Systemic Acquired Tolerance to Virulent Bacterial Pathogens in Tomato[1]

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Recent studies on the interactions between plants and pathogenic microorganisms indicate that the processes of disease symptom development and pathogen growth can be uncoupled. Thus, in many instances, the symptoms associated with disease represent an active host response to the presence of a pathogen. These host responses are frequently mediated by phytohormones. For example, ethylene and salicylic acid (SA) mediate symptom development but do not influence bacterial growth in the interaction between tomato (*Lycopersicon esculentum*) and virulent *Xanthomonas campestris* pv *vesicatoria* (*Xcv*). It is not apparent why extensive tissue death is integral to a defense response if it does not have the effect of limiting pathogen proliferation. One possible function for this hormone-mediated response is to induce a systemic defense response. We therefore assessed the systemic responses of tomato to *Xcv*. SA- and ethylene-deficient transgenic lines were used to investigate the roles of these phytohormones in systemic signaling. Virulent and avirulent *Xcv* did induce a systemic response as evidenced by expression of defense-associated pathogenesis-related genes in an ethylene- and SA-dependent manner. This systemic response reduced cell death but not bacterial growth during subsequent challenge with virulent *Xcv*. This systemic acquired tolerance (SAT) consists of reduced tissue damage in response to secondary challenge with a virulent pathogen with no effect upon pathogen growth. SAT was associated with a rapid ethylene and pathogenesis-related gene induction upon challenge. SAT was also induced by infection with *Pseudomonas syringae* pv *tomato*. These data show that SAT resembles systemic acquired resistance without inhibition of pathogen growth.

Multiple phytohormones are critical components of both local and systemic responses of a plant to pathogen invasion (Kunkel and Brooks, 2002). For instance, ethylene is necessary for resistance to certain fungal pathogens (Knoester et al., 1998, 1999) while salicylic acid (SA) is necessary for both resistance to some avirulent pathogens and generation of systemic acquired resistance (SAR; Gaffney et al., 1993). Mutants and transgenic plants compromised in phytohormone synthesis or perception have been important tools to demonstrate roles for ethylene, SA, and jasmonates in disease symptom development. For example, the ethylene-insensitive *ein2* mutant of Arabidopsis (*Arabidopsis thaliana*) has decreased symptom development in response to several virulent bacterial pathogens (Bent et al., 1992). Similarly, ethylene- and SA-deficient tomato (*Lycopersicon esculentum*) lines have decreased symptom development in response to virulent *Xanthomonas campestris* pv *vesicatoria* (*Xcv*; Lund et al., 1998; O'Donnell et al., 2001). Thus, reduced symptoms without reduced pathogen growth, termed tolerance, indicates that phytohormone-mediated defenses can be uncoupled from pathogen growth.

Disease development in tomato infected with virulent *Xcv* can be defined as occurring in two stages: a primary phase and a secondary phase. Primary disease development consists of localized lesion formation and is unaltered in the tolerant lines. Secondary disease development requires the cooperative action of ethylene and SA and is reduced in tolerant lines. Secondary disease development includes the formation of chlorosis and necrosis that spread from primary lesions (O'Donnell et al., 2003).

Our interest is in determining why plants would produce ethylene and SA in response to pathogens when their action does not limit pathogen growth. If pathogen growth is not inhibited by the extensive necrosis associated with secondary disease development, what purpose does this ethylene- and SA-mediated response serve? One possible function of extensive tissue death is induction of systemic responses. Based on results in other organisms, tomato may require ethylene or SA for SAR. SAR is the sensitization of systemic defense responses initiated by infection with certain pathogens, leading to resistance to subsequent pathogen infections (Ryals et al., 1996). Many factors influence the induction of SAR, including host cell death associated with an incompatible or compatible interaction (Hunt and Ryals, 1996). SAR

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results in the development of a broad-spectrum, systemic resistance. However, it is not effective against, or induced by, all pathogens. For example, infection of Arabidopsis with *Botrytis cinerea* fails to induce SAR and inoculation with *Pseudomonas syringae* does not affect subsequent *B. cinerea* challenge (Govrin and Levine, 2002). Thus, while SAR can be induced in tomato by pathogens such as tobacco necrosis virus and *Phytophthoria infestans*, there is neither systemic SA accumulation upon inoculation nor SA accumulation upon subsequent challenge by these pathogens (Enkerli et al., 1993; Jeun et al., 2000). In Arabidopsis, the evidence supports a model in which ethylene and jasmonates coordinately mediate one defense response while SA mediates a distinct and antagonistic response (McDowell and Dangl, 2000). Our work demonstrating cooperative interactions between jasmonates, ethylene, and SA in the susceptible response of tomato to *Xcv* indicates that the functions of these hormones may be species specific (O'Donnell et al., 2003).

Here, we investigate if *Xcv* is capable of inducing a systemic response in tomato as well as possible roles of SA and ethylene in systemic signal generation. We demonstrate that inoculation with either virulent or avirulent *Xcv* leads to an SA- and ethylene-dependent induction of defense genes and sensitizes the plant to subsequent pathogen challenge. However, instead of inducing SAR, *Xcv* generates tolerance to subsequent challenge with virulent *Xcv*. We term this response systemic acquired tolerance (SAT) and define it as prior pathogen exposure reducing host tissue damage in response to virulent pathogen infection without impacting pathogen growth. We further demonstrate that SAT is not unique to *Xcv* and can also be induced by *P. syringae* pv *tomato*.

RESULTS

Virulent and Avirulent *Xcv* Induce SAT in Tomato

To investigate systemic responses to *Xcv*, wild-type tomato plants at the three-leaf stage were mock inoculated, inoculated with virulent *Xcv* strain 93-1 or avirulent *Xcv* strain 87-7 (Bonas et al., 1993) on their lowest two leaves. The 87-7 strain contains the avirulence gene avrBs3-2 and is avirulent on all tomato varieties. The inoculations were then permitted to run their full course of approximately 14 d, at which point those leaves inoculated with virulent *Xcv* were fully necrotic and those inoculated with avirulent *Xcv* had developed lesions associated with the hypersensitive response (Fig. 1A).

