

# Tobacco Mosaic Virus Infection Results in an Increase in Recombination Frequency and Resistance to Viral, Bacterial, and Fungal Pathogens in the Progeny of Infected Tobacco Plants<sup>1[C][W][OA]</sup>

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Our previous experiments showed that infection of tobacco (*Nicotiana tabacum*) plants with Tobacco mosaic virus (TMV) leads to an increase in homologous recombination frequency (HRF). The progeny of infected plants also had an increased rate of rearrangements in resistance gene-like loci. Here, we report that tobacco plants infected with TMV exhibited an increase in HRF in two consecutive generations. Analysis of global genome methylation showed the hypermethylated genome in both generations of plants, whereas analysis of methylation via 5-methyl cytosine antibodies demonstrated both hypomethylation and hypermethylation. Analysis of the response of the progeny of infected plants to TMV, *Pseudomonas syringae*, or *Phytophthora nicotianae* revealed a significant delay in symptom development. Infection of these plants with TMV or *P. syringae* showed higher levels of induction of *PATHOGENESIS-RELATED GENE1* gene expression and higher levels of callose deposition. Our experiments suggest that viral infection triggers specific changes in progeny that promote higher levels of HRF at the transgene and higher resistance to stress as compared with the progeny of unstressed plants. However, data reported in these studies do not establish evidence of a link between recombination frequency and stress resistance.

Continuous exposure to stress leads to the evolutionary selection of adaptive traits beneficial in a particular environment. Such selection of the fittest of a population of plants grown under certain environmental conditions is believed to require a long time. However, it is known that plants also possess the ability to acclimate on much shorter time scales. A modification of homeostasis, also termed acclimatization, is a well-documented process that is used for adjusting metabolism to a new environment (Lichtenthaler, 1998; Mullineaux and Emlyn-Jones, 2005).

Pathogens represent one of a variety of stresses that plants are constantly exposed to. In nature, the evolution of plant resistance to a particular pathogen, virus, bacterium, or fungus has been the result of constant interactions with said pathogen (McHale et al., 2006;

Friedman and Baker, 2007). These interactions lead to a constant plant-pathogen arms race (Ingle et al., 2006).

Plants are able to tolerate or resist pathogens in a variety of ways, which could be broadly attributed to mechanisms of innate immunity and actual gene-for-gene-based resistance. The latter one depends on direct or indirect recognition of pathogen avirulence gene products by plant resistance gene products (Whitham et al., 1994; Durrant and Dong, 2004). Pathogen recognition during this incompatible interaction triggers complex events, including a local hypersensitive response that manifests itself as a booster of radical production and activation of the salicylic acid-dependent pathway and necrotic lesions, which working together restrict pathogen spread. It also results in a plant-wide systemic acquired resistance response that provides protection and tolerance to future pathogen attacks (Durrant and Dong, 2004; Park et al., 2007; Vlot et al., 2008).

If a functional pathogen resistance gene is absent (compatible interaction), then the interaction between a plant and a pathogen is more ambiguous. How do plants that lack a resistance gene respond to infection? We have previously reported that the compatible interaction between Tobacco mosaic virus (TMV) and tobacco (*Nicotiana tabacum* 'SR1') plants lacking the TMV resistance *N* gene results in the production of a systemic signal. The signal leads to an increase in the frequency of somatic homologous recombination (HRF; Kovalchuk et al., 2003a). Based on these observations, we hypothe-

<sup>1</sup> This work was supported by Natural Sciences and Engineering Research Council of Canada Discovery and Human Frontiers Scientific Program grants to I.K.

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www.plantphysiol.org/cgi/doi/10.1104/pp.110.157263

esized that these genomic changes could be inherited. Indeed, we found that the progeny of infected SR1 tobacco plants exhibited a higher frequency of RFLPs at the loci that have similarity (more than 60%) to the Leu-rich repeat region of the *N* gene (Boyko et al., 2007).

Although several reports have shown an increase in genome instability in plants exposed to pathogens and pathogen elicitors (Lucht et al., 2002; Kovalchuk et al., 2003a; Molinier et al., 2006; Boyko et al., 2007), many questions still remained unanswered. What is the mechanism of occurrence of a pathogen-induced systemic increase in HRF? What is the mechanism of inheritance of high-frequency homologous recombination? Are elevated levels of HRF maintained throughout generations? What other changes occur in progeny of infected plants?

Here, we attempted to answer the above questions by analyzing two consecutive progenies of TMV-infected tobacco cv SR1 plants. Both progenies of infected plants showed higher levels of somatic HRF, higher resistance to TMV infection and tolerance to methyl methane sulfonate (MMS), an increase in callose deposition, as well as a higher steady-state PATHOGENESIS-RELATED GENE1 (PR1) RNA level compared with the progeny of uninfected plants. Analysis of methylation patterns has revealed global genome hypermethylation in both progenies paralleled by hypomethylation in euchromatic areas.

## RESULTS

### Generating the Progeny of Virus-Infected Plants

For this study, we used transgenic tobacco cv SR1 carrying in the genome two nonfunctional truncated

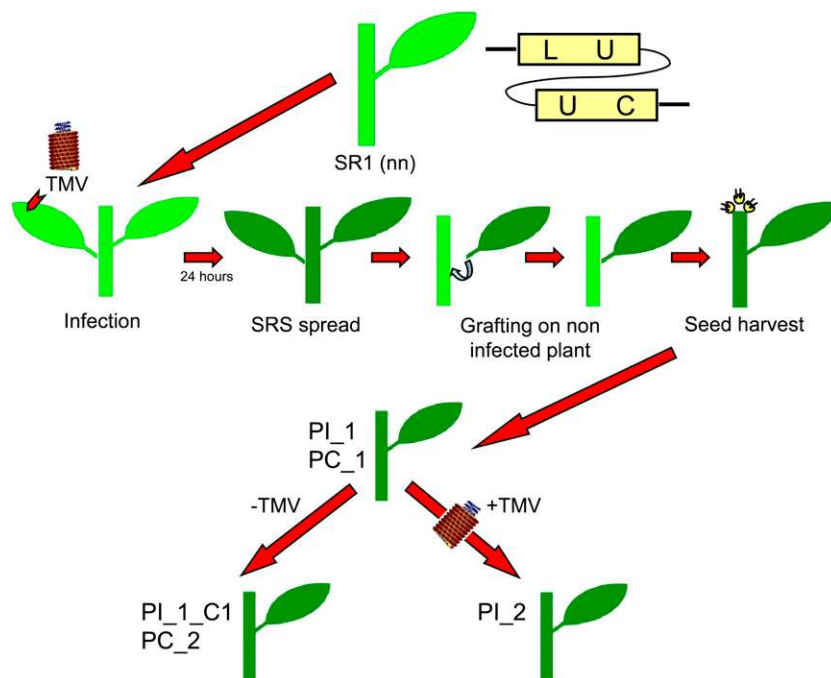
copies of the luciferase gene serving as a substrate for homologous recombination (Supplemental Fig. S1A). By using a special luciferase camera, recombination events can be visualized as bright spots (Supplemental Fig. S1B).

