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Expression of Defense-Related and Putative Signaling Genes During Tolerant and Susceptible Interactions of *Arabidopsis* with *Xanthomonas campestris* pv. *campestris*

C. R. Buell and S. C. Somerville

Carnegie Institution of Washington, Department of Plant Biology, 290 Panama Street, Stanford CA 94305

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The tolerance of *Arabidopsis thaliana* ecotype Columbia to *Xanthomonas campestris* pv. *campestris* 2D520 is conditioned by a dominant allele of the single, nuclear gene *RXC1*. Tolerance is exhibited by an asymptomatic response of Columbia to *X. campestris* pv. *campestris* 2D520 population levels greater than 10^6 CFU cm^{-2}. The establishment of similar bacterial population levels in the susceptible ecotype, Pr-0, results in spreading chlorosis within 3 to 4 days of inoculation. To better define the mechanism(s) that permit Columbia to tolerate infection by *X. campestris* pv. *campestris* 2D520, we examined mRNA levels of 10 genes proposed to have a role in either defense responses or signaling of stress events. These analyses revealed neither temporal nor quantitative accumulation of mRNA species unique to the tolerant interaction. Examination of mRNA levels of anthranilate synthase 1 (ASA1), anthranilate synthase 2 (ASA2), cinnamyl-alcohol dehydrogenase (CAD), cinnamate-4-hydroxylase (C4H), the ELI3 plant defense gene (ELI3), fatty acid desaturase 3 (FAD3), lipoxygenase (LOX1), myrosinase (MYR), and superoxide dismutase (SOD) revealed little to no accumulation in either Columbia or Pr-0 following inoculation with *X. campestris* pv. *campestris* 2D520. In contrast, mRNA levels encoding the pathogenesis-related protein 1 (PR1) accumulated to high levels beginning at 24 h postinoculation in both Columbia and Pr-0 tissues, with higher PR1 mRNA levels accumulating in Pr-0 than Columbia. The lack of mRNA accumulation for nine of ten mRNAs in tolerant and susceptible plants following inoculation with *X. campestris* pv. *campestris* was not due to an inability to accumulate these mRNAs. Inoculation of Columbia and Pr-0 plants with *Pseudomonas syringae* pv. *syringae* PSSD20, which elicits a hypersensitive response in both ecotypes, resulted in the accumulation of several mRNA species including ASA1, CAD, C4H, ELI3, LOX1, SOD, and PR1. The absence of preferential accumulation, either temporally or quantitatively, in Columbia tissues suggests these 10 defense-related and signaling mRNAs do not have a role in the establishment of tolerance.

Additional keywords: host-pathogen interactions, resistance mechanisms.

Corresponding author: C. R. Buell; E-mail: rbuell@andrew.stanford.edu

Arabidopsis thaliana, a member of the Brassicaceae, exhibits properties of the gene-for-gene hypothesis proposed by Flor (1955, 1971). Several *Arabidopsis* resistance loci and their corresponding avirulence genes in pathogens have been identified (Dangl et al. 1992; Davis et al. 1991; Debener et al. 1991; Innes et al. 1993; Kunkel et al. 1993; Parker et al. 1993; Wanner et al. 1993; Whalen et al. 1991; Yu et al. 1993). Well-documented systems involve interactions between *Arabidopsis* ecotypes and pathovars of *Pseudomonas syringae*. Avirulence genes from *P. syringae* pv. *glycinea*, *P. syringae* pv. *pisi*, *P. syringae* pv. *tomato*, and *P. syringae* pv. *maculicola* have been identified that interact with complementary resistance loci in *Arabidopsis*. Interestingly, homologs of *Arabidopsis* disease resistance loci have been demonstrated in crop species such as soybean, pea, and bean (Dangl et al. 1992; Innes et al. 1993; Kunkel et al. 1993; Whalen et al. 1991).

The precise mechanism(s) employed by *Arabidopsis* to deter pathogens has not been well defined. Studies with *Arabidopsis* and *P. syringae* pathovars suggest that products from the phenylpropanoid pathway may have a role in resistance. In *Arabidopsis* plants that recognize the *avrRpt2* gene from *P. syringae* pv. *tomato*, mRNA encoding phenylalanine ammonia-lyase (PAL), the initial biosynthetic step in the general phenylpropanoid pathway, accumulated more rapidly in resistant than susceptible plants (Dong et al. 1991). This pattern of PAL expression was seen also in *Arabidopsis* plants that recognize the *avrB* gene from *P. syringae* pv. *glycinea* (Wanner et al. 1993). Interestingly in both systems, there was no induction of chalcone synthase (CHS) mRNA, suggesting that flavonoids are not involved in resistance (Dong et al. 1991; Wanner et al. 1993). Limited, but delayed accumulation of PAL mRNA was observed in *Arabidopsis* plants challenged with a strain of *Xanthomonas campestris* pv. *campestris* that elicits a hypersensitive reaction (Lummerzheim et al. 1993). The gene *ELI3*, which has high identity to the alcohol dehydrogenase domain of cinnamyl-alcohol dehydrogenase (CAD) from *Arabidopsis* and tobacco, was rapidly activated in tissues that contain the dominant allele of the disease resistance locus *RPM1* (J. Dangl, personal communication; Kiedrowski et al. 1992). These data suggest that formation of phenolic compounds and subsequent synthesis of lignin are associated with the hypersensitive response in *Arabidopsis*.

The role of degradative enzymes has also been examined.

β-Glucanase mRNA levels were examined in host genotypes that respond to bacteria that contain the *avrRpt2* gene. β-Glucanase mRNA levels were induced in both the compatible and incompatible interaction, with higher levels of β-glucanase mRNA accumulation occurring in the compatible interaction at 24 and 48 h postinoculation (h.p.i.; Dong et al. 1991). In *Arabidopsis* tissues challenged with a *X. c.* pv. *campestris* strain that is able to elicit a hypersensitive reaction, β-glucanase mRNA accumulated in the incompatible interaction but this did not occur until 48 h.p.i., indicating this gene is not a component of the hypersensitive response (Lummerzheim et al. 1993). In contrast, chitinase mRNA levels in this system were similar in resistant and susceptible plants (Lummerzheim et al. 1993).