To determine if this inoculation with *Xcv* affected responses to subsequent pathogen exposure, a challenge was performed with virulent *Xcv* on uninoculated systemic leaves. Prior inoculations with either virulent or avirulent *Xcv*, but not mock inoculations, reduced the necrosis resulting from the challenge (Fig. 1B). The primary symptoms such as lesion formation were unaffected and secondary symptom development such as chlorosis and some confluent necrosis were still apparent. The major difference between challenged and unchallenged plants was reduced necrosis in plants with prior *Xcv* inoculation. As the response consists of two independent interactions between two biological entities, a high degree of variation is to be expected. With this in mind the level of necrosis was determined in large population groups by measuring ion leakage in leaf five at 16 d after challenge (Fig. 2). Percent ion leakage, an indicator of cell death, was 2-fold higher upon challenge in plants previously mock inoculated than those with prior *Xcv* inoculations. Prior virulent or avirulent *Xcv* inoculations led to similar reductions in ion leakage upon challenge with virulent *Xcv*.

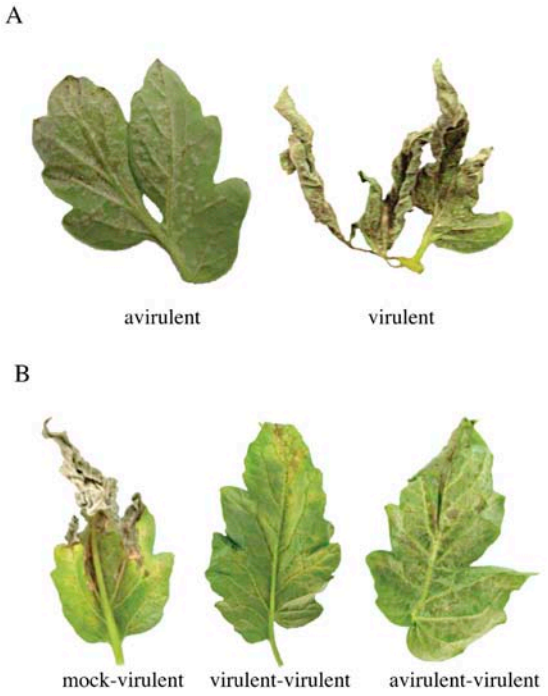

Figure 1. Symptom development of *Xcv*-infected tomato. Disease symptoms at 16 dpi of wild-type tomato plants (A) inoculated with avirulent and virulent *Xcv* and (B) following challenge with virulent *Xcv*. These pictures are representative of at least two independent biological experiments.

The reduction of symptom development due to previous pathogen exposure is consistent with SAR generation. Bacterial growth measurements confirmed that UC82B is resistant to avirulent *Xcv* but not virulent *Xcv*, as growth of avirulent *Xcv* was 10-fold lower than that of virulent *Xcv* (Fig. 3A). This difference is due to a gene-for-gene interaction (Bonas et al., 1993) and is consistent with levels of growth reported previously (Ciardi et al., 2000). However, when bacterial populations in challenge infections were determined, there was no difference in growth caused by prior inoculation with either virulent or avirulent *Xcv* when compared to plants with prior mock inoculations (Fig. 3B). This result leads to the conclusion that both virulent and avirulent *Xcv* induced SAT rather

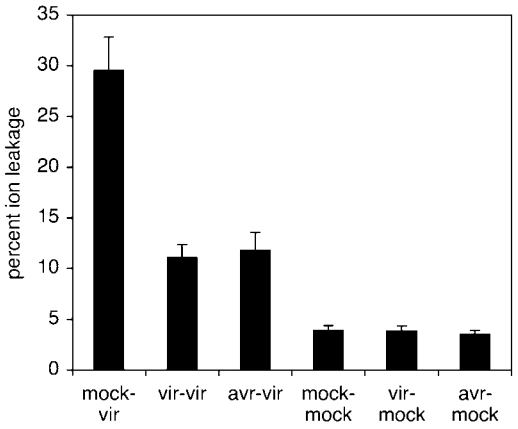

Figure 2. Cell death of tomato 16 d after challenge with virulent *Xcv*. Cell death was measured in the form of percent ion leakage in plants treated with a mock challenge or challenged with virulent *Xcv*. The plants were exposed to a mock inoculation or inoculation with virulent (vir) or avirulent (avr) *Xcv* prior to treatment. These data are representative of two independent biological experiments. Bars equal SE, $n = 30$.

than SAR, as they reduced symptom development but not bacterial growth during subsequent challenge with virulent *Xcv*. Note that there is an inherent age-dependent resistance in tomato with the first leaves supporting higher bacterial growth than later ones.

Prior Inoculation with *Xcv* Leads to Early Production of Ethylene upon Challenge with Virulent *Xcv*

Ethylene and SA are involved in the development of systemic responses, including SAR and induced systemic resistance in Arabidopsis (Lawton et al., 1995; Knoester et al., 1999). In order to examine potential roles for these hormones in the systemic response of tomato to *Xcv*, levels of ethylene and SA were measured during inoculation and challenge with *Xcv*. Inoculation with virulent *Xcv* led to ethylene accumulation in local tissues at 5 d post infection (dpi), while avirulent *Xcv* caused a greater accumulation of ethylene at 2 dpi (Fig. 4A). Virulent *Xcv* induced SA accumulation at 10 dpi, while avirulent *Xcv* induced SA accumulation at 4 dpi, peaking at 10 dpi (Fig. 4B). The accumulation of ethylene and SA was later and at reduced magnitude in response to virulent *Xcv* than avirulent *Xcv*. No systemic accumulation of ethylene was observed in response to *Xcv* inoculations and at 16 dpi no systemic accumulation of SA was observed in plants inoculated with *Xcv* (data not shown).

