of parent plants with avirulent *Pst*. At all time points post inoculation, the ColP and ColPP plants supported less bacterial growth compared to ColM plants (Fig. 4A). The restriction in bacterial growth correlated with reduced disease symptoms; ColM and ColPM plants showed more severe disease symptoms than the ColP and the ColPP plants (Fig. 4, B and C). Therefore, our data show that Arabidopsis plants exposed to same-generation priming by a necrotizing infection with avirulent bacteria transfer the experience of the encountered stress situation to their descendants. Interestingly, ColPM plants showed no significant reduction of disease symptoms and the same *PR1* transcript levels compared to ColM (Fig. 4B). As it was observed with BABA-treated progeny, transgeneration priming did not last after a priming-free generation, suggesting that BABA- and *PstavrRpt2*-induced transgenerational resistance may be temporarily maintained via similar mechanisms.

The differences in basal levels of resistance among the studied lines correlated with levels of transcript accumulation of *PR1* (Fig. 4D), *PR2* (Supplemental Fig. S5A), and *PR5* (Supplemental Fig. S5B).

Transgenerational Inheritance of Priming against *H. arabidopsidis*

The results described above demonstrate that transgeneration priming is associated with the sensitization of the SA-dependent response. To test whether this phenomenon was also effective against other SA-sensitive pathogens, we inoculated BABA- and water-treated progeny, as well as the progeny of plants mock or avirulent *Pst* inoculated, with virulent strains of the obligate biotrophic oomycete *H. arabidopsidis* (isolate Noco for Col-0 and isolate Emwa for Ws-0). Disease severity was assessed at 7 d post inoculation in trypan-blue-stained leaves (Fig. 5). The leaves were classified into five categories according to their degree of colonization (Fig. 5A). The control lines ColM and WsH (Fig. 5) plants consistently showed a higher degree of colonization by the oomycete than the primed descendants WsB and WsBB (Fig. 5B), ColP, and ColPP (Fig. 5C). Second generation descendants whose parents were not subjected to an additional priming treatment in the S$_1$ generation reverted to a more susceptible infection phenotype (Fig. 5, B and C). These results show that the progeny of Arabidopsis plants exposed to same-generation priming with avirulent bacteria or with BABA in the
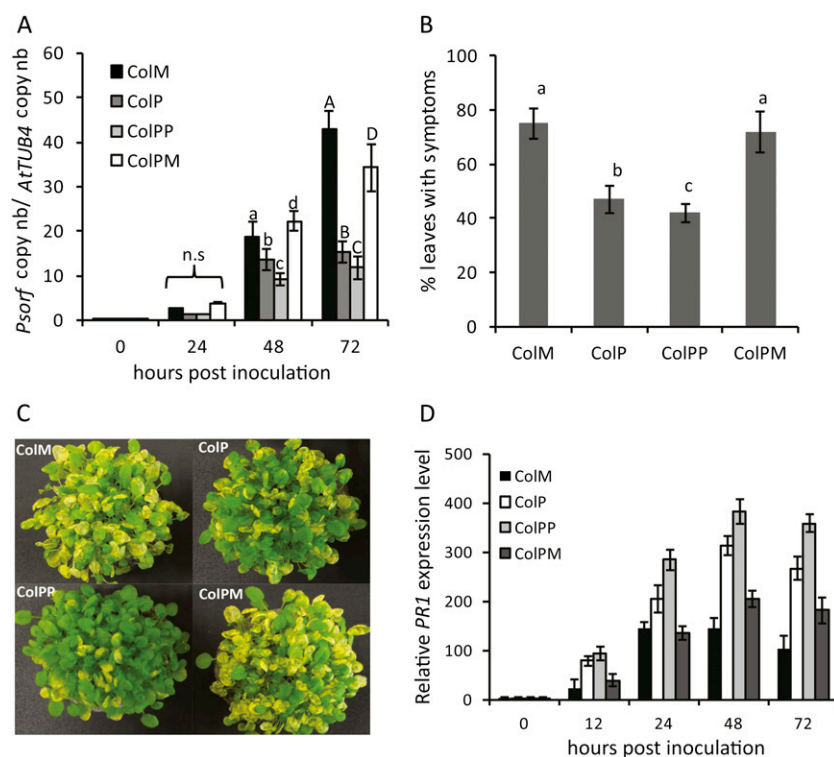
Figure 4. Inheritance of priming induced by avirulent *Pst* (*PstavrRpt2*). Single leaves of 4-week-old plants were infiltrated with *Pst avrRpt2* or mock infiltrated with buffer. A, Col-0 lines (see Fig. 1) were challenge inoculated with *Pst* DC3000 and bacterial growth was quantified by qRT-PCR as described for Figure 3. Small and capital letters above error bars represent statistically significant difference in bacterial growth within Col-0 lines at 48 and 72 h postinoculation (ANOVA, Student-Newman-Keuls, $n = 3$, $P < 0.001$). Values represent means $\pm$ SD of three biological replicates. B, Quantification of disease resistance of Col lines 5 d post inoculation. Letters represent statistically significant difference in the percentage of leaves with symptoms (ANOVA, Student-Newman-Keuls, $n = 30$, $P < 0.05$). C, Disease phenotype of Col lines. D, qRT-PCR analysis of *PR1* gene expression in Col lines. Expression was normalized to the sample treated with water at 0 h. The values represent means $\pm$ SD of three replicates. Similar results were obtained in three independent experiments.

