The Role of *NDR1* in Avirulence Gene-Directed Signaling and Control of Programmed Cell Death in Arabidopsis[1]

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Arabidopsis plants containing the *ndr1-1* mutation are incapable of mounting a hypersensitive response to bacteria carrying *avrRpt2*, but show an exaggerated cell death response to bacteria carrying *avrB* (Century et al., 1995). We show here that *ndr1-1* plants are severely impaired in induction of systemic acquired resistance and *PR1*-driven transcription of a reporter gene in response to *Pseudomonas syringae* strains carrying *avrRpt2* but not in response to *P. syringae* carrying *avrB*. The *ndr1-1* mutation also impaired salicylic acid (SA) accumulation in response to treatments that produced reactive oxygen species (ROS) and impaired induction of systemic acquired resistance in response to in situ production of ROS. Hydrogen peroxide accumulated in wild-type Arabidopsis leaves beginning 4 to 7 h postinoculation with *P. syringae* carrying either *avrRpt2* or *avrB*. In *ndr1-1* plants, *P. syringae* carrying *avrRpt2* elicited no detectable hydrogen peroxide production. Hydrogen peroxide production in response to bacteria carrying *avrB* was similar to that of Columbia in kinetics but of lesser intensity at early time points. These data are interpreted to indicate that *NDR1* links ROS generation to SA production and that the phenotypic consequences of the *ndr1-1* mutation are caused by a reduced ability to accumulate SA upon pathogen infection.

Exquisite specificity is a hallmark of gene-for-gene disease resistance. Individual plant lines carry a specific complement of disease resistance (*R*) genes. Plants resist infection only if the pathogen carries a specific avirulence (*avr*) gene that is the matched cognate of one of these plant *R* genes. Mutant plants with nonfunctional alleles of a particular *R* gene fail to recognize a pathogen carrying the corresponding *avr* gene, and disease ensues (Parker et al., 2000; Staskawicz, 2001). With bacterial pathogens, this specificity of molecular recognition in at least some cases is associated with direct binding of the plant R gene product to the bacterial avr gene product (Scofield et al., 1996; Tang et al., 1996). Recognition takes place inside the plant cell (Gopalan et al., 1996; Leister et al., 1996) following export of the avr gene product from the bacteria via a type III secretion system (Pirhonen et al., 1996; Mudgett and Staskawicz, 1998).

In contrast to the specificity of upstream molecular recognition processes, downstream plant responses to pathogen infection often bear strong similarities despite being elicited by vastly different types of pathogen. Gene-for-gene disease resistance is usually accompanied by rapid cell death (the hypersensitive response [HR]; Klement, 1982) in plant cells that are in direct contact with pathogen (Turner and Novacky, 1974). Uninoculated regions of the plant are induced to display an immunity to further pathogen challenge termed systemic acquired resistance (SAR). SAR protects plants from a broad spectrum of pathogens including those very different from the original (Ryals et al., 1994). A set of genes termed "pathogenesis related" (*PR*) are induced both locally and systemically (Lotan and Fluhr, 1990). Some PR gene products have been shown to possess antimicrobial activity (van Loon, 1997).

Previous analysis of disease resistance signaling has identified two second messengers: reactive oxygen species (ROS) and salicylic acid (SA). An NADPH oxidase activity related to that of mammalian neutrophils is thought to produce superoxide in an oxidative burst early in the response to pathogen (Doke and Ohashi, 1988; Mehdy, 1994). Both enzymatic and nonenzymatic steps subsequently will convert the superoxide to other types of ROS (Sutherland, 1991). It is not known which type of ROS is critical for disease resistance. However, exposing plants to treatments such as UV light or ozone that generate multiple types of ROS induces most facets of the resistance response (Levine et al., 1994; Yalpani et al., 1994; Green and Fluhr, 1995; Sharma et al., 1996).

SA is found in plants mostly as a β-glucoside (Enyedi et al., 1992; Malamy et al., 1992). Coincident

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with the disease resistance response, levels of free SA, and subsequently the conjugated forms of SA, rise dramatically (Yalpani et al., 1993). Transgenic plants engineered with *nahG*, a gene that encodes salicylate hydroxylase that degrades SA, show enhanced pathogen growth and impaired induction of SAR and *PR* genes (Gaffney et al., 1993; Delaney et al., 1994). Transgenic *nahG* plants show impaired gene-for-gene resistance to disease caused by some pathogens (Gaffney et al., 1993), but not others (Brading et al., 2000). Arabidopsis *nahG* plants do not show an HR to bacteria carrying *avrRpt2* but do show an HR to bacteria carrying *avrRpm1* (Rate et al., 1999). Both bacterial strains are avirulent on the Columbia parental line.