Accumulation of ethylene and SA were also measured following challenge with virulent *Xcv*. Results indicated that ethylene accumulated earlier and to a greater extent upon challenge during SAT than in equivalent plants that were previously mock inoculated (Fig. 4, C and D). The ethylene accumulation in response to a challenge with virulent *Xcv*, following prior mock inoculation, reached its maximum at 10 dpi. However, plants with prior *Xcv* inoculations accumulated additional ethylene around 5 dpi when challenged (Fig. 4C). Although at a reduced magnitude, this accumulation resembles a response to avirulent *Xcv*. Challenge with virulent *Xcv* caused ethylene accumulation at 8 to 10 dpi in all plants, the magnitude of which was dependent on the prior inoculation. Plants with prior avirulent *Xcv* inoculation accumulated the most ethylene when challenged. This ethylene accumulation was lower in plants with prior virulent *Xcv* inoculations and lowest in the mock-inoculated controls. It can be concluded that challenged leaves from plants previously infected with either avirulent or virulent *Xcv* synthesized ethylene earlier and to a greater magnitude than those previously mock inoculated.

Plants with prior mock or virulent *Xcv* inoculations accumulated similar amounts of SA in response to challenge with virulent *Xcv*. However, reduced SA accumulation was observed in plants with a prior avirulent *Xcv* inoculation. These plants also produced an early SA peak (Fig. 4D) that resembled the response to avirulent *Xcv* although reduced in magnitude. Subsequent accumulation of SA was delayed and resembled that caused by virulent *Xcv* although at reduced magnitude. That there is no significant difference in SA accumulation between the prior mock and the prior virulent *Xcv* inoculation in the challenged leaves seems to rule out a role in SAT for SA synthesized in the systemic leaves.

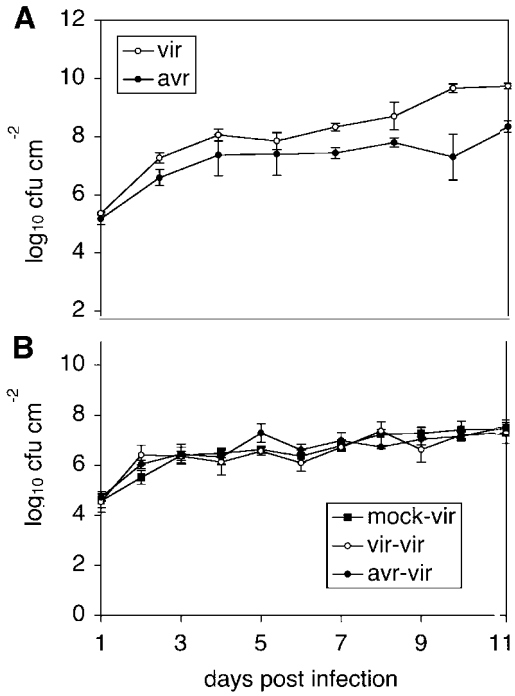

Figure 3. Bacterial growth during inoculation and challenge of tomato with *Xcv*. A, The growth of virulent (vir) and avirulent (avr) *Xcv* was measured during primary infections. B, A systemic challenge with virulent *Xcv* was then performed on these plants as well as mock-inoculated controls and the bacterial growth was measured. These data are representative of two independent biological experiments. Bars equal SE, $n = 5$.

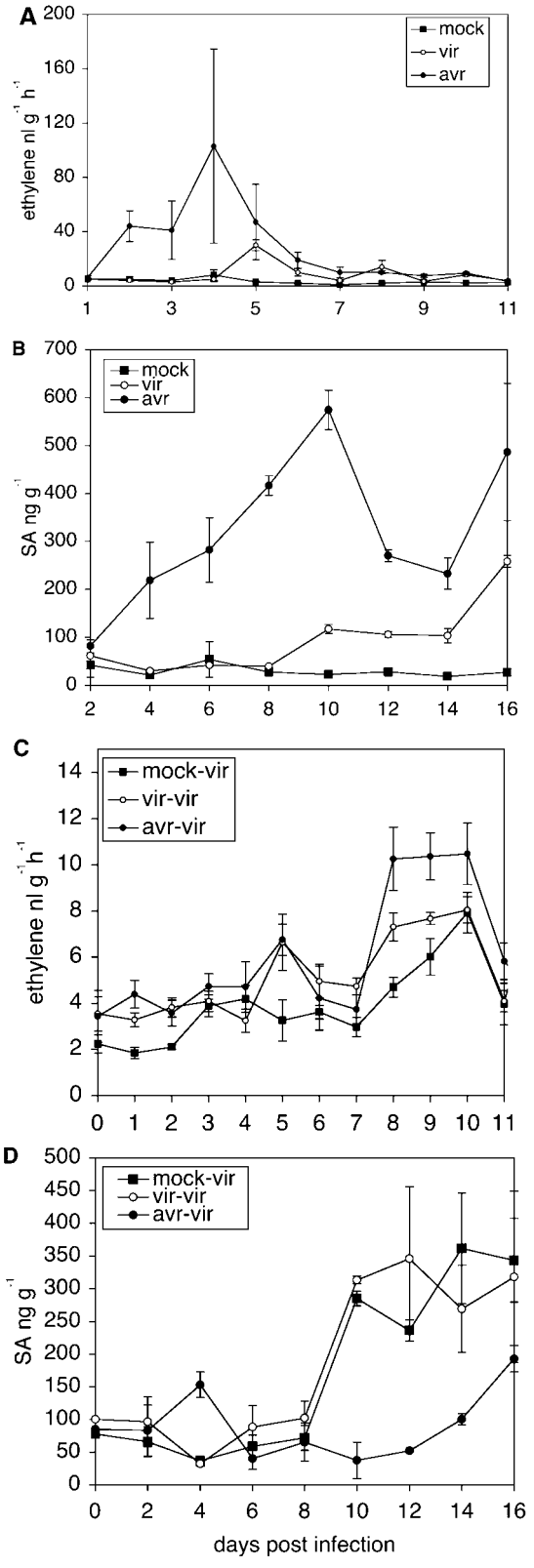

Figure 4. Local ethylene and SA accumulation following inoculation and challenge of tomato with *Xcv*. Tomato plants were mock inoculated or inoculated with virulent (vir) or avirulent (avr) *Xcv* and (A) ethylene and (B) SA accumulation following inoculation were measured. Fourteen days later, a challenge with virulent *Xcv* was performed on uninfected leaves and (C) ethylene and (D) SA accumulation following challenge was measured. These data are representative of two independent biological experiments. Bars equal SE, $n = 4$.