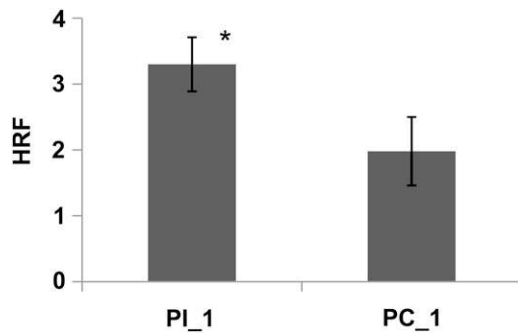
We have previously shown that infection of these transgenic tobacco plants with TMV increased HRF in infected leaves as well as in distant noninfected tissues (Kovalchuk et al., 2003a). Mock inoculation also led to an increase in HRF, but to a lesser degree (Kovalchuk et al., 2003a). For the experiments presented in this article, we used seeds collected from 20 independent infected and 20 independent mock-inoculated plants. These plants were named PI\_1 for “progeny of infected plants, generation one” and PC\_1 for “progeny of control plants, generation one” (Fig. 1). Mock-inoculated plants were used as controls.

### PI\_1 Plants Showed Higher Spontaneous and Induced Somatic HRF Compared with PC\_1 Plants

A previously observed (Boyko et al., 2007) increase in the frequency of rearrangements at *N* gene-like *R* gene loci is likely to reflect similar changes in other genomic loci. Therefore, we attempted to analyze genome stability in the progeny plants by measuring HRF at a transgene (LU-UC) locus. We analyzed the average number of somatic recombination events per plant in PI\_1 and PC\_1 plants and found that the HRF of  $3.3 \pm 0.4$  in PI\_1 plants was significantly (*t* test,  $P < 0.05$ ) different from that of  $1.98 \pm 0.5$  in PC\_1 plants (Fig. 2). Since recombination is a general DNA repair mechanism, it was possible that these changes would also be paralleled by changes in response to DNA-damaging agents and tolerance to stress.

**Figure 1.** Schematic representation of the experimental setup. Single leaves of 10-week-old SR1 tobacco plants were inoculated with 200 ng of TMV RNA (20 plants) or mock treated (20 plants). At 24 hpi, upper nontreated leaves (virus free) of these plants were grafted onto 10-week-old healthy plants (20 plants with leaves of virus-treated plants and 20 plants with leaves of mock-treated plants) from which the tops were previously removed. The darker green shows that these plants generate a systemic signal that can be transmitted via grafting. Seeds derived from newly emerged tissues were collected and designated PI or PC plants. To show that this is the first progeny, these plants were named PI\_1 and PC\_1. To analyze changes in the next generation, these plants were propagated with and without TMV. The second generation of plants was obtained and named PI\_2, PI\_1\_C1, and PC\_2 as described in the text. These seeds were used to analyze HRF and stress tolerance. SRS, Systemic recombination signal. [See online article for color version of this figure.]





**Figure 2.** Increased frequency of recombination in the first progeny of infected plants. Spontaneous HRF was analyzed in the population of 50 to 100 plants taken from each of 10 independent progenies of both infected (PI\_1) and mock-treated (PC\_1) lines, and the analysis was repeated three times. The data shown are average numbers of spots per plant with sd. The asterisk denotes a statistically significant difference between PC\_1 and PI\_1 plants ( $P < 0.05$ ).

To analyze whether stress exposure affects HRF in PI\_1 plants in a different way compared with PC\_1 plants, we exposed tobacco plants to 50, 75, and 100 mM NaCl or 4, 10, and 20  $\mu$ M rose Bengal, a photosensitizing dye that produces singlet oxygen (Ledford et al., 2007). Both chemicals induce radical production and were shown to increase HRF in plants (Puchta et al., 1995; Filkowski et al., 2004). This experiment showed that PI\_1 plants responded to stress with a higher increase in HRF ( $P < 0.05$ , two-factor ANOVA; Fig. 3). HRF decreased in plants exposed to higher doses of NaCl (100 mM) or rose Bengal (10 and 20  $\mu$ M); it was likely that a high concentration of these compounds resulted in a massive death of cells in which recombination events could have been observed.

#### The Progeny of Infected Plants Exhibit a Delay in the Onset of Symptoms of Viral Infection

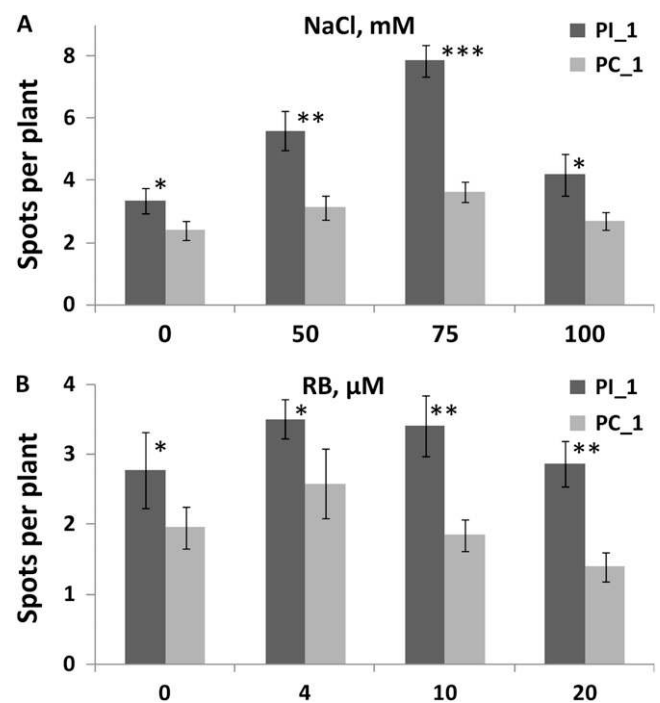
To analyze how the progeny of infected plants respond to TMV, we infected 5-week-old PI\_1 and PC\_1 plants with 200 ng of TMV U1 particles. At 5 d post infection (dpi), over half of PC\_1 plants exhibited the first symptoms of infection (light coloration between veins in young leaves). Further analysis of symptoms showed that PI\_1 plants had a significantly lower percentage of plants with these symptoms at 5, 6, 7, and 9 dpi ( $P < 0.01$  in all cases, single-factor ANOVA; Fig. 4A). At 12 and 15 dpi, the number of plants with the symptoms was statistically similar in PI\_1 and PC\_1 plants ( $P > 0.1$ , single-factor ANOVA; Fig. 4A), although several PI\_1 lines had plants with no symptoms up to 15 dpi. All PC\_1 plants exhibited the symptoms at 7, 9, 12, and 15 dpi.

The appearance of symptoms could probably correlate with the presence of virus in systemic tissues. Based on the data of symptom appearance, it was decided to check a virus titer at 6, 9, and 12 dpi. The analysis showed that at 6 dpi, the average viral titer in