It is thought that SA acts downstream of ROS production because the ability of exogenous ROS to induce resistance responses is dependent on SA accumulation (Bi et al., 1995; Neuenschwander et al., 1995). However, SA also appears to potentiate both ROS production and cell death in the response of cultured soybean cell suspensions to avirulent *Pseudomonas syringae* in what has been termed "agonist-dependent gain control" (Shirasu et al., 1997). Other experiments in Arabidopsis also support a role for SA in potentiating defense responses (Weymann et al., 1995; Mauch-Mani and Slusarenko, 1996; for discussion, see Shapiro, 2000). Nitric oxide has also been implicated in disease resistance signaling (Delledonne et al., 1998; Durner et al., 1998), and mammalian precedents suggest that it could function synergistically with ROS (Halliwell et al., 1999).

Mutations in *NDR1* compromise resistance to numerous strains of *P. syringae* and *Peronospora parasitica*. Mutant *ndr1* plants exhibit an exaggerated cell death response upon inoculation with bacteria carrying *avrB*, *avrRpm1*, or *avrPphB* despite being unable to restrict multiplication of these bacteria or resist disease caused by these bacteria. It is interesting that the HR of *ndr1* mutant plants is strain specific in that bacteria carrying *avrRpt2* do not elicit an HR (Century et al., 1995). *NDR1* encodes a small, highly basic, putative integral membrane protein (Century et al., 1997). Transcription of *NDR1* is induced by pathogen infection at very early time points. However, the sequence has not revealed a precise biochemical activity for NDR1.

Arabidopsis mutants are a valuable tool for understanding which of the myriad of responses correlated with gene-for-gene disease resistance make significant contributions to limiting pathogen growth and preventing disease (Shapiro, 2000). The strain specificity of the effects of the *ndr1* mutations on the HR led us to examine the strain specificity of other responses correlated with gene-for-gene disease resistance. These data suggest that the *ndr1-1* mutation blocks ROS-dependent SA accumulation. This hypothesis is further supported by results of experiments using in situ ROS generation. This reduced ability to accumulate SA appears to impair agonist-dependent gain control of the HR. These effects explain the phenotypic consequences of the *ndr1-1* mutation.

RESULTS

PR1-Driven Transcription in *ndr1-1* versus Columbia

The following approach was taken to determine the strain specificity of *ndr1* effects on responses correlated with gene-for-gene disease resistance: *P. syringae* bacteria carrying *avrB* were chosen as a representative of the class of bacteria that gave an exaggerated cell death response (Century et al., 1995). These strains were compared with *P. syringae* carrying *avrRpt2* for their effects on *ndr1-1* and Columbia plants. The *avr* genes were carried on the same stable plasmid vector. When informative, multiple bacterial strain backgrounds were used in an experiment. The strain backgrounds *P. syringae* pv tomato DC3000 (DC3000) and *P. syringae* pv *maculicola* 4326 (*P.s.m.* 4326) are pathogenic strains that multiply in Arabidopsis leaves. *P. syringae* pv *glycinea* Race 5 (*P.s.g.* Race 5) is a soybean pathogen that does not multiply in Arabidopsis leaves or cause disease on Arabidopsis (Century et al., 1995). The *ndr1-1* allele was chosen for these experiments because it was known to be a null allele (Century et al., 1997).

First, we investigated whether *ndr1-1* mutant plants showed differences from Columbia in *PR* gene transcription in response to bacteria carrying *avrRpt2* or *avrB*. The *PR1* gene is the most tightly regulated of the Arabidopsis *PR* genes (Uknes et al., 1992). A transgenic line carrying the β-glucuronidase (*GUS*) reporter gene under the control of the *PR1* gene promoter in a Columbia background was generated (see "Materials and Methods"). This line showed high uniform GUS expression in response to the same conditions that induce transcription of the native *PR1* gene (Uknes et al., 1992). This transgene was crossed into the *ndr1-1* background. These lines were designated *Col-0:PR1/GUS* and *ndr1-1:PR1/GUS*.

These lines were used to investigate *PR1*-driven transcription in response to bacterial inoculation. Leaves of either *Col-0:PR1/GUS* or *ndr1-1:PR1/GUS* plants were hand inoculated with 1×10^6 bacteria mL^{-1}, carrying *avrRpt2*, *avrB*, or the empty pVSP61 vector. Blank inoculations were performed using 10 mM $MgCl_2$. Bacteria-induced GUS activity was assessed using a fluorogenic substrate (see "Materials and Methods"). The data are presented in Figure 1.

The most striking result from this experiment was that *ndr1-1:PR1/GUS* plants inoculated with bacteria carrying *avrRpt2* showed negligible GUS activity. This impairment relative to Columbia plants was seen at both time points. Replicate experiments with *P.s.g.* Race 5 strains gave similar results (data not shown). All differences were judged significant at the $P < 0.01$ level using Student's *t* tests.