Pathogenesis-Related Gene Expression Demonstrates the Necessity of Ethylene and SA in Systemic Signal Generation

As ethylene- or SA-deficient plants develop less secondary disease symptoms in response to virulent *Xcv* than plants displaying SAT, a direct approach to determining any roles for these phytohormones in SAT is difficult. Therefore, pathogenesis-related (PR) gene expression was used as a marker for defense responses in ethylene- and SA-deficient plants. Induction of PR genes is often used as an indicator of early defense responses. They show both local and systemic induction during SAR (Ward et al., 1991). To determine if ethylene and SA are involved in systemic signal transduction, two transgenic lines were used. Loss of ethylene was achieved with transgenic plants expressing 1-aminocyclopropane-1-carboxylic acid deaminase (ACD). These plants do not accumulate the ethylene precursor 1-aminocyclopropane-1-carboxylic acid and thus underproduce ethylene. The ACD line was compared to its isogenic parent UC82B. The role of SA was determined using transgenic tomato plants expressing the bacterial salicylate hydroxylase, *nahG*, which does not accumulate SA. The NahG line was compared to its isogenic parent MoneyMaker (MM).

PR1a and *PR1b* were induced in local and systemic tissues of tomato during infection with virulent *Xcv* (Fig. 5). *PR1a* expression was lower than *PR1b*. The local expression of both genes was induced in all lines in response to virulent *Xcv*. The level of local *PR1a* induction varied between different cultivars, with an earlier and greater induction in MM than UC82B. The local expression of *PR1b* was also higher in MM than UC82B, although the timing of induction was the same. When compared to their isogenic parents, infected ACD and NahG plants had reduced local expression of both genes. However, this reduction did not affect pathogen growth (O'Donnell et al., 2001).

Virulent *Xcv* induced systemic expression of both genes in MM and UC82B. In MM systemic induction of *PR1a* and *PR1b* occurred at the same time, whereas in UC82B the expression of *PR1b* was later than *PR1a*. ACD and NahG showed no systemic induction of PR gene expression in response to virulent *Xcv*. Disease symptoms in ACD and NahG are comparable to their isogenic parents up to 8 dpi (O'Donnell et al., 2001). As there is no widespread necrosis before 8 dpi, the lack of systemic PR gene expression must be related to loss of ethylene or SA accumulation rather than to the loss of widespread necrosis in these lines. The lack of systemic PR gene induction indicates that ethylene- and SA-deficient lines may be compromised in their systemic signal transduction. However, the tolerant phenotype of these lines makes direct investigation difficult.