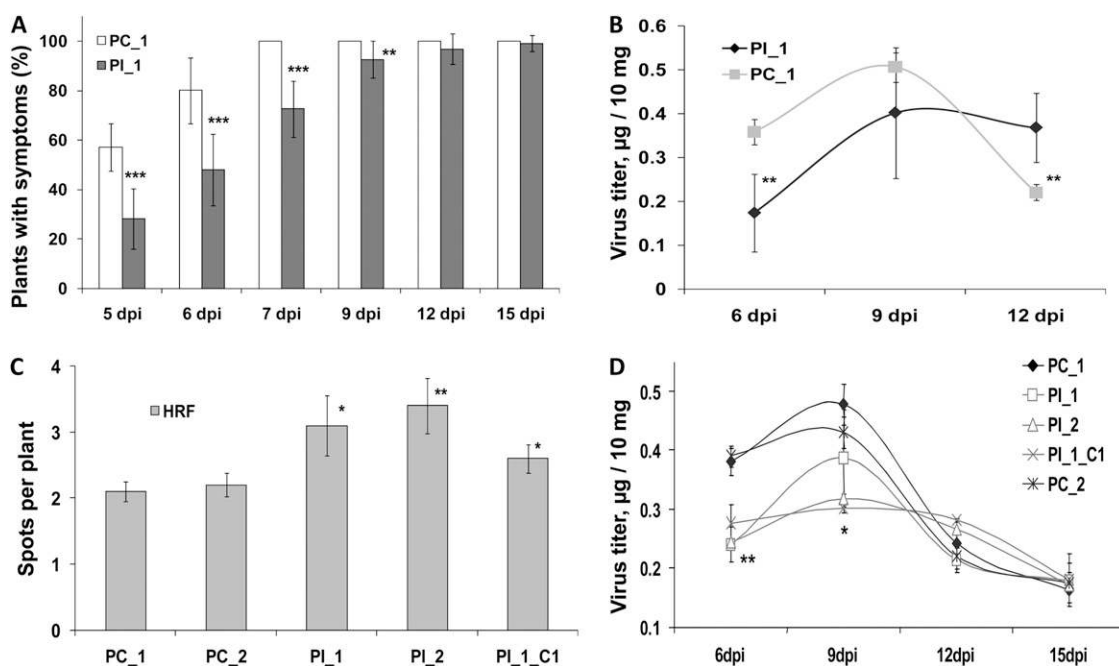
PI\_1 plants was significantly lower ( $P < 0.01$ ) than that in PC\_1 plants (Fig. 4B). A similar trend was observed at 9 dpi, although the difference was not significant ( $P > 0.1$ ; Fig. 4B). In most PI\_1 lines, virus concentrations were higher at 12 dpi compared with PC\_1 lines. An increase, peak, and decline in the viral titer is a known phenomenon observed before in tomato (*Solanum lycopersicum*) plants infected with TMV; virus concentrations peaked at 7 dpi and declined steadily on later days (Balogun et al., 2002). Thus, these experiments demonstrated that PI\_1 plants were able to significantly delay virus replication and the progression of symptoms.

#### The Progeny of Infected Plants Have Lower Bacterial Titers upon Infection with *Pseudomonas syringae* Compared with the Progeny of Uninfected Plants

Next, we attempted to analyze the response of PC\_1 and PI\_1 plants to *P. syringae*. Infection with avirulent strains of *P. syringae* pv *tomato* (Pst) DC3000 showed that PI\_1#9 and PI\_1#16 had lower bacterial counts compared with PC\_1. PI\_1#9 plants had significantly lower bacterial counts at 24 h post infection (hpi) and



**Figure 3.** PI\_1 plants exhibit a higher increase in HRF upon exposure to abiotic stress. PI\_1 and PC\_1 plants were germinated and grown for 1 week on sterile MS medium. At the age of 1 week, the plants were moved to MS plates containing various concentrations of either NaCl (A) or rose Bengal (RB; B). Recombination frequency was assayed at 3 weeks post germination. The data shown are average HRF (with sd) per single plant scored in a population of approximately 100 plants taken from each of 10 independent PI\_1 and PC\_1 lines. Asterisks denote significant differences in exposed plants as compared with control nonexposed plants: \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .



**Figure 4.** PI\_1 and PI\_2 plants exhibit delayed symptoms and a lower viral titer upon TMV infection. Single leaves of 5-week-old PI\_1 and PC\_1 plants were infected with 200 ng of TMV (for details, see “Materials and Methods”). For analysis, 100 PC\_1 plants (10 plants from each of 10 lines) and 360 PI\_1 plants (20 plants from each of 18 different PI lines) were infected. The experiment was performed in three independent sets. Asterisks denote statistically significant differences: \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ . A, Symptoms were monitored daily, and the data for 5, 6, 7, 9, 12, and 15 dpi are presented. The graph shows the average percentage (from three independent experiments with SD) of plants with symptoms (out of the total number of infected plants). B, Virus concentrations were measured in infected plants at 6, 9, and 12 dpi. The graph depicts the average viral concentrations (from 18 different PI\_1 lines and 10 different PC lines) as calculated from three independent repeats (in  $\mu\text{g mL}^{-1}$  TMV with SD). C, Spontaneous noninduced HRF analyzed in 3-week-old plants is shown as the average number of recombination events (from three independent experiments with SD) in a population of 100 to 200 plants (10–20 plants per individual line) in each experimental group. Statistical analysis was performed to identify differences between PI\_1 and PC\_1, PI\_2, and PC\_2 as well as PI\_1\_C1 and PC\_2 plants. D, Single leaves of 5-week-old plants (20 plants per treatment) were infected with 200 ng of TMV. The viral titer was analyzed at 6, 9, 12, and 15 dpi. The graph shows the average viral concentrations as calculated from three independent experiments (in  $\mu\text{g mL}^{-1}$  TMV with SD). Statistical analysis was performed to identify differences between PI\_1 and PC\_1, PI\_2, and PC\_2 as well as PI\_1\_C1 and PC\_2 plants.

72 dpi ( $P < 0.05$  for both; Fig. 5A). PI\_1#16 plants had significantly lower bacterial counts at 48 hpi (Fig. 5A).

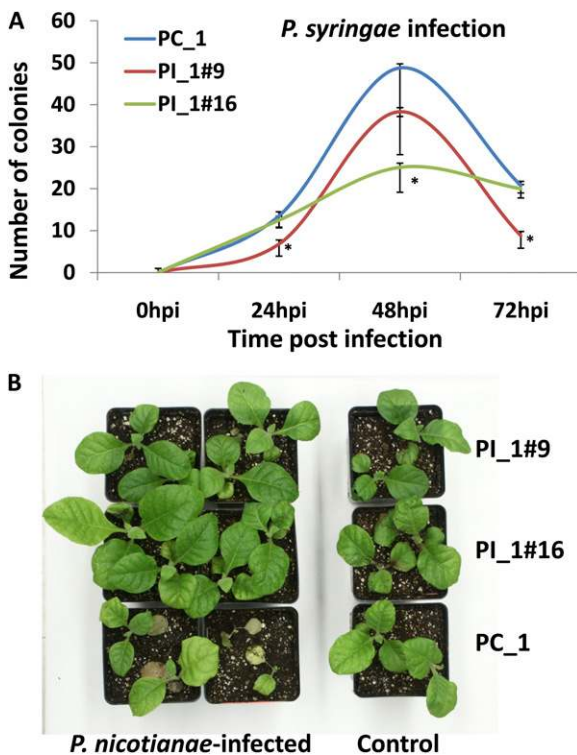
#### The Progeny of Infected Plants Have Higher Resistance to *Phytophthora nicotianae* Infection

To analyze whether PI\_1 plants also have a different response to a fungal pathogen, we infected PC\_1 plants and plants from two PI\_1 lines (PI\_1#9 and PI\_1#16) with *P. nicotianae*. Analysis of symptom appearance showed dramatic differences between PC\_1 plants and either of two PI\_1 plants (Fig. 5B). To analyze infection progression, a wilt index was calculated (0–5, with 5 being the most severe; for details, see “Materials and Methods”). The assay showed that PC\_1 plants had an average disease severity of 3.4, whereas in PI\_1#9 and PI\_1#16 it was only 0.3 and 0.5, respectively (Table I). Also, PI\_1 plants had a much lower number of plants with symptoms as compared with PC\_1 plants: whereas 87% of PC\_1 plants showed

symptoms of infection, only 26% and 33% of PI\_1#9 and PI\_1#16 plants exhibited any infection symptoms (Table I).