parental generation are more resistant to *H. arabidopsidis*.

Priming in the Progeny of BABA-Treated Plants Is Not Due to Transfer of BABA to the Next Generation through the Seeds

To assess whether the priming observed in the progeny of BABA-treated plants might be due to a transfer of BABA to the next generation through the seeds, we measured BABA concentrations in untreated plants and plants soil drenched to a final concentration of 40 ppm (Supplemental Fig. S6A) and 25 ppm BABA (Supplemental Fig. S6B), respectively. No BABA was present in untreated plants. The highest concentration of BABA was detected within days after treatment. Traces of BABA were found in the flowers of BABA-treated plants and in the derived S_1 seeds (Supplemental Fig. S6A). We also analyzed BABA content in 7-d-old S_1 seedlings and 5-week-old S_1 rosette stage descendants of BABA-treated Col-0 plants. The traces of BABA detected (<2.2 μg/g dry weight) were considerably below the concentration threshold for resistance induction that corresponds in planta with 50 μg/g dry weight (calculated empirically from experiments performed over the years in our lab). Hence, the BABA level in the progeny of BABA-treated plants is far too low to be responsible for the observed transgenerational priming. Our conclusion is further supported by the fact that transgenerational priming is also observed in the absence of BABA in the progeny of plants inoculated with avirulent *Pst*.

Changes in DNA Methylation Levels in the PR1 Promotor

Since the *PR1* gene has been shown to be very responsive to priming and this effect is transferred to the next generation (this article) we decided to assess the DNA methylation level in the region of the *PR1* promotor where many of the elements possibly involved in such an increased induction are located. To this end we subjected plant DNA to bisulfite treatment and quantified the pattern of methylation of CGN, CHG, and CHH sites over a 503-bp-long region in the generated clones using the CyMate program (Hetzl et al., 2007). The CyMate output for methylation analysis is depicted in Supplemental Figures S7 and S8. This analysis was performed on samples of WsH, WsB, WsBB, and WsBH as well as on samples of *ibs1*H, *ibs1*B, *ibs1*BB, and *ibs1*BH. As shown in Supplemental Figure S9, only minimal changes in overall c-methylation frequency were observed in the sequenced clones. This is also true for the methylation pattern of specific motives (WRKY/W boxes; TGA binding sites; ERF binding site; PR-box and NF-κB binding site) within the *PR1* promotor section tested (data not shown).