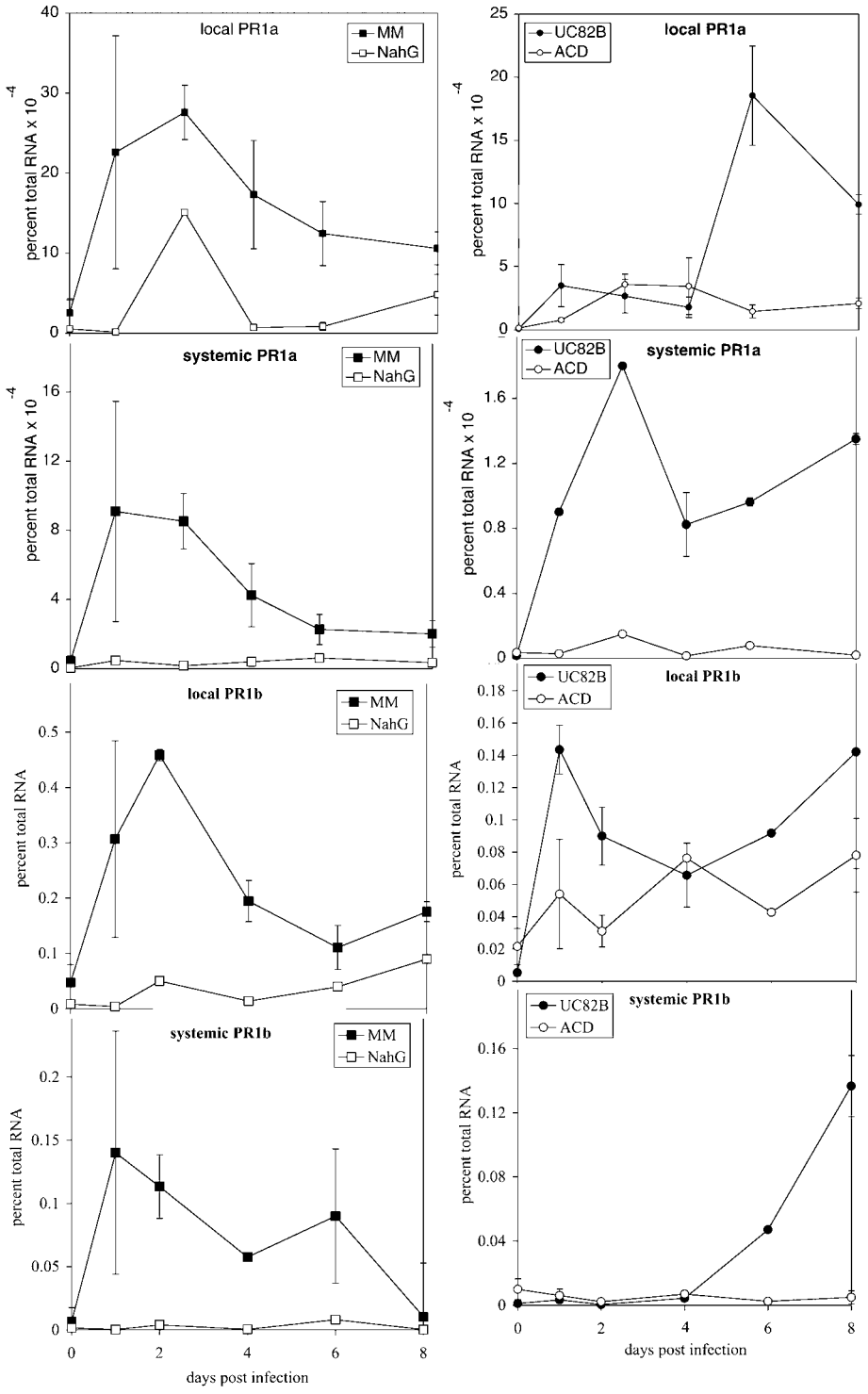

Figure 5. Local and systemic PR gene induction in ethylene- and SA-deficient tomato lines in response to inoculation with virulent *Xcv*. Ethylene-deficient ACD, SA-deficient NahG, and their isogenic parents were inoculated with virulent *Xcv*. *PR1a* and *PR1b* expression levels were determined by real-time RT-PCR in local and systemic tissues. These data are representative of two independent biological experiments. Bars equal SE, $n = 4$.

Greater PR Gene Expression Was Induced by Avirulent Than Virulent *Xcv*

The accumulation of ethylene and SA is more rapid in response to avirulent than virulent *Xcv*, yet the resulting systemic response to these two pathogens is the same. To determine if the early defense responses follow this trend, induction of PR gene expression by avirulent and virulent *Xcv* inoculations was assayed. Avirulent *Xcv* induced higher and earlier local expression of PR genes than virulent *Xcv* (Fig. 6). The timing of systemic *PR1b* induction was similar in response to each pathogen, but systemic *PR1a* induction was faster

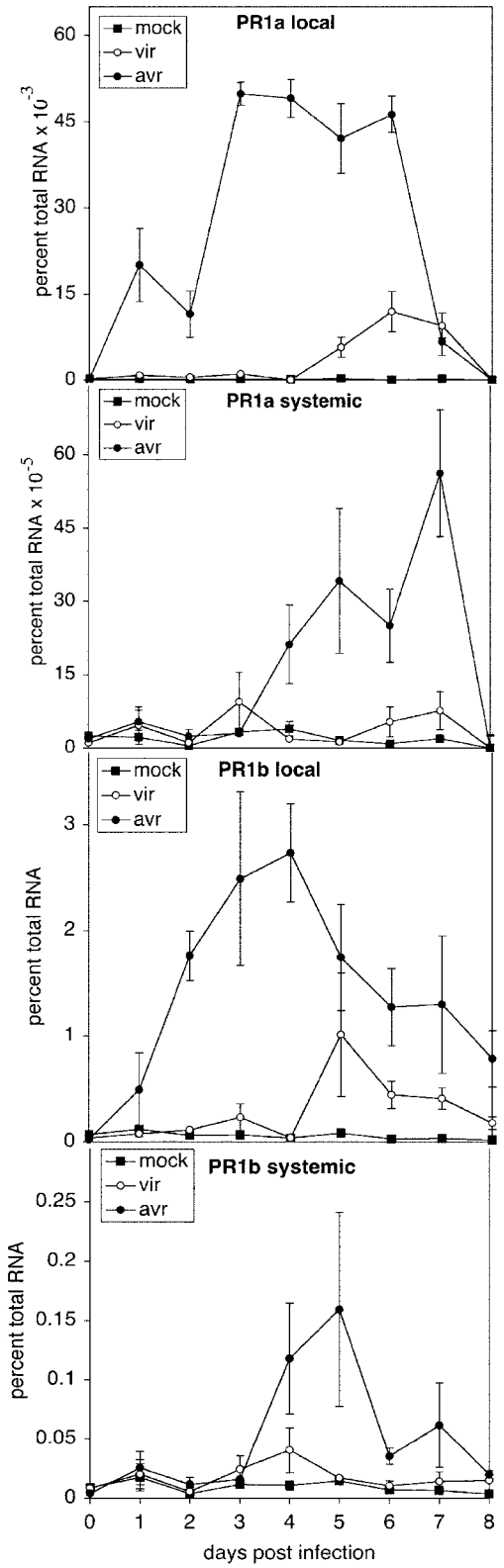

Figure 6. Local and systemic PR gene expression during inoculations with virulent or avirulent *Xcv*. Wild-type (UC82B) plants were mock inoculated or inoculated with virulent (vir) or avirulent (avr) *Xcv* and the local and systemic expression of *PR1a* and *PR1b* was determined by real-time RT-PCR. These data are representative of two independent biological experiments. Bars equal SE, $n = 4$.

in response to avirulent *Xcv*. Faster and stronger local PR gene response to avirulent than virulent *Xcv* may be an indication of increased defense responses that limit pathogen growth.

Prior avirulent and virulent *Xcv* inoculations produced similar inductions of ethylene and reductions in symptom development during challenge. The two prior *Xcv* inoculations induced similar PR gene expression upon challenge. Both caused an early peak of PR gene expression at 1 dpi upon challenge that was absent in plants with prior mock inoculation (Fig. 7). These results led to the hypothesis that inoculation with *Xcv* sensitized systemic defenses and caused their faster induction upon challenge, leading to tolerance. Sensitization or priming to the presence of pathogens is also observed in the generation of SAR (Conrath et al., 2002), therefore SAT may use similar mechanisms to SAR.