#### The Second Generation of Plants Exposed to Virus Also Exhibited Higher Levels of Spontaneous HRF Compared with PC\_1 Plants

Previously, Molinier et al. (2006) found that plants exposed to UV-C light maintained a high frequency of recombination for several generations. Thus, the question arises whether PI\_1 plants will exhibit a different frequency of homologous recombination if they are propagated for further generations being or not being exposed to virus. To obtain the second generation, PI\_1 plants of lines 9 and 19 were used. Plants of both lines showed significant changes in methylation patterns and in the frequency of rearrangements at the *N* gene-like resistance gene loci (Boyko et al., 2007). To obtain the second generation of control plants, PC\_1 plants of



**Figure 5.** PI\_1 plants exhibit higher resistance to *P. syringae* and *P. nicotianae*. Plants were germinated on soil and infected with the pathogen either at 4 weeks (*P. syringae*) or 6 weeks (*P. nicotianae*) post germination. A, The number of bacterial colonies (with sd) recovered from infected PC\_1 or PI\_1 plants is shown (for details, see “Materials and Methods”). Asterisks represent significant differences between PC\_1 and either PI\_1 line ( $P < 0.05$ ). B, Disease response of tobacco inoculated with an aggressive isolate of *P. nicotianae*. PC\_1 plants (bottom row) exhibited significantly more symptoms of black shank disease than PI\_1#9 plants (middle row) and PI\_1#16 plants (top row). On the far right, uninoculated plants of each line are shown. [See online article for color version of this figure.]

lines 1 and 3 were used (Fig. 2). PI\_1 plants were either mock inoculated or infected with TMV, and PC\_1 plants were mock inoculated only.

The analysis of spontaneous noninduced somatic recombination in the progeny of infected PI\_1 plants (named PI\_2) revealed a similar high level of HRF as compared with PI\_1 plants (Fig. 4C). The progeny of PI\_1 propagated under control conditions (named

PI\_1\_C1) also exhibited a high level of HRF, although it was lower than in PI\_1 and PI\_2 plants. Recombination in PC\_1 and PC\_2 plants was comparably low. These facts suggest that the propagation of plants in the presence of virus maintains a higher level of HRF, while when not being exposed to virus the propagation of plants decreases the level of HRF, albeit not to the level exhibited by control plants (Fig. 4C).

#### PI\_2 Plants Showed Lower Viral Titers Compared with PC\_1 Plants

To analyze whether the second generation of exposed plants still maintains higher tolerance to viral infection, we infected PI\_1, PC\_1, PI\_2, PI\_1\_C1, and PC\_2 plants (20 plants per treatment). The analysis of viral titers showed that all PI\_1, PI\_2, and PI\_1\_C1 plants had lower titers at 6 and 9 dpi as compared with either PC\_1 or PC\_2 plants ( $P < 0.01$ ). Significant differences were observed in all lines at 6 dpi and in all but PI\_1 at 9 dpi. The virus titers of PI\_1, PI\_2, and PI\_1\_C1 plants did not differ from each other ( $P > 0.1$ ; Fig. 4D). PC\_1 and PC\_2 plants also had a similar viral titer ( $P > 0.1$ ).

#### PI\_1, PI\_2, and PI\_1\_C1 Plants Were More Tolerant to MMS Compared with PC\_1 Plants

Next, we analyzed whether the progeny of infected plants exhibit cross-tolerance to other stresses. PC\_1, PI\_1, PI\_2, and PI\_1\_C1 plants were exposed to MMS, a DNA-damaging agent. A pilot experiment established the concentration of  $120 \mu\text{L L}^{-1}$  MMS that was ideal for the observation of potential differences in stress tolerance (Fig. 6A). We found that despite the fact that PC\_1 plants had longer roots under normal conditions than PI\_1 plants, PC\_1 plants grown in the presence of MMS had significantly shorter roots as compared with PI\_1, PI\_2, and PI\_1\_C1 plants ( $P < 0.05$ ; Fig. 6B). The analysis of plant biomass showed similar differences (data not shown).

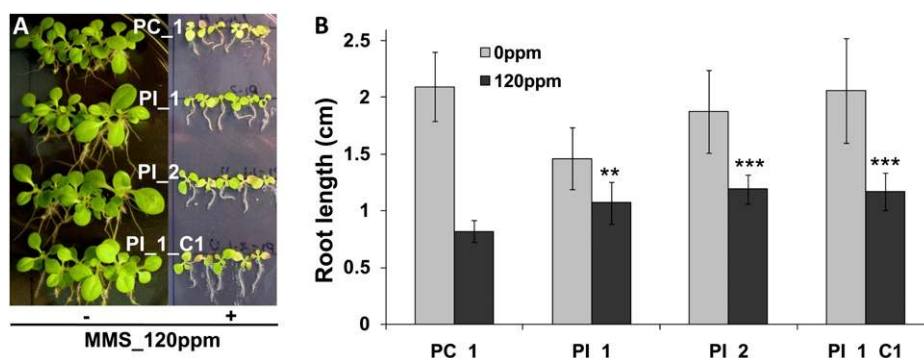
#### The Progeny of Infected Plants Exhibited Global Genome Hypermethylation

We assumed that epigenetic mechanisms might be responsible for an increase in stress tolerance. Herita-

**Table 1.** Black shank disease assay shows higher resistance of PI\_1 plants to *P. nicotianae*

The number of plants with wilt symptoms of different severity is shown, 0 indicating no symptoms and 5 indicating the appearance of the most severe symptoms. Average severity represents the appearance of average symptoms for the population of infected PC\_1 or PI\_1 plants. Incidence shows the percentage of infected plants. The difference between PC\_1 and PI\_1 lines is statistically significant ( $P < 0.01$  in all cases).

Plant	Severity						Average Severity	Incidence
	0	1	2	3	4	5		
PC_1	0	0	0	57	33	0	3.4	87%
PI_1#9	66	21	3	0	0	0	0.3	26%
PI_1#16	57	24	3	3	3	0	0.5	33%



**Figure 6.** Progeny of virus-infected plants exhibit higher tolerance to MMS. Plants were germinated on either normal medium or medium supplemented with 120  $\mu\text{L L}^{-1}$  MMS. A, Representative phenotypes of plants grown with and without MMS are shown. B, Root length was measured at 2 weeks after exposure. The average root length (in cm with SD) of 20 plants (four plates with five plants in each) was calculated. Asterisks denote statistically significant differences as analyzed between PI\_1 and PC\_1, PI\_2 and PC\_1, or PI\_1\_C1 and PC\_1 plants exposed to 120  $\mu\text{L L}^{-1}$  MMS: \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ . [See online article for color version of this figure.]

ble DNA methylation changes in the genome could allow differential gene expression in the progeny, which in turn may provide more protection for plants (Kovalchuk et al., 2003b). Analysis of global genome methylation by a cytosine extension assay showed that all PI\_1, PI\_2, and PI\_1\_C1 plants had significant genome hypermethylation compared with PC\_1 plants ( $P < 0.05$ ; Fig. 7A; data for PC\_2 were similar).

In order to analyze methylation in situ, we performed immunohistochemical staining of cross-sectioned tobacco leaves using an anti-5-methyl cytosine antibody (Fig. 7, B–D). PI\_1 plants showed higher methylation at the periphery (the brighter red intensity at the nuclear periphery) and lower methylation in the center of the nucleus (a low level of red coloring in the center of the nucleus; Fig. 7, C–E). The analysis of the ratio of red (5-methyl cytosine) to blue (4',6-diamino-phenylindole [DAPI]) confirmed hypomethylation in the center of the nucleus (Fig. 7, C–E). A higher intensity of red coloring observed in PI\_1 plants as compared with PC\_1 plants could suggest hypermethylation in the nuclear envelope; however, it was difficult to measure it quantitatively (Fig. 7, C and D).

The above-mentioned experiments revealed that PI\_1 and PC\_1 plants differ not only in global genome methylation but possibly in the distribution of methylation across the genome.