DISCUSSION

Our results show that subjecting Arabidopsis plants to a treatment with the chemical BABA or an inoculation with avirulent bacteria induces a primed state
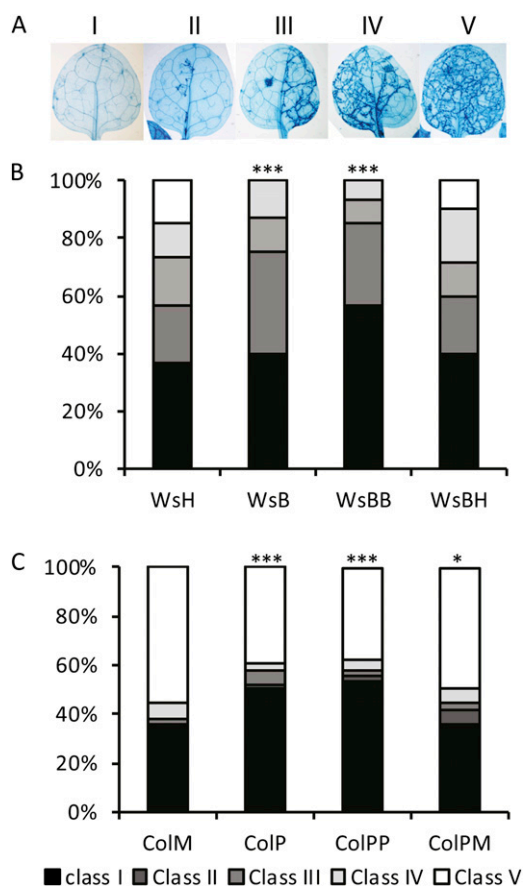
Figure 5. Inheritance of priming against *H. arabidopsidis*. A, Disease ratings were classified based on the percentage of leaf colonization by mycelium. Class I: 0%; class II: 1% to 25%; class III: 26% to 50%; class IV: 51% to 75%; and class V: 76% to 100%. Disease ratings are shown as the percentage of leaves in each resistance class. B, Disease resistance in Ws-0 lines inoculated with the Emwa strain of *H. arabidopsidis*. Asterisks indicate statistically significant differences in the percentage of leaves in the different classes compared to WsH (Fisher test, *** = $P < 0.001$). C, Disease resistance in Col lines inoculated with the Noco strain of *H. arabidopsidis*. Asterisks indicate statistically significant differences in the percentage of leaves in the different classes compared to ColM (Fisher test, * = $P < 0.05$; *** = $P < 0.001$).

that is transmitted to the progeny. The progeny of primed plants has a higher basal level of disease resistance and an enhanced capacity to react to additional priming treatments.

To study the transmission of the BABA-primed state in WsB, WsBB, and WsBH plants, we analyzed levels of transient accumulation of *PR* gene transcripts upon BABA treatment. Basal levels of *PR* expression were not altered previous to BABA treatment (Fig. 2), however WsB was sensitized to additional BABA treatments, resulting in higher *PR1* transcript levels compared to the progeny of water-treated plants (WsH). Additional BABA treatment of the WsB generation further increased the priming capacity in the WsBB generation (Fig. 2). Furthermore, the enhanced disease resistance in the progeny of primed lines

against *Pst* DC3000 correlated with increased levels of SA-dependent gene transcripts of *PR1*, *PR2*, and *PR5* upon infection, indicating general changes in the regulatory mechanisms of defense gene expression (Fig. 3). Interestingly, our data also show that priming induced by inoculation with avirulent *Pst* (*PstavrRpt2*) can also be inherited by the descendants. The progeny of plants treated with BABA or inoculated with avirulent *Pst* were significantly more resistant to virulent *Pst* and *H. arabidopsidis*, respectively, than the progeny of control-treated plants (Fig. 4). Transgeneration priming of *PR1* expression was lost in the S_2 generation (WsBH) in the absence of new BABA treatments, whereas the resistance phenotype was still partially detected.