SAT Is Induced by Both *Xcv* and *P. syringae* pv *tomato* DC3000

We have demonstrated that infection with either virulent or avirulent *Xcv* leads to the production of

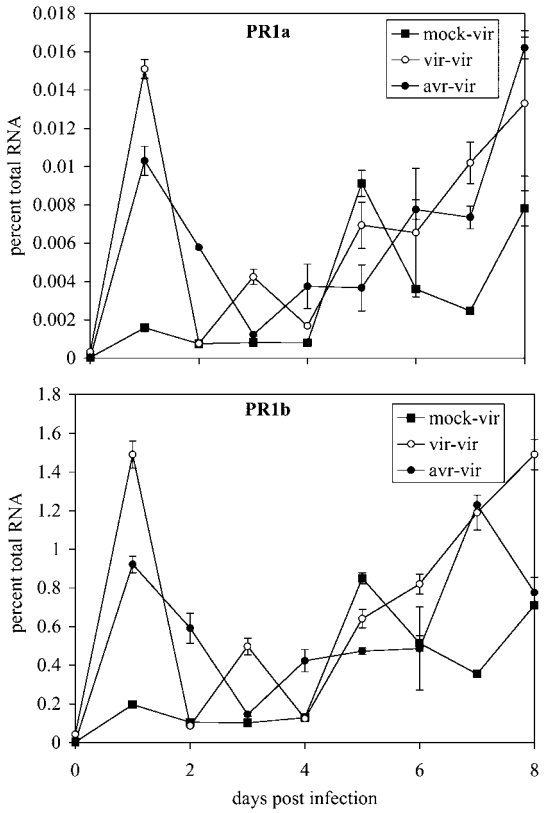

Figure 7. Induction of PR genes during challenge with virulent *Xcv* in the presence or absence of SAT. Wild-type plants were mock inoculated or inoculated with virulent (vir) or avirulent (avr) *Xcv*. Fourteen days later, a challenge was performed with virulent *Xcv*. The expression of *PR1a* and *PR1b* was determined with real-time RT-PCR following challenge. These data are representative of two independent biological experiments. Bars equal SE, $n = 4$.

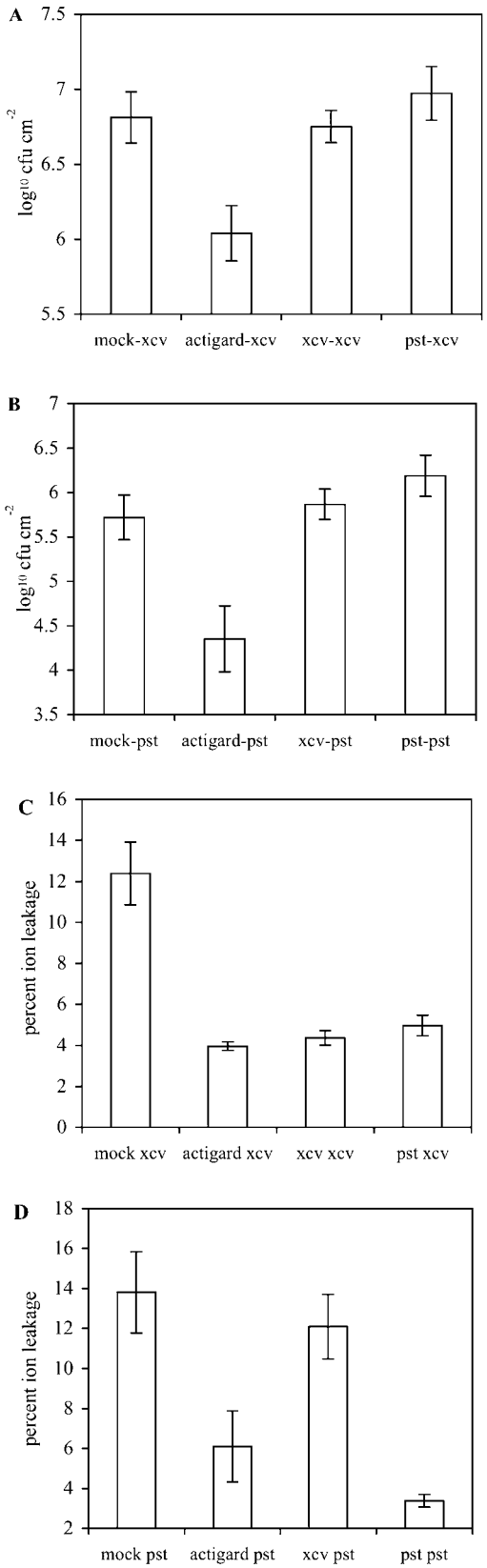

Figure 8. SAT induction by *Xcv* and *Pst*. Wild-type plants were mock inoculated, treated with the SAR inducer Actigard, or inoculated with virulent *Xcv* or *Pst*. Fourteen days later, a challenge was performed with virulent *Xcv* or *Pst*. Populations of (A) *Xcv* and (B) *Pst* were determined at 5 d after challenge. Bars equal SE, $n = 5$. Percent ion leakage was determined 16 d after challenge in plants challenged with (C) *Xcv* or (D) *Pst*. Bars equal SE, $n = 30$. Each experiment was repeated at least twice.

tolerance to subsequent infections with virulent *Xcv*. It may be that SAT is due to an inability of tomato to induce resistance to virulent *Xcv*. To test this hypothesis, tomato plants were treated with the SAR inducer Actigard at 10 and 4 d prior to challenge with virulent *Xcv*. Ion leakage and bacterial population measurement following challenge with virulent *Xcv* (Fig. 8, A and C) demonstrated that treatment with Actigard reduced *Xcv* growth and tissue damage following challenge. These data demonstrate that tomato can be induced to mount systemic resistance to *Xcv*. However, under normal circumstances SAT is the default response to *Xcv*.