#### Infection with TMV and *P. syringae* Results in a Stronger Induction of the *PR1* Gene in PI\_1 Plants

Since we observed higher resistance of PI plants to viral infection, we hypothesized that these plants, while exposed to a pathogen, would also have higher expression of pathogenesis-related genes. Expression of *PR1* is an indicator of the attempted resistance response (Mitsuhara et al., 2008). We exposed plants of two PI\_1 lines, PI\_1#9 and PI\_1#16, and PC\_1 plants to TMV and *P. syringae* and analyzed steady-state RNA

levels of the *PR1* gene at 0, 24, and 48 hpi. Northern-blot analysis confirmed that PI\_1 plants responded with a higher increase in steady-state levels of *PR1* RNA at 24 and 48 hpi (Fig. 8).

#### PI\_1 Plants Showed a Higher Level of Callose Deposition Compared with PC\_1 Plants

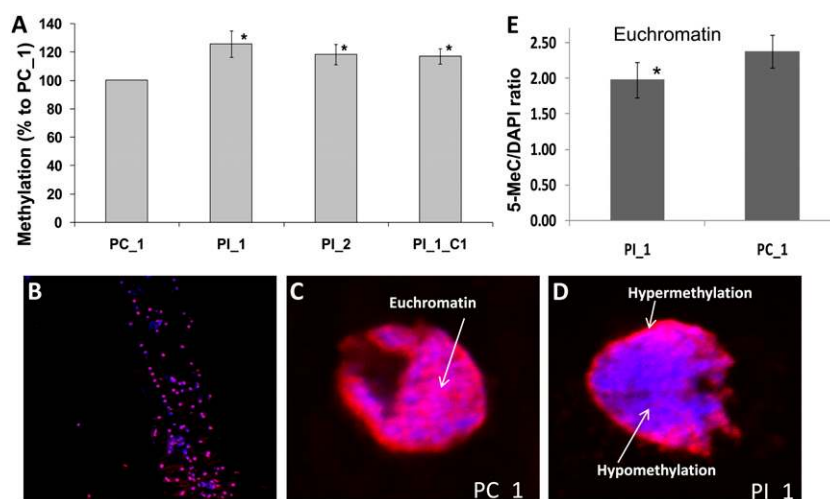
One of the mechanisms of pathogen resistance is the accumulation of callose in plasmodesmata. Callose is an amorphous  $\beta$ -1,3-D-glucan found in numerous locations in higher plants and induced by abiotic and biotic stresses (Flors et al., 2008). Rapid deposition of callose at the point of attempted penetration by fungal pathogens has been observed (Ton and Mauch-Mani, 2004). To analyze callose deposition at plasmodesmata, we performed immunohistochemical staining with anti-callose antibodies (Fig. 9). We analyzed callose deposition at 12, 24, 36, and 48 hpi with *P. syringae*. We found that PI\_1 plants from lines 9 and 16 had significantly more ( $P < 0.01$ ) callose depositions compared with PC\_1 plants (Fig. 9).

## DISCUSSION

In this article, we report that the progeny of infected plants exhibit a higher level of spontaneous and stress-induced somatic HRF as well as higher resistance to viral, bacterial, and fungal infection and tolerance to MMS exposure as compared with the progeny of uninfected plants. We demonstrate that PI plants have higher induction of the *PR1* gene and a higher level of callose deposition than PC plants.

#### Local Infection of SR1 Tobacco Plants with TMV Leads to Heritable Changes in Genome Stability

Here, we found that the progeny of infected SR1 plants (PI\_1) had higher spontaneous somatic HRF



**Figure 7.** PI plants exhibit methylation changes. A, Analysis of methylation using the cytosine extension assay. The data shown are percentages of methylation relative to methylation in PC<sub>1</sub> plants (with sd). Asterisks denote significant differences ( $P < 0.05$ ). B, Analysis of methylation via immunohistochemistry using anti-5-methyl cytosine antibodies. The image depicts a cross-sectioned leaf at low magnification. Methylated cells are in red. C, The cell nucleus of PC<sub>1</sub> plants; different degrees of methylation in the center of the nucleus (red staining for methylated areas and blue staining for DNA) and at the periphery (the margin of the nucleus, mostly red) are observed. D, The cell nucleus of a PI<sub>1</sub> plant. E, The intensity of staining in the center of the nucleus was analyzed using ImageJ software. The data shown are average ratios (with sd) of 5-methyl cytosine (5-MeC) to DAPI staining in the cells of PI<sub>1</sub> and PC<sub>1</sub> plants. The asterisk indicates a significant difference between PI<sub>1</sub> and PC<sub>1</sub> plants ( $P < 0.05$ ). [See online article for color version of this figure.]

than PC<sub>1</sub> plants. The progeny of PI<sub>1</sub> plants also exhibited a higher level of HRF, although it was less pronounced when PI<sub>1</sub> plants were propagated under normal conditions. Previously, Molinier et al. (2006) reported five consecutive generations of UV-C light-exposed plants that showed a significant increase in HRF. Unfortunately, those authors did not obtain similar results with flagellin-treated plants; they found an increase in HRF in the first progeny of flagellin-treated plants but did not profile further generations (Molinier et al., 2006).

A recent report by Pecinka et al. (2009) suggests that transgenerational changes in HRF in response to stress are rather stochastic and do not occur in response to all stresses. The authors proved that four out of 10 stresses tested resulted in changes in HRF in the progeny. Unfortunately, they did not test exposure to biotic stress (Pecinka et al., 2009).

#### The Progeny of Infected Plants Exhibit Higher Resistance to TMV Infection and MMS Compared with the Progeny of Uninfected Plants

The fact that the progeny of infected plants have higher resistance to viral infection suggests that plants possess the mechanisms that allow certain adaptations to pathogen pressure. Single-generation adaptations/acclimations have been reported, although they have primarily been described for temperature stress.

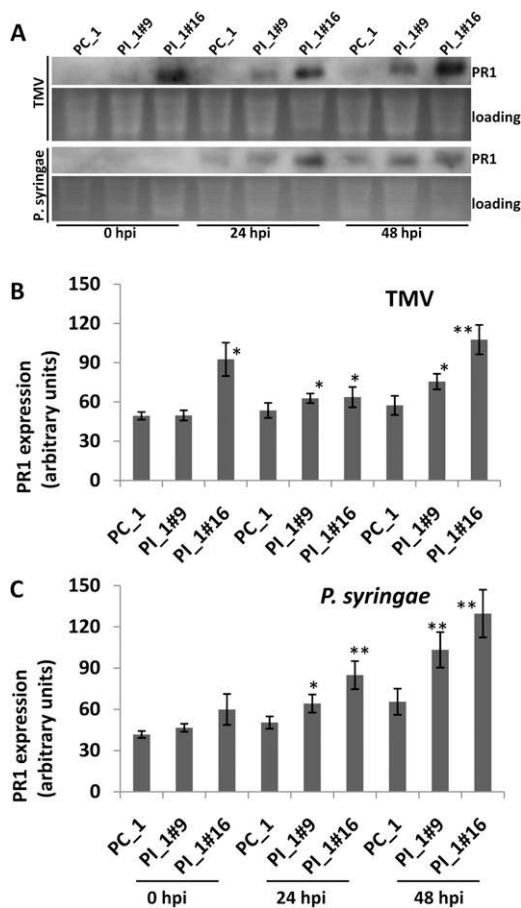
It has been shown that during the development of maternal and embryonic tissues in *Picea abies*, warm temperatures are associated with better performance of

offspring grown at the same elevated temperature (Johnsen et al., 2005). Similar findings have recently been reported for Arabidopsis (*Arabidopsis thaliana*; Blodner et al., 2007). There has been demonstrated the effect of temperature treatment memory on subsequent bud set and height growth in *P. abies* during somatic embryogenesis, thereby eliminating the influence of maternal effects per se (Kvaalen and Johnsen, 2008).