Elicitation of a hypersensitive response in *Arabidopsis* results in synthesis of the phytoalexin camalexin, a sulfur-containing indole derivative (Tsuji et al. 1992). Camalexin is synthesized following challenge with the incompatible wheat pathogen, *P. syringae* pv. *syringae*, but not with the black rot pathogen, *X. campestris* pv. *campestris* (Tsuji et al. 1992). Camalexin is also synthesized in response to *Cochliobolus carbonum* (R. Hammerschmidt, personal communication) and abiotic stress (Tsuji et al. 1992). Messenger RNA encoding one enzyme involved in indole synthesis, anthranilate synthase 1 (ASA1), accumulated following pathogen challenge but was not preferentially induced in resistant plants (Niyogi and Fink 1992).

Production of volatile bactericidal compounds can occur via the separate enzymatic activities of lipoxygenase and myrosinase. In the bean–*P. syringae* pv. *phaseolicola* interaction, lipoxygenase generates volatile compounds with bactericidal activity (Croft et al. 1993). In *Arabidopsis*, more rapid accumulation of lipoxygenase mRNA levels was observed in resistant genotypes that recognized either the *avrB* gene from *P. syringae* pv. *glycinea* or the *avrRpt2* gene from *P. syringae* pv. *tomato* (Melan et al. 1993; Wanner et al. 1993). Myrosinase cleaves glucosinolates to form glucose and unstable aglycones that are able to either spontaneously yield sulfate and a substituted isothiocyanate or under low pH conditions decompose to sulfur and a substituted nitrile (Chapple et al. 1994). Myrosinase breakdown products have the potential to influence the interactions of crucifers with pathogens (Greenhalgh and Mitchell 1976; Mithen et al. 1986).

Other genes are induced in *Arabidopsis* tissues challenged with pathogenic organisms. Although functions are known for some of the pathogenesis-related (PR) proteins, the function of PR1 remains unknown (Uknes et al. 1992, 1993). Pathogenesis-related protein 1 mRNA levels are much higher in tissues challenged with the bacterial pathogen, *P. syringae* pv. *tomato*, or with the viral pathogen, turnip crinkle virus, than in control plants (Uknes et al. 1992, 1993). Increased expression of a PR1a cDNA in transgenic tobacco resulted in increased resistance to two fungal pathogens, *Peronospora tabacina* and *Phytophthora parasitica* var. *nicotianae* (Alexander et al. 1993).

Recognition of potential pathogens and subsequent signaling of a resistance response through putative signal transduction pathway components is not well documented in *Arabidopsis*–pathogen systems. However, other plant–pathogen systems provide a foundation to investigate signaling pathways in *Arabidopsis*. In addition to producing potential bactericidal compounds, lipoxygenase can generate octadecanoid precursors of jasmonic acid, which are potent elicitors of proteinase inhibitors in tomato (Farmer and Ryan 1992). Jasmonic acid, 12-oxo-phytodienoic acid, 13(S)-hydroperoxylinolenic acid, and the fatty acids, linoleic (18:2) acid and linolenic (18:3) acid, are able to elicit accumulation of proteinase inhibitors in tomato (Farmer and Ryan 1992). Although this work was done in tomato and involved induction of proteinase inhibitors, lipoxygenase mRNA levels in *Arabidopsis* can be induced by pathogens, abscisic acid, and methyl jasmonate, indicating that a similar lipoxygenase-mediated signaling cascade may be present (Melan et al. 1993). An initial, rapid plant response to pathogens is an oxidative burst in which there is enhanced production of activated oxygen molecules that are hypothesized to have a role either in the defensive response or in signaling of stress responses (Atkinson 1993; Tzeng and DeVay 1993). Messenger RNA levels of superoxide dismutase accumulate in *Arabidopsis* following challenge with both the compatible *P. syringae* pv. *maculicola* ES4326 strain and incompatible *P. syringae* pv. *maculicola* ES4326 transconjugant that contains the *avrRpt2* gene (Ausubel et al. 1993).

Resistance in *Arabidopsis* to the black rot pathogen, *X. campestris* pv. *campestris* 2D520, is governed by a dominant allele of the single, nuclear gene *RXC1* (Tsuji et al. 1991). Plants of the tolerant ecotype Columbia display no symptoms after infiltration of a bacterial suspension into the intercellular space. This is in sharp contrast to spreading chlorosis that develops on the susceptible ecotype, Pr-0, within 6 days after infiltration of the bacteria into the intercellular space. Although inoculated Columbia plants remain asymptomatic, bacterial multiplication in planta is indistinguishable from that of the susceptible ecotype, indicating tolerance by Columbia to *X. campestris* pv. *campestris* multiplication (Tsuji et al. 1991). The precise cellular mechanism(s) by which Columbia is able to tolerate a *X. campestris* pv. *campestris* 2D520 population in the intercellular leaf space, yet remain asymptomatic, is not understood.

Models for the physiological and biochemical mechanisms of resistance in *Arabidopsis* to bacterial pathogens have focused on those in which a hypersensitive response is elicited and pathogen growth is arrested. A temporal accumulation of mRNA species involved in formation of defense and signaling molecules is correlated with the hypersensitive response and restriction of pathogen levels. These defense and signaling molecules may have a role in tolerance as well. In one model, *X. campestris* pv. *campestris* could fail to elicit these mRNAs in tolerant tissue and thus avoid synthesis of deleterious molecules in planta. In an alternative model, these mRNAs could accumulate in the tolerant interaction and have a role in establishing tolerance. This model suggests that these mRNAs may function in preventing symptom development but not influence *X. campestris* pv. *campestris* growth in tolerant tissue. To test these two hypotheses, we examined the relative accumulation of mRNAs of genes involved in formation of antimicrobial compounds and signaling of stress responses. Examined were mRNA levels of genes responsible for synthesis of phenylpropanoid compounds, lignin precursors, indole glucosinolates, as well as phytoalexins, and PR proteins, and mRNAs of two genes responsible for the formation of potential bactericidal breakdown products of glucosi-

nolates and lipids. Enzymes examined that may have a role in formation of signaling molecules include fatty acid desaturase, lipoxygenase, and superoxide dismutase.