To assess if SAT is a specific response to *Xcv*, we tested the ability of virulent *Xcv* to induce tolerance to subsequent challenge with the virulent bacterial pathogen *P. syringae* pv *tomato* DC3000 (*Pst*). We first confirmed that tomato was capable of inducing resistance to *Pst*. This was accomplished by treatment with Actigard at 10 and 4 d prior to challenge. Ion leakage and bacterial growth were measured following challenge with *Pst* (Fig. 8, B and D). Both tissue damage and bacterial growth were reduced compared to mock-treated controls, demonstrating that tomato can induce resistance to *Pst*. Ion leakage and bacterial growth were then measured following challenge with *Pst* in plants with prior exposure to virulent *Xcv* (Fig. 8, B and D). These data show that there is no significant difference in tissue damage or bacterial growth following challenge with *Pst* between plants with prior mock inoculations and prior inoculation with virulent *Xcv*. Therefore, virulent *Xcv* does not induce tolerance to *Pst*.

We then examined if *Pst* can itself induce SAT. Mock-inoculated and *Pst*-inoculated plants were challenged with either virulent *Xcv* or *Pst*. Ion leakage and bacterial populations following challenge were determined (Fig. 8). Prior exposure to *Pst* reduced tissue damage but not bacterial growth following both *Pst* and virulent *Xcv* challenge. Therefore, *Pst* can induce SAT both to itself and to virulent *Xcv*, indicating that this response is not specific to *Xcv*.

DISCUSSION

In this paper, the systemic responses of tomato to both virulent and avirulent *Xcv* were studied. Inoculation with avirulent or virulent *Xcv* led to local and systemic PR gene induction. Systemic PR gene induction by virulent *Xcv* was ethylene and SA dependent. The systemic signal, as well as inducing PR gene expression, altered the response to challenge with virulent *Xcv*. This altered defense response is tolerance and not resistance. SAT is manifested by rapid PR gene expression and ethylene induction in response to subsequent challenge, leading to suppressed symptom

development. SAT is not specific to *Xcv* since it was also induced by the virulent bacterial pathogen *Pst*.

The ability of a plant to send a systemic signal in response to a biological stimulus is well established. This communication allows systemic tissues to act in concert to the many stimuli they perceive. Systemic signals have been characterized in terms of responses to pathogens, symbionts, and wounding. The phytohormones SA, ethylene, jasmonates, and systemin are all involved in generating systemic defense-related signals (Pearce et al., 1991; Gaffney et al., 1993; Pieterse et al., 1998). SA is a key player in the development of SAR in many species (Ryals et al., 1996) and NahG plants are unable to mount SAR to bacterial, viral, or fungal pathogens (Gaffney et al., 1993; Friedrich et al., 1995; Lawton et al., 1995). The role of SA in systemic responses of tomato appears to differ somewhat from that in other plant species. For example, the induction of SAR against *P. infestans* showed neither systemic SA accumulation upon inoculation nor SA accumulation upon challenge, both of which are common in other plant species (Jeun et al., 2000). We have previously observed local SA accumulation in tomato in response to virulent Xcv, but only at 8 d following infection (O'Donnell et al., 2001), well after the systemic PR gene induction reported here.

In tomato, ethylene and SA accumulate in response to virulent and avirulent *Xcv*, although neither phytohormone is involved in limiting bacterial growth. Ethylene deficiency in tomato causes tolerance to virulent *Xcv*. It also reduces cell death and lesion size in response to infection with avirulent *Xcv*. SA deficiency in tomato also leads to tolerance to virulent *Xcv*. However, it increases cell death and lesion size in response to infection with avirulent *Xcv* (Lund et al., 1998; Ciardi et al., 2001; O'Donnell et al., 2001, 2003). As reported in Ciardi et al. (2000), avirulent *Xcv* induces a rapid and stronger expression of PR genes than virulent *Xcv*. Here, we show the same effect systemically.

The systemic signal generated by infection with avirulent but not virulent *Xcv* inoculation suppressed SA accumulation in response to challenge (Fig. 4D). This SA suppression apparently has no major impact on SAT. Despite the differences in SA accumulation following challenge, systemic PR gene expression was reduced in both ethylene and SA-deficient plants, suggesting that these phytohormones are important for generation of systemic signals in response to *Xcv*. The patterns of PR gene induction and ethylene emissions indicated an earlier and stronger response during challenge in plants displaying SAT. Prior *Xcv* exposure apparently primes systemic defenses, as measured by PR gene expression. The priming of PR genes and ethylene by prior *Xcv* exposure suggests that SAT could be a partial SAR that sensitizes systemic defenses sufficiently to repress symptom development but not pathogen growth. Systemic induction of defense responses that do not lead to SAR have been observed in the interaction of Arabidopsis and the necrotizing fungal pathogen *B. cinerea* (Govrin and Levine, 2002). Our experiments with *Xcv*, Actigard, and *Pst* demonstrate the flexibility of systemic responses to pathogen attack. For example, treatment with Actigard leads to resistance to *Xcv* and *Pst*, whereas prior inoculation with *Pst* leads to tolerance. Prior inoculation with *Xcv* leads to tolerance to *Xcv* yet has no visible impact on *Pst* challenge. This plasticity of the systemic response appears to depend on both inducer and challenge. Tolerant plants demonstrate that bacterial growth and symptom development can be uncoupled. Alternately, it may be argued that distinctions between susceptibility, tolerance, and resistance are artificial and that all of these outcomes are part of a continuum of plant defense responses. Similar signaling events could coordinate both SAT and SAR. This would imply that SAT and SAR share common signals including those that repress symptom development, but additional or stronger signals may be responsible for repression of pathogen growth in SAR.