Interestingly, BABA treatment of a parental generation sensitizes its descendants to be primed more effectively: The progeny is primed to be primed. In contrast, the *ibs1* mutant that is defective in priming (Ton et al., 2005), was compromised in the primed to be primed response (Fig. 3). Transgenerational enhancement of basal resistance was, however, not affected in *ibs1*, suggesting the involvement of different underlying mechanisms in the two phenomena.

We had previously shown that BABA was able to prime plants to react faster to biotic and abiotic stress (Zimmerli et al., 2000; Jakab et al., 2005; Flors et al., 2008; Gális et al., 2009) and it is accepted that priming occurs in many plant species and with various priming agents (Agrawal, 2002; Conrath et al., 2002; Prime-A-Plant Group et al., 2006). Despite numerous reports on the same generation, examples pointing to transgenerational priming are scarce. Wild radish (*Raphanus sativus*) that had been fed on by *Pieris rapae* or treated with jasmonate, respectively, yielded progeny that displayed an increase in resistance to herbivores (Boyko et al., 2007). Enhanced virus resistance was reported in the progeny of virus-induced tobacco (*Nicotiana tabacum*) plants (Boyko et al., 2010; Kathiria et al., 2010). In addition, other studies describe transgeneration effects in the progeny of plants infected with the virulent *Pst* DC3000 or damaged by herbivory attack, which are mediated by the up-regulation of the SA- or jasmonic-acid-related signaling pathway, respectively (Luna et al., 2011; Rasmann et al., 2011). Therefore, this suggests that the molecular mechanisms underlying induced defense inheritance may depend on the stress or the priming agent to which parental lines were exposed to. The capacity for transgenerational priming on the other hand is clearly not accession specific since we observed it in both Arabidopsis accessions with Ws-0 and with Col-0 background, respectively.

In our study, for both BABA- and *Pst*-primed plants the primed state was only upheld in the immediate progeny. Without renewed priming treatment of the parent generation, in the progeny, the disease resistance phenotype and defense gene expression reverted to an unprimed state. In contrast, Luna et al. (2011) and Rasmann et al. (2011) demonstrated that transgenerational priming can be maintained over one stress-free

generation. However, nonpersistent transgenerational adaptive effects to further generations have been described previously (Pecinka et al., 2009). Furthermore, Luna et al. (2011) exposed parental plants to recurrent pathogen stress by *Pst* DC3000 whereas our experiments consisted in single inoculations. The timeframe in which transgenerational priming or resistance is upheld seems to be dependent on the inducer and the severity of the disease in the parental generation.

The possibility that the transgenerational priming observed in progeny from BABA-treated parents might be due to a direct transfer of BABA through the seeds to the new generation is improbable. Our BABA measurements show that already 3 weeks after treating Arabidopsis with BABA, the remaining quantities of BABA in the plant tissues were far below the threshold needed to induce resistance (Supplemental Fig. S6). BABA was undetectable in the progeny of BABA-treated plants at the time of challenge inoculation.