RESULTS

In planta growth studies.

Previous studies with *X. campestris* pv. *campestris* 2D520 and *Arabidopsis thaliana* employed high inoculum concentrations of the bacterium and substantially different growth conditions (Tsuji et al. 1991, 1992). We have modified the growth conditions for *Arabidopsis* to obtain more leaf material per plant and more reproducible symptoms. Infiltration of *X. campestris* pv. *campestris* 2D520 (10^6 to 10^7 CFU ml^{-1}) into the intercellular leaf space of Pr-0 resulted in chlorosis that was detected first at 3 to 4 days postinoculation (d.p.i.), with complete chlorosis of the leaf occurring by 6 d.p.i. (Fig. 1). In contrast, infiltration of *X. campestris* pv. *campestris* 2D520 (10^6 to 10^7 CFU ml^{-1}) into the intercellular space of Columbia resulted in asymptomatic plants at 3 d.p.i., with no to limited symptoms present at 6 d.p.i. (Fig. 1). Leaves treated with phosphate buffer remained asymptomatic (data not shown). Use of higher concentrations of *X. campestris* pv. *campestris* 2D520 (>10^7 to 10^8 CFU ml^{-1}) on Columbia and Pr-0 plants grown under the short light cycle described here result in more rapid symptom development on Pr-0 and slight chlorosis on Columbia, implying that a high inoculum of *X. campestris* pv. *campestris* 2D520 can overcome the tolerance mechanism present in Columbia (data not shown). Infiltration of *X. campestris* pv. *campestris* 2D520 into the intercellular space resulted in an increase in bacterial population levels of both Columbia and Pr-0 (Fig. 2). Bacterial population levels in Columbia and Pr-0 increased 100-fold from ~10^4 CFU cm^{-2} after infiltration to ~10^6 to 10^7 CFU cm^{-2} at 3 to 4 d.p.i. and remained at ~10^6 to 10^7 CFU cm^{-2} until 6 d.p.i. (Fig. 2).

Northern analyses.

Quantitation of the signal observed in Northern blots allowed for a precise measurement of mRNA levels in tolerant and susceptible *Arabidopsis* plants infected with *X. campestris* pv. *campestris* 2D520. Defense gene mRNA levels were examined throughout an extended time course starting at 3 h and terminating at 72 h after challenge with *X. campestris* pv. *campestris* (Figs. 3 to 6). During this time both susceptibility in Pr-0 and tolerance in Columbia are established, as Pr-0 exhibits chlorosis within 3 to 4 days of inoculation while Columbia remains asymptomatic (Fig. 1). In addition, the two ecotypes were inoculated with *P. syringae* pv. *syringae* PSSD20 to provide a reference to mRNA levels incurred during a hypersensitive response (Table 1). With the exception of PR1, mRNA levels in the bacterial treatments were expressed as a ratio of buffer-treated tissues rather than percent maximal accumulation to eliminate bias attributable to the infiltration method and allow direct comparison of mRNAs levels among replicates. As PR1 mRNA was not detected in all buffer-treated control samples, expression of PR1 mRNA accumulation as a ratio of treatment versus control was not possible. Thus, mRNA accumulation was expressed as total radioactivity in the treatment minus the buffer control (Fig. 6).

Messenger RNA of ASA1, ASA2, MYR (Fig. 3), CAD, C4H, ELI3 (Fig. 4), FAD3, LOX1, and SOD (Fig. 5) did not accumulate to substantial levels throughout the extended time course in either Columbia or Pr-0 inoculated with *X. campestris* pv. *campestris* 2D520. In contrast, mRNA encoding PR1 accumulated to high levels in both Columbia and Pr-0 tissues

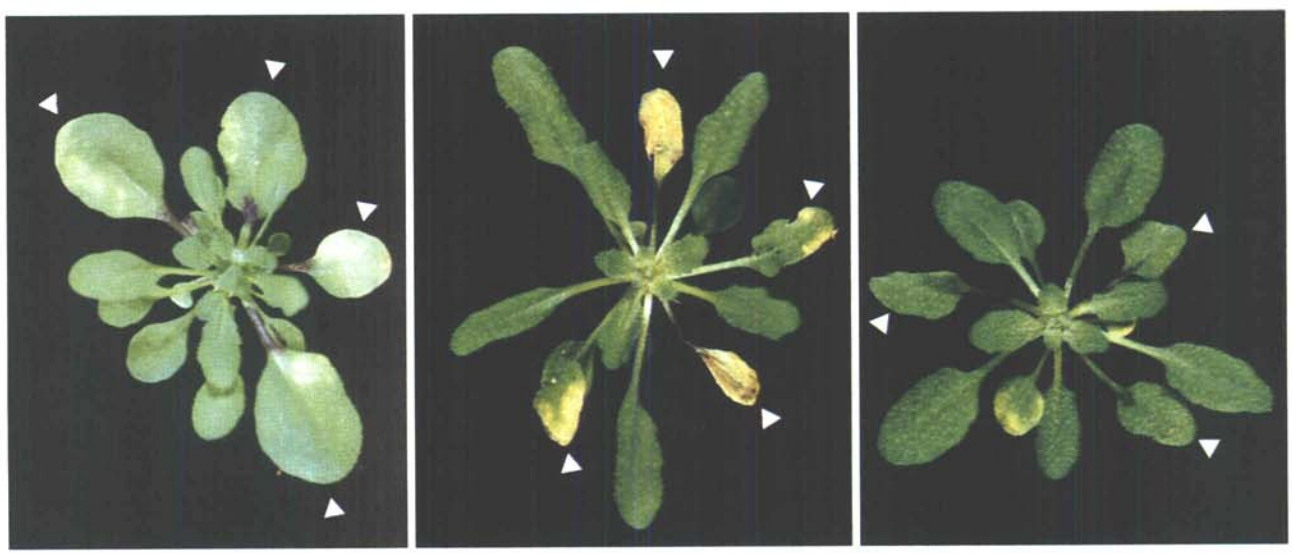

Fig. 1. Symptom formation in Columbia and Pr-0 following infiltration with *Xanthomonas campestris* pv. *campestris* 2D520. Leaves of *Arabidopsis* ecotype Columbia (tolerant) or Pr-0 (susceptible) were infiltrated with a suspension of *X. campestris* pv. *campestris* 2D520 as described in Materials and Methods. The inoculation densities were 6.2×10^6 CFU ml^{-1} in panel A and 1.5×10^7 CFU ml^{-1} in panels B and C. Inoculated leaves were marked on their petiole with a black felt pen and denoted on the figure with an arrow. Only half of each leaf was infiltrated with the bacterial suspension. **A,** Pr-0 at 3 d.p.i.; **B,** Pr-0 at 6 d.p.i., and **C,** Columbia at 6 d.p.i.

beginning at 24 h.p.i. and continuing until 72 h.p.i. (Fig. 6). PR1 mRNA levels accumulated to higher levels in Pr-0 in comparison to Columbia. These results were seen in three replicates.