Cross-protection against MMS in the progeny of infected tobacco plants also deserves some attention. Exposure to abiotic and biotic stresses often triggers as a response a set of commonly regulated genes (Glombitza et al., 2004). It is common knowledge that plants with certain mycorrhizal associations are less sensitive to cadmium stress than nonmycorrhizal plants (Schutzendubel and Polle, 2002). Several recent reports have shown that plants exposed to UV-C light acquire protection against pathogen infection (Kunz et al., 2006, 2008). Moreover, one of the reports proved that plants treated with salicylic acid exhibited higher tolerance to UV-B and UV-C light (Mahdavian et al., 2007).

#### Higher Resistance to Stress Could Be a Result of Enhanced Innate Immunity

Higher resistance to pathogen stress could be a result of various events. Innate immunity is one of the types of broad-spectrum protection against pathogen infection. In this case, plant resistance is associated with a plant's ability to slow pathogen progression

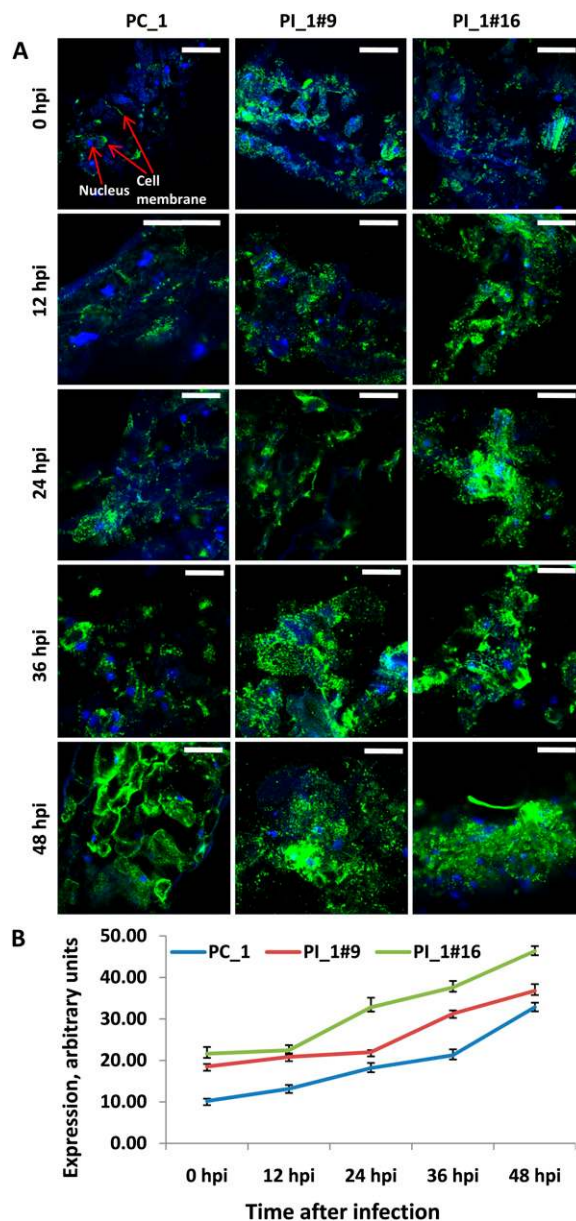


**Figure 8.** PI<sub>1</sub> plants exhibit higher levels of *PR1* expression than PC<sub>1</sub> plants. A, Northern-blot analysis of *PR1* expression in PI<sub>1</sub>#9, PI<sub>1</sub>#16, and PC<sub>1</sub> plants that were either infected with *P. syringae* or TMV or mock treated. Samples were taken at 24 and 48 hpi. Electrophoresis of total RNA from each sample is shown as a loading control. B, Quantification analysis of *PR1* expression in TMV-infected samples (see A). The data shown are averages of four independent repetitions (in arbitrary units with SD). Asterisks denote significant differences between infected PI<sub>1</sub> plants and infected PC<sub>1</sub> plants: \*  $P < 0.05$ , \*\*  $P < 0.01$ . C, Quantification analysis of *PR1* expression in *P. syringae*-infected samples (see A). The data shown are averages of four independent repetitions (in arbitrary units with SD). Asterisks indicate significant differences between infected PI<sub>1</sub> plants and infected PC<sub>1</sub> plants: \*  $P < 0.05$ , \*\*  $P < 0.01$ .

and/or to withstand a higher level of damage by a pathogen (de Wit, 2007). This is in part due to the function of secondary metabolites that could serve as signaling molecules, could be toxic to a pathogen, and could physically impair pathogen progression (Jansen et al., 2001; Asselbergh et al., 2008).

Having analyzed the amount of total phenolics, we found much higher levels of phenolics in nonstressed PI plants as compared with PC plants (Supplemental Fig. S2). Many phenolic compounds were shown to be effective protectants against stress (Jansen et al., 2001; Kovacik and Klejdus, 2008). The total phenolic contents of injured poplar (*Populus* spp.) and maple (*Acer*

spp.) tree seedlings and noninjured seedlings cohabiting with them were significantly higher than those in sequestered controls (Baldwin and Schultz, 1983). Since we observed an elevated level of phenolics in PI<sub>1</sub> plants, we assumed that these plants would also have changes in other metabolites. Using NMR spectroscopy, we analyzed metabolites in PI<sub>1</sub> and PC<sub>1</sub> plants and found that PI<sub>1</sub> plants had an increase of



**Figure 9.** PI<sub>1</sub> plants show higher levels of callose deposition than PC<sub>1</sub> plants. A, Immunohistochemical staining for callose deposition (green fluorescence). Stained with DAPI, DNA appeared as blue fluorescence. Bars = 40  $\mu$ m. B, Quantification of the intensity of callose-positive staining (with SE; as in A). The data were analyzed in three independent experiments (three leaves from each of five plants). [See online article for color version of this figure.]

about 80% in the total amount of metabolites as compared with PC\_1 plants (Supplemental Fig. S3).

In our experiments, the progeny of infected plants also showed a high level of callose deposition. This plant polysaccharide is known to accumulate in response to exposure to various abiotic and biotic stresses (Jones et al., 2006). In fact, TMV movement through plasmodesmata was substantially delayed in plants with a high level of callose (Iglesias and Meins, 2000).

Induced *PR1* gene expression is one of the hallmarks of a hypersensitive response and systemic acquired resistance (Chamnongpol et al., 1998). High expression of *PR1* genes in rice (*Oryza sativa*) is in good correlation with higher tolerance to *Xanthomonas oryzae* (Ponciano et al., 2006). Plants that had the spontaneously activated *PR1* gene displayed higher tolerance to Pst DC3000 (Morita-Yamamuro et al., 2005). In our experiments, we also found that PI\_1 plants had lower bacterial counts upon infection with this pathogen.