For the offspring to remember a past experience from the parents, the latter have to be able to perceive the specific stress, they have to store this information, retain and transmit it to the descendants. To benefit from this information the descendants have to be able to retrieve the information and translate it into appropriate reactions. Although the molecular mechanisms of same-generation priming have recently started to be better understood (for review, see Conrath, 2011), the molecular details underlying transgenerational priming are still under study. However, recent groups have demonstrated that epigenetic mechanisms play a role in plant defense and several review articles comment on the possible involvement of epigenetic mechanisms in transgenerational phenomena (Chinnusamy and Zhu, 2009; Alvarez et al., 2010; Sano, 2010). Histone modifications are examples of epigenetic mechanisms; indeed, such modifications in the promoters of defense genes have been shown to correlate with transgenerational induced resistance in Arabidopsis against abiotic (Boyko et al., 2010) and biotic stresses (Luna et al., 2011). In addition, small RNA molecules are known to be important components of gene regulation (Vaucheret, 2006) and have been described that they play a role in transgenerational effects in plants against abiotic stress (Boyko et al., 2010). In agreement with these results, Rasmann et al. (2011) describe that mutants blocked in the enzymatic activity responsible for the biosynthesis of small RNA lacked to express transgeneration-enhanced resistance to herbivores. Finally, it has been described that different defense situations such as pathogen infection (Pavet et al., 2006) or chemical treatment with jasmonic acid and SA (Verhoeven et al., 2010) resulted in changes in DNA methylation patterns. These changes are yet the most likely signal to be transmittable through meiosis. Indeed, Luna et al. (2011) showed that reduced DNA methylation in the *drm1drm2cmt3* (for *domains rearranged methyltransferase1, -2, cytosine methyltransferase3*) triple mutant mimics transgenerational priming of SA-dependent defenses. In combination with previous findings that *Pst* bacteria

induce large-scale hypomethylation in Arabidopsis (Pavet et al., 2006), it is plausible that the transmission of this defense priming is mediated by hypomethylated DNA. Although DNA methylation has long been considered to be stable, spontaneous gain or loss of DNA methylation, leading to metastable heritable changes in methylated cytosines have been described recently (Schmitz et al., 2011). Such mechanisms might explain why the priming state is not transmitted to a further generation in our system or in the one described by Rasmann et al. (2011) when the priming pressure is not upheld.

Since we observed a constant and strong priming of *PR1* expression in both BABA- and *Pst*-primed plants we tested whether demethylation in the *PR1* promotor might explain the priming behavior in our Ws-0 parental and descendant lines as well as in the corresponding *ibs1* lines lacking the strong priming of *PR1* expression. Notable changes in DNA c-methylation were found for neither of the lines and treatments in the section of the promotor sequenced nor in the specific motives corresponding to cis-elements known to be important for *PR1* expression (Supplemental Figs. S7–S9). In contrast, Pavet et al. (2006) observed a demethylation of nearly 38% following infection of Arabidopsis with *Pst*.

Inheritance of alterations in DNA methylation being the most plausible explanation for the observed transgenerational inheritance of priming, it is tempting to conclude from our results that transgenerational regulation of *PR1* expression in this case could be based on the activity of hypomethylated transacting elements of transcription factors that regulate the expression of *PR1* and related genes. Whether this is the case in our system is currently under investigation.

CONCLUSION

The capacity of a plant to express primed resistance depends on multiple signal transduction pathways (Zimmerli et al., 2000; Jakab et al., 2005; Gális et al., 2009). The emerging picture is that priming improves a plant's ability to cope with a given stress situation. Hence, a plant's capacity for priming is an important survival parameter. Inheritance of the primed state as observed in transgenerational priming is expected to contribute to improved adaptation of the progeny to environmental conditions. Our findings have obvious implications for natural and agronomical ecosystems. Especially in the latter field, transgenerational priming suggests the possibility of producing disease-resistant offspring by intentionally exposing parent plants to a priming treatment.

MATERIALS AND METHODS

Biological Material

Arabidopsis (*Arabidopsis thaliana*) accession Col-0, Ws-0, and the *ibs1* mutant were grown in Jiffy peat pellets maintained at 20°C day/18°C night temperature with 8 h light (150 μE m^{-2} s^{-1}) and 70% relative humidity. About 30 seeds per

pot were stratified at 4°C in the dark for 2 d and grown for 3 weeks before being used for *Pst* DC3000 bioassays. For the *Hyaloperonospora arabidopsidis* bioassays, 2-week-old plants were used. When the seedlings had reached the cotyledon stage, the number was reduced to 15/Jiffy (*n* = 3 in figure legends stands for *n* = 3 Jiffys with each 15 plants). One 4-week-old plant per Jiffy was used for stress induction with *Pst avrRpt2* and for BABA-priming experiments. Bacterial strains were grown as described (Zimmerli et al., 2000) with the exception that the avirulent strain *Pst avrRpt2* was grown with the addition of 25 μg mL^{-1} kanamycin for selection. *H. arabidopsidis* strains Noco and Emwa were maintained on Arabidopsis Col-0 and Ws-0, respectively.