In a fourth replicate, high basal levels of PR1 mRNA were detected in buffer-treated samples, suggesting previous exposure of the plants to an eliciting agent such as a pathogen. In this fourth replicate, some accumulation of ASA1, CAD, C4H, ELI3, FAD3, LOX1, and SOD mRNA was observed in both Columbia and Pr-0 tissues at 72 h.p.i. (data not shown). Due to the high basal levels of PR1 mRNA, these accumulations cannot be attributed solely to the presence of *X. campestris* pv. *campestris* and the data from this replicate were discarded.

Treatment with *P. syringae* pv. *syringae* PSSD20 resulted in accumulation of mRNAs (Table 1). At 12 h.p.i., CAD, C4H, and ELI3 mRNA levels were induced 1.83- to 2.19-fold in Columbia tissue and 1.32- to 1.65-fold in Pr-0 tissue infiltrated with *P. syringae* pv. *syringae* PSSD20. SOD was also induced in the two ecotypes (1.33-fold in Columbia and 1.43-fold in Pr-0), whereas LOX1 was induced only in Columbia (1.71-fold). Although ASA1 was induced in both ecotypes in the two replicates, the magnitude of induction was different in the two replicates with 1.57- and 4.87-fold induction in Columbia and 2.39- and 11.34-fold induction in Pr-0, respectively. PR1 mRNA levels were dramatically induced in both Columbia and Pr-0 by *P. syringae* pv. *syringae* PSSD20 in the two replicates.

The basal levels of nine of the 10 mRNA species were similar between Columbia and Pr-0 (data not shown). Interestingly, the basal levels for myrosinase were substantially different in Columbia versus Pr-0. Myrosinase levels in 0 h, untreated or buffer-treated samples were 1.4- to 3.2-fold higher in Columbia compared to Pr-0 (data not shown).

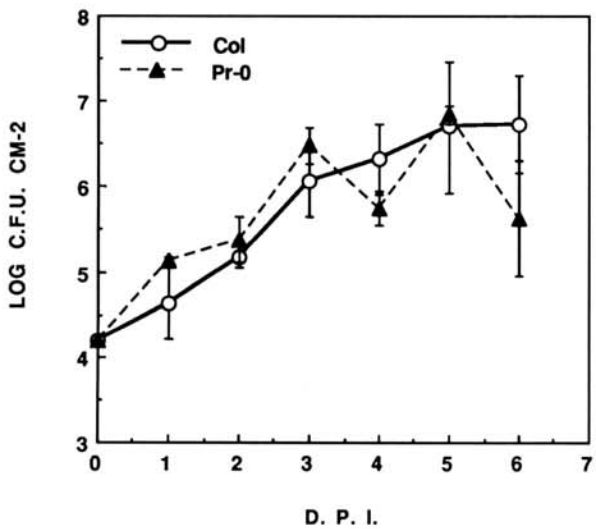

Fig. 2. Growth of *Xanthomonas campestris* pv. *campestris* 2D520 in planta. Leaves of *Arabidopsis* ecotype Columbia or Pr-0 were infiltrated with a suspension of *X. campestris* pv. *campestris* 2D520 (9×10^6 CFU ml^{-1}) as described in Materials and Methods. Leaf disks were excised and bacterial populations determined as described in Materials and Methods. Data are the mean and standard deviation of two replicates.