The high resistance of PI\_1 plants to *P. nicotianae* deserves separate attention. Although resistance of PI plants to viral (TMV) and bacterial (*P. syringae*) infection was rather marginal, the response to the fungal pathogen *P. nicotianae* was quite drastic. Whereas 87% of all infected PC\_1 plants developed infection symptoms, only about one-quarter of PI\_1 plants developed them (Table I). One of the possible explanations of such an effect could be higher spontaneous and induced levels of the *PR1* gene. It was previously demonstrated that tobacco plants that overexpressed the *PR1* gene exhibited higher tolerance to the aforementioned fungal pathogen (Alexander et al., 1993). Curiously, Shin et al. (2002) held the opinion that transgenic pepper (*Capsicum annuum*) plants overexpressing the tobacco stress-induced gene *Tsi1*, which is involved in regulation of the *PR1* gene, exhibited higher resistance to viral, bacterial, and fungal pathogens. The fact that PI plants also acquire higher resistance to viral, bacterial, and fungal pathogens may suggest that plants with higher resistance can be obtained by local transient infection with TMV; only single leaves of parental generation plants were infected with TMV, and infected leaves were removed 24 hpi. It remains to be proved whether other pathogens can trigger the same response and whether PI plants acquire similar resistance to other pathogens.

### Possible Mechanisms of Transgenerational Changes in the Progeny of Stressed Plants

Our previous work showed a systemic increase in HRF upon local infection (Kovalchuk et al., 2003a). We hypothesized that upon local infection, signaling molecules arrive at noninfected tissues faster than a virus. Love et al. (2005) showed that infection of Arabidopsis plants by *Cauliflower mosaic virus*, a compatible pathogen of Arabidopsis, led to the activation of multiple defense signaling mechanisms. This suggests the existence of signaling even in response to a compatible

interaction in plants. It is possible that this signal reaches the gametes and establishes different methylation patterns, which possibly leads to differential gene expression, including expression of *PR1*, higher levels of HRF, increases in the levels of phenolics and metabolites, and thus higher stress tolerance. Although changes in HRF and stress resistance could be the outcome of changes in methylation pattern and gene expression, there is no evident link between the level of recombination and stress resistance. We hypothesize that these two events possibly represent features of ongoing adaptive processes. Increases in HRF and stress tolerance are likely to represent transient events that would not last for more than a couple generations. Several recent publications suggest the existence of transgenerational memory in the response of plants to stress (Molinier et al., 2006; Pecinka et al., 2009; Boyko et al., 2010). It is suggested that a transgenerational response depends on the transgenic plants used, the type and intensity of stress, the mode of stress application, and even the conditions under which plants are grown. More recent work suggests the involvement of small interfering RNA biogenesis pathways in the establishment of a transgenerational response to abiotic stress (Boyko et al., 2010). It remains to be shown whether these notions can be applied to biotic stress.

Collectively, our findings suggest the existence of adaptive inheritance, possibly of epigenetic nature. This indicates that biotic stress of viral origin can induce genetic and epigenetic molecular changes and also suggests the possibility of multigenerational adaptive epigenetic phenomena in progeny of infected plants.

## MATERIALS AND METHODS

### Generation and Use of Transgenic Tobacco Plants Carrying a Homologous Recombination Substrate

The generation of transgenic tobacco (*Nicotiana tabacum* 'SR1') plant line A carrying in the genome a single copy of the luciferase recombination substrate was described previously (Kovalchuk et al., 2003a).

### Infection of SR1 Plants with TMV and Generation of Infected Plant Progeny

In these experiments, single leaves of 20 SR1 plants containing the recombination substrate (line A) were infected with 200 ng (100 ng  $\mu\text{L}^{-1}$  in phosphate buffer) of TMV U1 strain, and 20 plants were mock-inoculated with phosphate buffer only. Carborundum was used as an abrasive. The experiments were done in two independent rounds, with 10 plants from each group in each round. Infected and mock-inoculated plants were kept at 32°C. At 24 h after infection, upper noninfected leaves (as checked by PCR; Kovalchuk et al., 2003a) were grafted to noninfected 6-week-old plants (Kovalchuk et al., 2003a). The apical meristem of these grafted plants was removed to promote the development of lateral buds. Seeds from these plants were collected individually and named PI\_1 (20 different lines; PI\_1#1, PI\_1#2, etc.) or PC\_1 (20 different lines; PC\_1#1, PC\_1#2, etc.; Fig. 1). All these progenies had comparable numbers and weights of seeds produced (data not shown). Plants germinated from these seeds never developed any viral infection, unless infected. Similarly, the progeny of infected plants never showed any presence of viral RNA (data not shown).

## Analysis of Plant Resistance to TMV Infection: Plant Inoculation, Monitoring of Infection Symptoms, and Analysis of Viral Titers

For analyzing resistance to TMV, single leaves of 400 5-week-old PI\_1 plants (20 plants each per 20 lines) and 100 PC\_1 plants (10 plants from 10 different lines) were infected with 200 ng ( $100 \text{ ng } \mu\text{L}^{-1}$ ) of TMV U1 particles. Infection was carried out by rub inoculation with Carborundum powder as an abrasive. The experiments were done in two rounds, 10 plants per each PI group and five plants per each PC group for each experimental round. After infection, plants were grown at 32°C under 16/8-h day/night conditions. PC plants were propagated at 32°C without infection. Symptom appearance (light coloration between veins in young leaves followed by leaf darkening and curling) was monitored daily. For the analysis of the viral titer, tissue samples of noninoculated leaves from each infected plant (20 plants per each line), regardless of whether plants showed symptoms or not, were taken at 6, 9, and 12 dpi. Viral RNA was extracted, and virus concentration was analyzed as published previously (Asurmendi et al., 2007). In brief, the plant tissue was homogenized and divided into aliquots of 500 mg per sample. A total of 400  $\mu\text{L}$  of 0.5 M phosphate extraction (pH 7.0) buffer was added to 500 mg of tissue homogenate (4.1 g of  $\text{Na}_2\text{HPO}_4$ , 2.5 g of  $\text{NaH}_2\text{PO}_4$ , and 100  $\mu\text{L}$  of  $\beta$ -mercaptoethanol per 100 mL). Samples were purified using equal volumes (400  $\mu\text{L}$ ) of chloroform and 1-butanol. Viral particles were precipitated using separate volumes of 50  $\mu\text{L}$  of 40% polyethylene glycol 6000 and a 10% NaCl solution. The pellet was resuspended in 20  $\mu\text{L}$  of a 1:50 dilution of phosphate buffer that lacked  $\beta$ -mercaptoethanol. The viral titer was estimated by measuring the optical density at 260 nm ( $\text{OD}_{260}$ ) using a spectrophotometer. For TMV, the extinction coefficient is assumed to be 3. The titer is expressed in micrograms of TMV per 10 mg fresh weight. Individual readings for each plant are shown in Supplemental Figure S4.

## Black Shank Disease Assays

*Phytophthora nicotianae* (synonym *Phytophthora parasitica* Dastur) assays were performed with an aggressive isolate (LA0921) using 6-week-old plants of line PC\_1, PI\_1#9, and PI\_1#16 grown in a 1:1:1 soil:peat moss:vermiculite mixture (sterilized by autoclaving) in 4-inch pots. About 80 plants per each experimental group were used. Plants were watered, allowed to drain, and inoculated by applying 10 mL of a zoospore and sporangia suspension containing 1,000 propagules per mL to the soil. Inoculated plants were incubated in plexiglass trays at 22°C and 100% relative humidity. A wilt index was calculated for the assay at 5 dpi as follows: 0 = no symptoms; 1 = some signs of wilting and stunting with reduced turgidity; 2 = advanced wilting and stunting but no chlorosis or necrosis; 3 = advanced wilting with stunting and chlorosis but no necrosis; 4 = severe wilting with necrosis; 5 = advanced necrosis with plants near death or dead. An average incidence was calculated by relating the number of plants with signs of wilting (any scale from 1–5) to the total number of plants used. All screening was blind with plants assayed in a random design.