The timing of treatments in the various bioassays is schematically presented in Supplemental Figure S1.

Generation of First and Second Progeny of BABA-Primed Lines and *Pst avrRpt2* Lines

Tap water (pH 8.2) as a control treatment or an aqueous solution of BABA (pH 8.2; Sigma; final concentration of 25 ppm in the soil) were applied as a soil drench to 4-week-old Ws-0 and *ibs1* plants. Each Jiffy stood in a small petri dish to guarantee that each plant received exactly the same amount of BABA. Flowering was induced by transferring plants to long-day conditions (16 h of light). S$_1$ plants grown from the seeds of those parent plants were designated as WsB, *ibs1*B, WsH, and *ibs1*H, respectively (see Fig. 1). They were again treated with water or BABA at 4 weeks post germination. Seeds of these plants gave rise to the S$_2$ generation WsBB, *ibs1*BB and WsBH, and *ibs1*BH lines, respectively.

Pst DC3000 carrying the avirulence gene *avrRpt2* was used to generate the *Pst avrRpt* lines (Fig. 1). Five leaves of 4-week-old Col-0 plants were syringe infiltrated with a bacterial suspension of 10^8 colony-forming units mL^{-1} in 10 mM MgSO$_4$. Mock-infiltrated plants were injected with 10 mM MgSO$_4$ (pH 8.6). Plants were maintained at 100% relative humidity for 1 week. Flowering was induced by transferring plants to long-day conditions. The resulting S$_1$ seeds (ColP and ColM, respectively) were again syringe infiltrated with bacteria or mock infiltrated to give rise to the S$_2$ generation (ColPP and ColPM, respectively). For the generation of the transgeneration seeds we started with 15 mother plants each for every line and treatment; 15 single plants each were randomly chosen to produce the subsequent generation for each treatment.

Plant Inoculation and Sample Processing

BABA (25 ppm final concentration in the soil) or water (control) was applied as soil drench to 3-week-old plants 2 d prior to inoculation with *Pst* (Zimmerli et al., 2000). BABA treatment (15 ppm final concentration in the soil) or water was applied similarly on 2-week-old plants for *H. arabidopsidis* bioassay (Ton et al., 2005).

Plants were dip inoculated with bacteria (Zimmerli et al., 2000) and disease symptoms were scored (Jakab et al., 2005).

Seedlings were spray inoculated with *H. arabidopsidis* and tissue colonization was quantified (Ton et al., 2005). Colonized leaves were assigned to five different classes based on hyphal proliferation (Fig. 5A). Fisher's exact test using R.2.11.0 software was done to show significant differences between lines and treatments.

Quantification of *Pst*

Quantification of *Pst* DC3000 growth was performed by qRT-PCR as described (Boachon et al., 2009). Genomic DNA from 3-week-old plants was extracted using the NucleoSpin plant II kit (Macherey-Nagel) and bacterial growth was quantified as transcript levels of *Psorf* normalized to the transcript level of the Arabidopsis gene *AtTUB4*. This method was used because it allows to accurately process large number of samples in a short time and to have enough material to perform gene expression analyses on the same material.

Gene Expression Analyses

Total RNA was isolated from 3-week-old plants using the NucleoSpin RNA plant kit (Macherey-Nagel) according to the manufacturer's instructions. cDNA was obtained from 1 μg of total RNA using the PrimeScript RT kit (Takara). qRT-PCR quantification of gene expression was performed in a Rotor Gene 6000 (Qiagen). Gene-specific primer sequences were designed using Primer 3 software. Three biological replicates were collected per time point. The software program Gene-X was used to calculate the mean normalized (to the housekeeping gene *SAND*) expression of the genes (Vandesompele et al.,

2002). Ct values $\pm$ sD for *PR1*, *PR2*, and *PR5* for all the lines and treatments at time zero can be found in Supplemental Table S2.