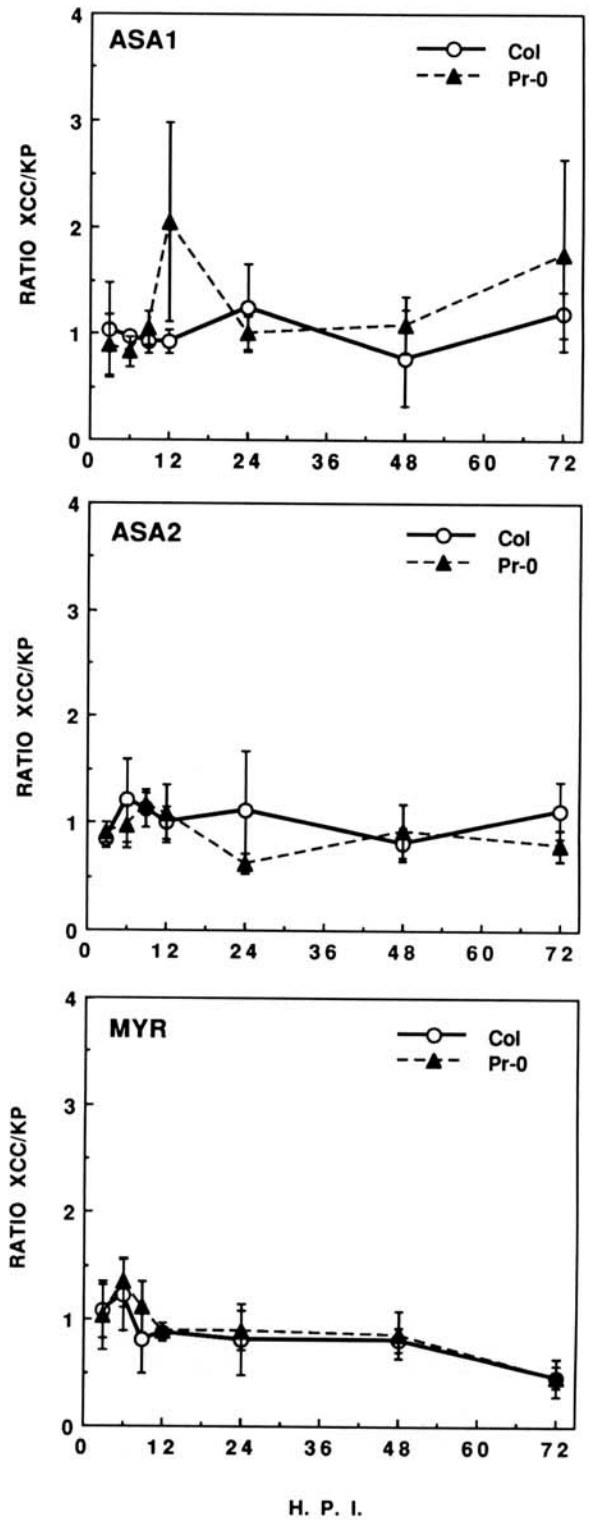

Fig. 3. Quantitation of mRNA levels of genes involved in synthesis of phytoalexins and catabolism of glucosinolates from Columbia and Pr-0 leaves inoculated with *Xanthomonas campestris* pv. *campestris* 2D520. Anthranilate synthase 1 (ASA1), anthranilate synthase 2 (ASA2), and myrosinase (MYR) mRNA levels were examined. Data represented are the mean ± the standard deviation from three replicates. Treatments, RNA extractions, and Northern hybridizations of samples were as described in the Materials and Methods.

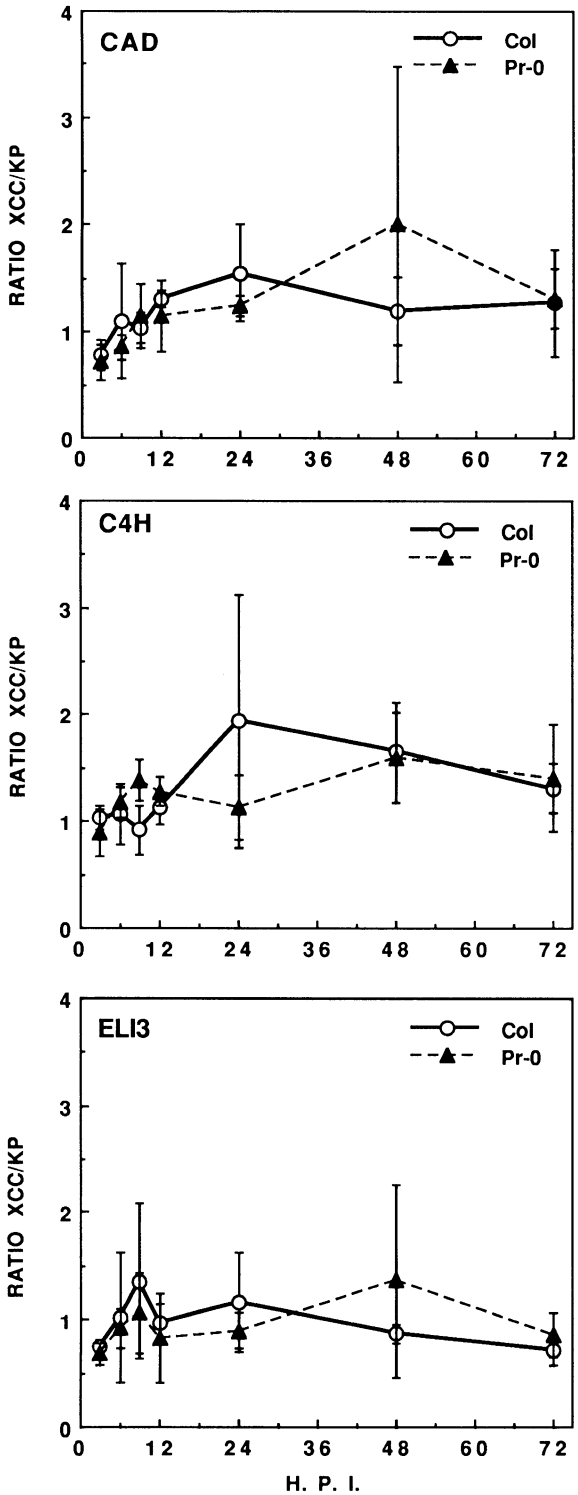

Fig. 4. Quantitation of mRNAs levels from Columbia and Pr-0 leaves inoculated with *Xanthomonas campestris* pv. *campestris* 2D520 of three genes involved in phenylpropanoid metabolism. Probes examined were cinnamyl-alcohol dehyrogenase (CAD), cinnamate-4-hydroxylase (C4H), and the ELI3 plant defense gene (ELI3). Data represented are the mean ± the standard deviation from three replicates. Treatments, RNA extractions, and Northern hybridizations of samples were as described in the Materials and Methods.