Evidence for low level of defense responses causing tolerance is provided by tobacco plants (*Nicotiana tabacum*) that overexpress PR1a and exhibit enhanced tolerance to *Peronospora tabacina* (Alexander et al., 1993) as well as catalase-deficient tobacco with low-level activation of defense responses by H_2O_2 that have enhanced pathogen tolerance (Chamnongpol et al., 1998). These experiments suggest that low-level defense responses can repress symptom development without affecting bacterial growth. It may be that the systemic defense responses initiated by a particular pathogen are sufficient to cause either resistance or tolerance to a different pathogen depending on its ability to circumvent the induced defenses. In some cases, for instance inoculation with *Xcv* followed by challenge with *Pst*, the challenging pathogen can bypass the induced defense responses leading to full susceptibility. It can be concluded that systemic defense responses, and perhaps resistance itself, are more complex than previously suspected.

MATERIALS AND METHODS

Plant Materials and Treatments

Tomato (*Lycopersicon esculentum*) cultivars MM and UC82B are the parental lines for NahG (Oldroyd and Staskawicz, 1998) and ACD (Klee et al., 1991), respectively. Wild type refers to UC82B in all experiments except those associated with Figure 5. Plant growth and treatments were performed under ambient temperature and lighting in a greenhouse. To avoid confusion, we use the terms "inoculation" for the first treatment of a tomato plant and "challenge" for the subsequent treatment on distal leaves. Plants were inoculated by submersion of the leaves for 15 s in a bacterial suspension of 1×10^7 colony forming units (cfu) mL^{-1} of *Xcv* strain 93-1 (virulent) or 87-7 (avirulent) containing 10 mM $MgCl_2$ and 0.02% (v/v) Silwet L-77. Mock inoculations were performed by dipping plants in buffered Silwet. For analysis of the systemic response to infection, primary inoculations were performed on 3-week-old plants by dipping the first and second leaves in infection media. Challenges were then performed 14 d later by dipping leaves 5 and 6 in the infection media containing 1×10^7 cfu mL^{-1} of virulent *Xcv*. For PR gene analysis, the first and second leaves of 6-week-old plants were

inoculated, and tissue from the local inoculation (first and second leaves) and the systemic response (fifth and sixth leaves) were removed at the indicated time points and flash frozen in liquid nitrogen. *Pst* inoculations were performed with 1×10^7 cfu mL^{-1} *Pst* DC3000. Actigard (0.03 gL^{-1}) treatments were sprayed on the first and second leaves of tomato plants at 10 and 4 d prior to challenge. Each experiment was repeated at least twice.

Bacterial Culture

Xcv and *Pst* cultures were grown as previously described by O'Donnell et al. (2001). Leaf colony counts were determined on two independent biological repeats as previously described by Lund et al. (1998). Briefly, five 1-cm^2 leaf discs were sampled from each line at each time point indicated. The discs were ground in 10 mM $MgCl_2$, and serial dilutions were incubated at room temperature (*Pst*) or at 30°C (*Xcv*) for 2 d on solid media. The average cfu per square centimeter (cfu cm^{-2}) for each sample was determined by counting individual colonies ($n = 5$).

Ion Leakage

Amount of cell death was estimated by measuring percentage of ion leakage on two independent biological repeats. For challenge inoculations at 16 dpi the fourth leaf of each plant was placed in 6-mL deionized water and a vacuum of 20 psi applied for 5 min. The samples were then shaken at room temperature for 1 h. Three milliliters of the water was then removed and its conductivity was measured. The samples were then placed in a boiling water bath for an hour and the conductance of the remaining 3 mL of water measured. Percent ion leakage was determined by conductivity of first 3 mL divided by conductivity of second 3 mL multiplied by 100. $n = 30$ for challenge inoculations with a pathogen and $n = 10$ for mock challenges. Each experiment was repeated at least twice.

Ethylene Measurements

Ethylene measurements were performed on a minimum of two independent biological replicates with $n = 4$. Ethylene production was determined by sampling the headspace above a single leaf enclosed in 5-cm^3 tubes for 1 h as described by Lund et al. (1998). Ethylene concentration in a 1-mL sample was determined by gas chromatography (model 5890; Hewlett-Packard, Palo Alto, CA).

SA Measurements

SA was extracted from tomato tissue and derivatized using trimethylsilyldiazomethane. The volatile SA methyl ester was collected from the complex matrix using vapor phase extraction and quantified by isobutene chemical ionization gas-chromatography/mass spectrometry as described in Schmelz et al. (2004). Each experiment was repeated at least twice; $n = 4$.

Real-Time RT-PCR

PR1a (M69247) and PR1b (M69248) mRNA levels were quantified by real-time quantitative RT-PCR using Taqman one-step RT-PCR reagents (Applied Biosystems, Foster City, CA) and an Applied Biosystems GeneAmp 5700 sequence detection system. Each determination was performed using 250 ng of DNase-1 treated total RNA isolated using RNeasy Plant mini kit (Qiagen, Valencia, CA) in a 25-μL reaction volume. RT-PCR conditions were: 48°C for 30 min, 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Absolute mRNA levels were quantified using synthesized sense strand RNAs as standards. Primers and probes were designed using PRIMER EXPRESS software (Applied Biosystems) and were as follows: *PR1b* probe 5′-/56-FAM/CAACGGATGGTGGTTCATTTCTTGCA/3BQH_1/-3′; *PR1a* probe 5′-/56-FAM/TGTGGGTGTCCGAGAGGCCAGA/3BHQ_1/-3′; *PR1b* forward primer 5′-GGTCGGGCACGTTGCA-3′; *PR1b* reverse primer 5′-GATCCAGTTGCCTACAGGACATA-3′; *PR1a* forward primer 5′-GAGGGCAGCCGTGCAA-3′; *PR1a* reverse primer 5′-CACATTTTTCCACCAACACATTG-3′ (Intergrated DNA Technologies, Coralville, IA). Each experiment is representative of two independent biological replicates; $n = 2$.

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