Determination of BABA Levels

Plants were frozen in liquid nitrogen and lyophilized. Dry plant material was extracted on ice with a methanol:water mixture (10:9) containing 0.01% formic acid. After centrifugation the supernatant was recovered and filtered through a 0.22-μm cellulose filter. Aliquots were injected into an ultraperformance liquid chromatograph coupled to a triple quadruple mass spectrometer (Aquity TQD, Waters). The amount of BABA in the samples was calculated by using an external calibration curve of BABA by scoring the eluting peaks using a standard C-18 Ultra Performance Liquid Chromatography column. The mass spectrometer transition selected in positive electrospray ionization (ESI) was 104 > 44 mass-to-charge ratio. The gradient, mobile phases, and other chromatographic conditions were as described (Flors et al., 2008).

DNA Extractions and Bisulphite Sequencing

Total genomic DNA was extracted from 3-week-old leaf tissue of each WsH, WsB, WsBB, WsBH, *ibs1*H, *ibs1*B, *ibs1*BB, and *ibs1*BH lines using the Qiagen DNeasy plant kit (Qiagen) according to the manufacturer's protocol. Approximately 2 μg of genomic DNA were used for bisulphite conversion. Bisulphite modification and desulfonication of genomic DNA were performed using the MethylDetector bisulfite modification kit (Active Motif, www.activemotif.com) according to the manufacturer's instructions.

Based on Johnson et al. (2003), the primer pair used was as follows: 5'-TCGGAGGAGTATATGTTATTGCTTAGAATCA-3' and 5'-TTGTTTCGTA-TCGGTAGCTTTGCCAT-3' (PCR conditions are available upon request). This allowed the amplification by PCR of a 672-bp fragment of the *PR1* promotor containing *as-1* sequences. The PCR products were inserted into pGEM-T easy vector and at least eight to 20 clones for each WsH, *ibs1*, and their respective transgenerational descendant lines were selected for plasmid isolation. The clones were sequenced (www.macrogen.com) and 503-bp segments of each sequence starting at position −679 relative to transcription initiation site of the *PR1* gene were then aligned using CLUSTAL version 1.83. The alignment files were saved, then submitted to CyMATE (http://www.gmi.oeaw.ac.at/research-groups/cymate/cymate) and analyzed with this program.

Supplemental Data

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

Supplemental Figure S1. Experimental design of resistance tests.

Supplemental Figure S2. *PR1* expression levels in Ws-0 and *ibs1* lines control treated with water.

Supplemental Figure S3. Descendants of BABA-treated Ws-0 plants are more resistant to virulent *Pst*.

Supplemental Figure S4. qRT-PCR analysis of *PR2* and *PR5* transcript levels in Ws-0 and *ibs1* lines upon inoculation with virulent *Pst*.

Supplemental Figure S5. qRT-PCR analysis of *PR2* and *PR5* transcript levels in Col-0 lines upon inoculation with virulent *Pst*.

Supplemental Figure S6. Priming of the progeny of BABA-treated lines is not due to direct transfer of BABA to the next generation.

Supplemental Figure S7. Graphical output generated by CyMate of the Ws transgenerational lines.

Supplemental Figure S8. Graphical output generated by CyMate of the *ibs1* transgenerational lines.

Supplemental Figure S9. Bilsulfite sequencing data showing percentage of CGN, CHG, and CHH methylation, as computed by the CyMate program, in a 503-bp region of the *PR1* promotor of Ws-0 and descendants and *ibs1* and descendants, respectively.

Supplemental Table S1. Comparison of priming efficiency of different generations of Ws-0 and *ibs1* lines in response to inoculation with virulent *Pst*.

Supplemental Table S2. Ct values for the transgenerational lines and treatments at 48 h.

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