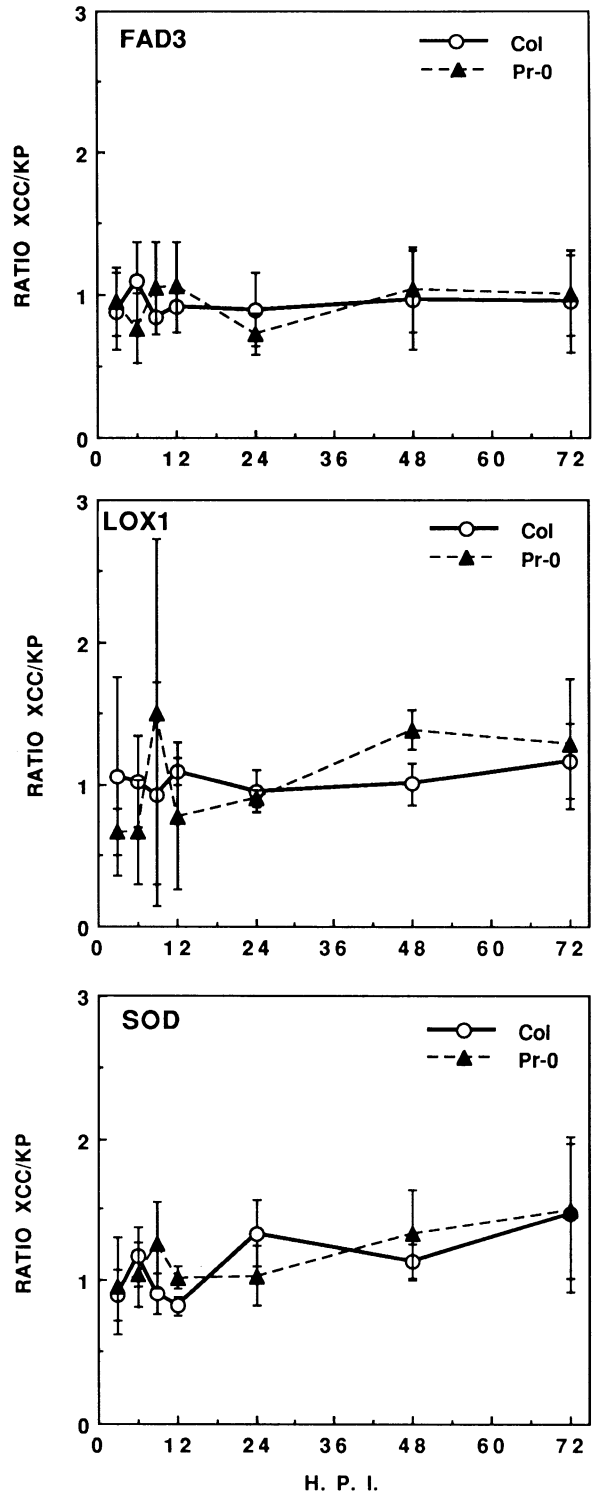

Fig. 5. Quantitation of mRNA levels from Columbia and Pr-0 leaves inoculated with *Xanthomonas campestris* pv. *campestris* 2D520 of three genes involved in formation of signaling molecules. Probes examined were fatty acid desaturase 3 (FAD3), lipoxygenase (LOX1), and superoxide dismutase (SOD). Data represented are the mean ± the standard deviation from three replicates. Treatments, RNA extractions, and Northern hybridizations of Columbia or Pr-0 samples were as described in the Materials and Methods.

DISCUSSION

Columbia and Pr-0 are two ecotypes of *A. thaliana* that differ at the *RXC1* locus, with Columbia having the dominant allele, *rxc1-1*, and Pr-0 having the recessive allele, *rxc1-2* (Tsuji et al. 1991). The presence of the dominant allele of *RXC1* permits Columbia to remain asymptomatic when infected with *X. campestris* pv. *campestris* 2D520 but does not result in suppression of *X. campestris* pv. *campestris* population levels. The phenomenon of tolerance in the *Arabidopsis–X. campestris* pv. *campestris* interaction is distinguished from other incompatible interactions that are characterized by the rapid appearance of a hypersensitive response and restricted bacterial population levels. In these incompatible interactions, several genes that encode defensive or signaling molecules are preferentially induced in a manner consistent with their role in formation of a hypersensitive response. Thus, as an initial step in understanding the molecular basis for tolerance in Columbia to *X. campestris* pv. *campestris* 2D520, we examined mRNA levels of genes previously suggested to have a role in resistance in *Arabidopsis* as well as other genes that may have a role in manifestation of resistance or signaling of stress events.

Of the 10 genes examined for mRNA accumulation, only one, PR1, accumulated to substantial levels in either interaction with *X. campestris* pv. *campestris*. The lack of mRNA accumulation of nine of 10 defense-related and signaling genes supports the hypothesis that *X. campestris* pv. *campestris* 2D520 does not elicit typical defensive responses in the tolerant ecotype, Columbia. The substantial accumulation of the PR1 transcript suggests that this gene is a particularly sensitive indicator of pathogen invasion relative to the other nine genes tested. However, this accumulation was not unique to the tolerant interaction, with substantially more PR1 mRNA accumulating in the compatible interaction than the tolerant interaction. Thus, the alternate hypothesis that lack of symptom development in the tolerant host, Columbia, is due to the preferential accumulation of defense-related and signaling mRNAs is not supported by the data.

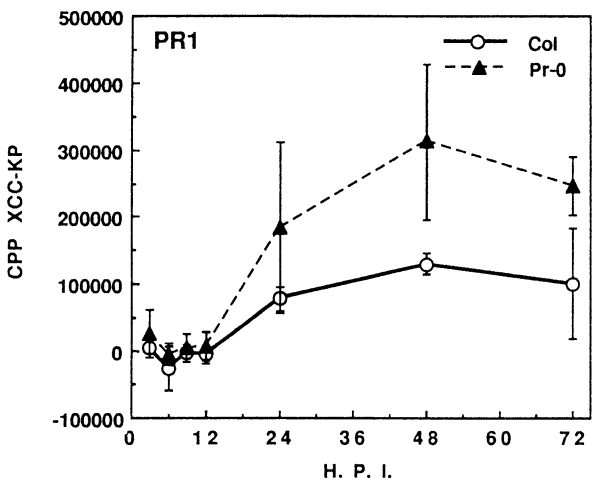

Fig. 6. Quantitation PR1 mRNA populations from Columbia and Pr-0 leaves inoculated with *Xanthomonas campestris* pv. *campestris* 2D520. Treatments, RNA extractions, and Northern hybridizations of samples were as described in Materials and Methods. PR1 mRNA levels are expressed as total counts per pixel of the treatment minus the buffer-treated control and are presented as the mean ± standard deviation of two replicates which were hybridized together with the same PR1 probe. Similar results were obtained with a third replicate.