## Analysis of Plant Resistance to *Pseudomonas syringae*

*P. syringae* pv *tomato* DC3000 avirulent strain (Pst-avr) was used for the experiment. Bacteria were cultured at 28°C on King's medium (King et al., 1954) containing 25 mg  $\text{L}^{-1}$  rifampicin and 50 mg  $\text{L}^{-1}$  kanamycin (Pst-avr). Bacterial growth in the leaves of tobacco plants from PC\_1, PI\_1#9, and PI\_1#16 lines was determined by syringe infiltrating the bacterial suspension in vivo into leaves as described (Whalen et al., 1991). Briefly, the fresh overnight bacterial culture was centrifuged at 3,200 relative centrifugal force for 5 min and washed twice with 10 mM  $\text{MgCl}_2$ . The  $\text{OD}_{600}$  of the suspension was determined by spectrometry and further diluted to  $\text{OD}_{600} = 0.05$  with 10 mM  $\text{MgCl}_2$ .

Four-week-old plants were incubated at 30°C for 24 h prior to infection. Plants were infiltrated with the above-mentioned *Pseudomonas* culture, and the infiltrated area was marked. Tissues were harvested at 0, 24, 48, and 72 hpi. At each time point, five samples were taken from five different plants from each line. Each of these samples consisted of three leaf discs (3 mm diameter) from independent infiltrations from a single plant. The samples were macerated in 10 mM  $\text{MgCl}_2$ , and the content was diluted 3,000 and 5,000 times. From each sample, two plates containing Murashige and Skoog (MS) medium were inoculated using 50  $\mu\text{L}$  of extract. The number of colonies formed was counted after 2 d. The experiment was repeated twice. Each experimental data point is

an average of two experiments, each consisting of 10 individual data points (two plates from each of five samples).

## Generating the Second Progeny of Infected Plants

PI\_1 plants from lines 9 and 19 were used to produce the second generation of plants exposed to virus. At the age of 5 weeks, 20 PI\_1 plants from line 9 and 20 PI\_1 plants from line 19 were either infected with 200 ng ( $100 \text{ ng } \mu\text{L}^{-1}$ ) of TMV U1 particles or mock inoculated. Next, 10 plants (those that showed an increase in HRF and looked healthy) from each group were propagated to seeds. For propagating PC\_1 plants, we used lines 1 and 3. Similarly, 10 plants from each PC\_1 line were propagated to generate PC\_2 plants.

## Visualization of Luciferase Activity

Recombination events in transgenic plants were visualized on living plants with a CCD camera 2 h after the cleavage substrate, luciferine, was applied (Supplemental Fig. S1B).

## Exposure to MMS

Progenies of tobacco plants were exposed to various concentrations of MMS by growing plants on MS medium supplemented with this chemical. First, in the pilot experiment, it was shown that the concentration of 120  $\mu\text{L L}^{-1}$  led to a significant change in the plant phenotypic appearance (data not shown). That is why the same concentration was used to analyze the resistance of PC\_1, PI\_1, PI\_2, and PI\_1\_C1 plants.

## Exposure to NaCl and Rose Bengal

Plants from individual PI\_1 and PC\_1 lines were germinated and grown on sterile MS medium. At the age of 1 week, they were moved to MS medium supplemented with various amounts of NaCl or rose Bengal. Four NaCl concentrations (0, 50, 75, and 100 mM) were used, whereas the concentrations of rose Bengal used were 0, 4, 10, and 20  $\mu\text{M}$ . Approximately 100 plants per each plate and each line were sampled. Recombination frequency was tested at 3 weeks post germination.

## Analysis of the Phenolic Content

For the analysis of total polyphenolic compounds, fresh tissues of PI\_1 plants from lines 9 and 19 and PC\_1 plants from lines 1 and 3 were ground in liquid nitrogen. A total of 500 mg of tissue powder of each sample was weighed and used for extraction using 500  $\mu\text{L}$  of phosphate-buffered saline (PBS). After centrifugation of the extract, 1.0 mL of Folin-Ciocalteu reagent (Sigma-Aldrich) and 0.8 mL of 7.5% sodium carbonate were added to 20  $\mu\text{L}$  of the extract in a tube. The solution was mixed and incubated for 30 min. Absorbance was measured at 765 nm using a spectrophotometer. Gallic acid was used as a standard, and values were expressed as relative arbitrary units.

## Analysis of Callose Deposition

For the analysis of callose deposition, leaves were harvested at 0, 12, 24, 36, and 48 h after *Pseudomonas* infiltration. Transverse sections (15  $\mu\text{m}$ ) were obtained from leaves using a cryomicrotome. After postfixation and washing, the tissue was blocked in 5% goat serum. The primary anti-callose antibody (Biosupplies) was diluted 1:250 in blocking solution and incubated at 4°C overnight. After washing, anti-mouse Alexa 488 (Invitrogen) was diluted 1:500 in blocking buffer and incubated for 3 h at room temperature. DNA counterstaining was done using DAPI (in blue). After washing, the slides were mounted and analyzed using a confocal microscope (Nikon). Images were quantified using ImageJ software ([www.rsby.info.nih.gov/ij](http://www.rsby.info.nih.gov/ij)). Each time point is the average of five different readings stemming from sections of three different leaves.

## Analysis of Global Genome Methylation Using a Cytosine Extension Assay and the Anti-5-Methyl Cytosine Antibody

Global genome methylation was analyzed as described previously (Boyko et al., 2007). For immunohistochemical staining, plants were fixed in 4% paraformaldehyde, and leaf cross-sections (15  $\mu\text{m}$  thick) were obtained by cryosectioning. The slides were postfixated in 4% paraformaldehyde and washed in 1× PBS. Blocking was carried out in a 5% bovine serum albumin

solution in PBS, and the primary anti-5-methyl cytosine antibody (Eurogentec) was applied overnight. After subsequent washes, DAPI was applied as a counterstain, and laser scanning electron microscopy was carried out. The intensity of staining at the center of the nucleus (Fig. 6, C and D) of PI\_1 and PC\_1 plants was analyzed by comparing the average ratio of red to blue in 10 independent cells from each of three independent leaves taken from three independent plants.

### Analysis of *PR1* Expression in Tobacco Infected with TMV or *P. syringae*

Single leaves of 1-month-old tobacco plantlets from PC\_1 and PI\_1 groups were rub inoculated with either 200 ng of TMV or *P. syringae* ( $OD_{600} = 0.05$ ). The control group was mock treated. Tissues were collected at 24 and 48 hpi. Northern-blot analysis for *PR1* RNA was conducted using tissues from tobacco plants infected with either TMV or *P. syringae*, as described previously (Kwon and An, 2001). The *PR1* probe was prepared using the PCR digoxigenin probe synthesis kit (Roche) with the following primers: forward primer 5'-TAGTCATGGGATTGTCTCT-3' and reverse primer 5'-CTAGACCAT-CAACACATGATT-3'. Fragment intensity was quantified using ImageJ software.

### Statistical Treatment of Data

In all cases, average and SD values in three independent experiments were calculated. The statistical significance of experiments was confirmed by performing either Student's *t* test (two-tailed paired or nonpaired) or single-factor or two-factor ANOVA. Statistical analyses were performed using MS Excel software and Microcal Origin 6.0.

### Supplemental Data

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

**Supplemental Figure S1.** Scheme of the recombination reporter, and detection of recombination events.

**Supplemental Figure S2.** Analysis of phenolic compounds in PI\_1 and PC\_1 plants.

**Supplemental Figure S3.** Analysis of metabolites.

**Supplemental Figure S4.** Viral titers in individual PI\_1 and PC\_1 plants infected with TMV.

### ACKNOWLEDGMENT

We thank Valentina Titova for proofreading the manuscript.

Received April 3, 2010; accepted May 20, 2010; published May 24, 2010.

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