Columbia and Pr-0 tissues were challenged with *P. syringae* pv. *syringae* PSSD20 to allow comparison of mRNA accumulation in a hypersensitive response versus the tolerant interaction. Induction of CAD, C4H, and ELI3 mRNA levels are consistent with previous findings, which suggests a role for increased accumulation of phenylpropanoid mRNAs in the hypersensitive response (Dong et al. 1991; Lummerzheim et al. 1993; Wanner et al. 1993). The preferential induction of ASA1, but not ASA2, is consistent with the findings of Niyogi and Fink (1992). Also, the accumulation of ASA1 mRNA is correlated with the production of camalexin, an indole-containing phytoalexin (Tsuji et al. 1992). The accumulation of specific mRNAs in Columbia and Pr-0 during a hypersensitive response provides evidence that both ecotypes are capable of accumulating mRNAs of defense-related genes in response to attack by a potential pathogen. However, neither the specificity nor the magnitude of mRNA accumulation observed in *P. campestris* pv. *syringae*-treated tissue at 12 h.p.i. was observed in *X. campestris* pv. *campestris*-treated tissues. These differences in mRNA accumulation between HR-mediated resistant and tolerant interactions are consistent with differences in bacterial growth in planta. In the hypersensitive response, there is active host cell necrosis associated with a reduction of bacterial population levels. In the *X. campestris* pv. *campestris* 2D520–*Arabidopsis* system, a null phenotype occurs in ecotype Columbia and no substantial reduction in bacterial population is observed in comparison to the susceptible ecotype Pr-0.

Although Columbia and Pr-0 differ in their *RXC1* allele, they are ecotypes of the same species. Their similarity is documented by having similar basal mRNA levels of at least

Table 1. Messenger RNA accumulation in response to infiltration with *Pseudomonas syringae* pv. *syringae* PSSD20

	Ratio of mRNA levels[a]	
Clone	**Columbia**	**Pr-0**
ASA1	3.22 ± 2.33	6.87 ± 6.33
ASA2	1.13 ± 0.24	1.02 ± 0.09
CAD	2.19 ± 0.21	1.59 ± 0.15
C4H	1.83 ± 0.57	1.32 ± 0.08
ELI3	2.17 ± 0.66	1.65 ± 0.11
FAD3	0.87 ± 0.04	0.98 ± 0.14
LOX1	1.71 ± 0.31	0.67 ± 0.21
SOD	1.33 ± 0.10	1.43 ± 0.28
MYR	0.77 ± 0.15	0.97 ± 0.21
CPP Treatment-Buffer Control[b]		
PR1-RepI	19528	76721
PR1-RepII	130338	600995

[a] Quantitation of mRNAs levels in Columbia and Pr-0 tissue at 12 h.p.i. with *P. syringae* pv. *syringae* PSSD20. Genes examined were anthranilate synthase (ASA1), anthranilate synthase 2 (ASA2), cinnamyl-alcohol dehydrogenase (CAD), cinnamate-4-hydroxylase (C4H), ELI3 plant defense gene (ELI3), fatty acid desaturase 3 (FAD3), lipoxygenase (LOX1), myrosinase (MYR), and superoxide dismutase (SOD). Treatments, RNA extractions, and Northern hybridizations and quantitation of samples were as described in the Materials and Methods. The mean and standard deviation from two replicates is reported.

[b] PR1 mRNA levels were expressed as the counts per pixel of the treatment minus the control tissues. Data from two replicates are shown. The determination of the mean could not be derived due to differences in signal strength between the replicates.

nine genes and by a similar hypersensitive response to *P. syringae* pv. *s. syringae* PSSD20. In contrast, a substantial difference in basal myrosinase mRNA levels exists between Columbia and Pr-0. In *Arabidopsis*, myrosinase is encoded by a small gene family of three members (Xue et al. 1992). Examination of restriction fragment length polymorphisms between Columbia and Pr-0 suggests the myrosinase gene structure is highly conserved (Buell and Somerville, unpublished). Thus, the difference in myrosinase mRNA basal levels between the two ecotypes may be due to differences in gene expression rather than the size of the myrosinase gene family.

This study does not implicate increased levels of defense and/or signaling molecules in establishment of the tolerant phenotype and suggests that tolerance depends on other molecular mechanisms. The plant hormone ethylene has been implicated in symptom development in other plant-bacterial interactions and may have a role in tolerance of *Arabidopsis* to the black rot pathogen (Bent et al. 1992; Stall and Hall 1984). Future experiments focused on the potential role of ethylene in *RXC1*-mediated symptom development will provide information on the mechanism(s) of tolerance and susceptibility to the black rot pathogen. In addition, cloning *RXC1* using map-based cloning approaches is in progress and characterization of *RXC1* will provide further insight into why Columbia is tolerant to *X. campestris* pv. *campestris* 2D520.

MATERIALS AND METHODS

Bacterial strains and plasmids.

Plasmids used in this study are listed in Table 2. *X. campestris* pv. *campestris* 2D520 (C. Kado, University of California, Davis CA) was stored at –80°C in 20% glycerol and streaked on 523 medium (Fahy and Persley 1983) supplemented with rifampicin (50 μg ml^{-1}). *P. syringae* pv. *syringae* PSSD20 (D. Fulbright, Michigan State University, East Lansing, Mich.) was stored at –80°C in 20% glycerol and streaked onto King's Medium B (King et al. 1954) containing nalidixic acid (50 μg ml^{-1}). *X. campestris* pv. *campestris* 2D520 suspensions used for plant inoculations were obtained from cultures grown overnight at 30°C and 250 rpm in 523 medium supplemented with rifampicin (50 μg ml^{-1}). *P. syringae* pv. *syringae* PSSD20 suspensions were obtained from cells grown at 23°C and 150 rpm in King's medium B. *X. campestris* pv. *campestris* 2D520 and *P. syringae* pv. *syringae* PSSD20 cells were washed twice in 10 mM potassium phosphate, pH 6.9, and suspended to give the stated concentrations.

Plant growth conditions and infection conditions.

Seeds of *Arabidopsis thaliana* ecotype Columbia and Pr-0 were surface sterilized by immersion in 70% ethanol for 2 min, followed by vigorous agitation in 1.6% sodium hypochlorite/0.02% Triton X-100 for 15 min. Seeds were rinsed four times in sterile deionized water, suspended in 0.15% agar, and plated onto Gamborg's B5 medium (Gibco BRL, Gaithersburg, Maryland). Seeds were allowed to germinate for approximately 2 weeks at 23°C with an 8 h light (~120 μE m^{-2} s^{-1}; cool white fluorescent lamps)/16 h dark cycle. Seedlings were transplanted into sterilized *Arabidopsis* soil mix as described previously (Somerville and Ogren 1982). Plants were then grown for 3 to 4 weeks under conditions described above but with 130 to 180 μE m^{-2} s^{-1} light and ~65 to 75% relative humidity.

Leaves (four to five per plant) of well-formed rosettes were inoculated by hand infiltration of a suspension of *X. campestris* pv. *campestris* 2D520 (inoculum concentrations in the three replicates were: 4.3, 8.8, and 8.9 × 10^6 CFU ml^{-1}), *P. syringae* pv. *syringae* PSSD20 (inoculum concentrations were 2.6 and 2.8 × 10^6 CFU ml^{-1}), or 10 mM potassium phosphate buffer, pH 6.9, using a plastic, disposable 1-ml syringe (Tsuji et al. 1991). Excess liquid was removed from the leaf surface with Kimwipes. For in planta growth studies, three leaf disks (diameter = 0.6 cm) from separate plants were excised using a hole punch and ground in 10 mM potassium phosphate pH 6.9. Serial dilutions of the extracts were plated on 523 medium containing rifampicin. For RNA extractions, the entire rosette was collected, immersed in liquid nitrogen, and stored at –80°C until RNA was extracted. Buffer or treated tissue was harvested at 3, 6, 9, 12, 24, 48, and 72 h.p.i. The 0-h tissue was untreated. Each treatment consisted of treated leaf tissue from 12 to 14 plants.

Molecular techniques.

Leaf tissue was homogenized in an equal volume of phenol and buffer (0.18 M Tris-Cl, pH 8.2, 4.5 mM sodium ethylenediamine tetraacetic acid (EDTA), 1% sodium dodecyl sulfate [SDS]) and RNA was extracted as described by Palmiter (1974) and modified by Haffner et al. (1978). RNA samples, 5 μg, were electrophoresed on formaldehyde gels and transferred to Hybond-N nylon membranes (Amersham, Arlington Heights Ill.; Ausubel et al. 1987; Sambrook et al. 1989). Two

Table 2. Clones used in this study

Probe	Relevant characteristics	Source or reference
ASA1	pKN41; *Arabidopsis thaliana* anthranilate synthase 1 cDNA	Niyogi and Fink 1992
ASA2	pKN408A; *A. thaliana* anthranilate synthase 2 cDNA	Niyogi and Fink 1992
CAD	33E10T7P; *A. thaliana* cinnamyl-alchohol dehydrogenase cDNA expressed sequence tag	*Arabidopsis* Biological Resource Center[a]
C4H	SCD12-T7P; *A. thaliana* cinnamate-4-hydroxylase cDNA expressed sequence tag	*Arabidopsis* Biological Resource Center[a]
ELI3	ELI3-2; *A. thaliana* ELI3-2 cDNA	Kiedrowski et al. 1992
EIF4A	p1175-34; *A. thaliana* eIF4A translation initiation factor clone	Taylor et al. 1993
FAD3	pBNDES3; *Brassica napus* fatty acid desaturase 3 cDNA	Arondel et al. 1992
LOX1	LOX1; *A. thaliana* lipoxygenase cDNA	Melan et al. 1993
MYR	34B7T7; *A. thaliana* myrosinase cDNA expressed sequence tag	*Arabidopsis* Biological Resource Center[a]
PR-1	PR-1; *A. thaliana* pathogenesis related protein 1 cDNA	Uknes et al. 1992
SOD	pcSODRH; *A. thaliana* superoxide dismutase cDNA	Hindges and Slusarenko 1992

[a] Ohio State University, Columbus Ohio.

conditions for Northern blot hybridizations were employed. For the first protocol, Northern blots were prehybridized in 0.12 M Tris-Cl, pH 7.4; 0.6 M sodium chloride; 8 mM EDTA; 0.1% sodium pyrophosphate; 0.2% SDS; 100 μg ml^{-1} heparin at 65°C overnight (Sambrook et al. 1989). The hybridization buffer was identical to the prehybridization buffer but was supplemented with 10% (w/v) dextran sulfate, and the heparin concentration was increased to 1 mg ml^{-1}. Probes were synthesized from isolated inserts using a random primed reaction kit supplied by Boehringer Mannhein Biochemicals (Indianapolis, Ind.). Northern blots were washed by placing filters at 23°C in 0.2× SSC (3 M sodium chloride, 0.3 M sodium citrate)/ 0.2% SDS and incubating at 65°C for 1 h and followed by a second wash in 0.1× SSC/0.1% SDS under the same temperature regime. In the second protocol, Northern blots were prehybridized and hybridized at 65°C in 5× SSPE, 0.5 % SDS, 5× Denhardt's, and 100 μg ml^{-1} salmon sperm DNA (Sambrook et al. 1989). Blots were washed three times for 15 min in 1× SSPE, 0.1% SDS at 55°C. All Northern blots were stripped of radioactive probe by placing the filters in 95°C 0.1× SSPE/0.1% SDS and then washing for 30 min at 21°C. Blots were checked for complete removal of radioactivity by autoradiography.

Northern blots were exposed to X-ray film as well as Phosphorimager cassettes. Northern blots were quantitated using a Molecular Dynamics ImageQuant Program (Sunnyvale, Calif.). Equal loading of the lanes was confirmed by ethidium bromide staining of the RNA and Northern hybridization with the control mRNA, EIF4A (Taylor et al. 1993). Due to differences in signal strength among different hybridizations, raw pixel counts could not be compared directly. Thus, with the exception of PR1 mRNA, the amount of hybridization for each band was quantitated, adjusted for loading differences as determined by representative hybridizations with the translation initiation factor clone, EIF4A, and expressed as a ratio of treatment to the buffer-treated control from the same time point. Due to the lack of PR1 basal expression in a majority of samples, ratios of PR1 to buffer-treated control samples could not be derived. Thus, the amount of hybridization to PR1 was quantitated, adjusted for loading differences relative to the control mRNA, EIF4A, and expressed as counts per pixel in the bacterial treatment minus the buffer control.

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