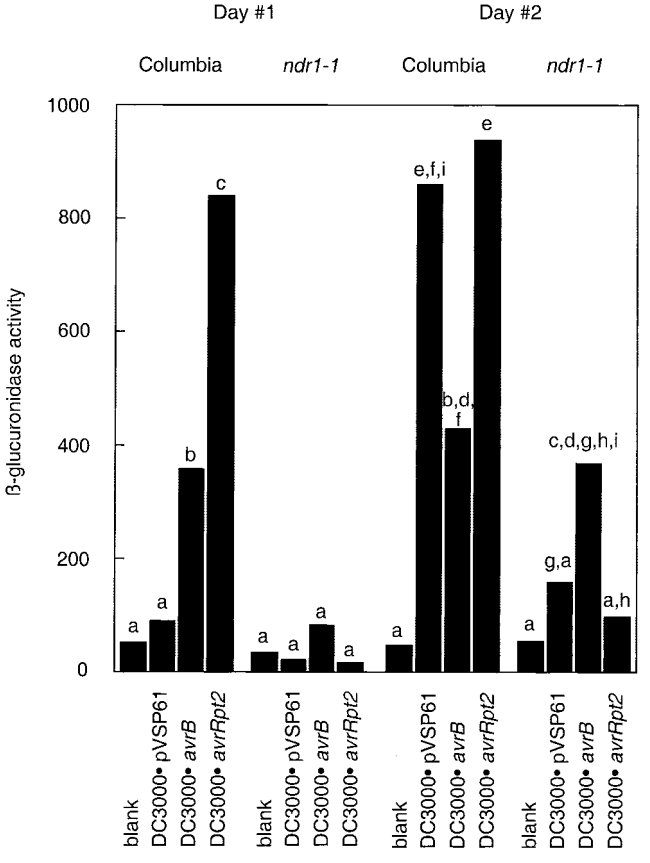

Figure 1. Bacteria-induced *PR1*-driven transcription in *ndr1-1* versus Columbia. 1×10^6 bacteria mL^{-1} were hand inoculated into leaves of Arabidopsis carrying a transgene expressing GUS under control of the *PR1* promoter in either the Columbia or the *ndr1-1* background. Each bar represents a mean of data from 11 to 12 leaves. All data are expressed in arbitrary fluorescence units. Data are normalized in that an identically sized leaf area was sampled in each case. Differences between means were assessed for statistical significance using Student's *t* tests. Lowercase letters indicate which differences between means were judged significant at the $P < 0.01$ level. These comparisons were made separately for the two experimental time points. *PR1*-driven transcription induced by DC3000·*avrB* was significantly greater than that induced by DC3000·*avrRpt2* on *ndr1-1* plants at both time points if significance was judged at the $P < 0.05$ level (not pictured).

Experiments using bacteria carrying *avrB* had very different results. DC3000·*avrB* elicited comparable GUS activity in the two lines at the 2-d time point, although the *ndr1-1:PR1/GUS* response was significantly lower (Student's *t* test, $P < 0.01$) at the 1-d time point. Replicate experiments with *P.s.g.* Race 5 strains failed to show significant differences due to the *ndr1-1* mutation at either time point (data not shown). Thus, the bacterial strains that did not elicit an HR on *ndr1-1* plants caused negligible induction of *PR1*-driven transcription, whereas the bacterial strains that could give a vigorous cell death response on *ndr1-1* induced *PR1*-driven transcription.

The behavior of the strains carrying the empty vector was also worthy of note. DC3000·pVSP61 induced only low-level GUS activity in *Col-0:PR1/GUS* plants at the 1-d time point, but high level activity by the 2-d time point. The data from both time points appear to show impairment in the response of the *ndr1-1:PR1/GUS* line. Differences between the Arabidopsis lines were significant at the $P < 0.01$ (Student's *t* test) level only with the data from the 2-d time point. *P.s.g.* Race 5·pVSP61 induced only low-level GUS activity in both lines at both time points (data not shown).

Biological Induction of SAR in *ndr1-1* versus Columbia

Next, we investigated the relative ability of the two bacterial strains to induce SAR on *ndr1-1* and Columbia plants. SAR can be assessed in Arabidopsis as the ability of a primary inoculation with bacteria to inhibit multiplication of bacteria introduced later as a secondary challenge (Cameron et al., 1994). *P. syringae* do not move systemically in Arabidopsis; therefore, excision of primary inoculated leaves prior to secondary challenge allows monitoring growth of only the challenge bacteria.

The data from one representative set of experiments is shown in Figure 2. In all cases, data from plants that had received a primary inoculation with bacteria (squares) is shown in comparison with data from plants where 10 mM $MgCl_2$ was used as a blank primary inoculation (circles). SAR was judged to be occurring if comparisons of means showed, at minimum, significant differences (Student's *t* test, $P < 0.1$) at either the d-2 or day-4 time point in all replicates of the experiment. Growth of challenge bacteria in plants that received blank primary inoculations was statistically indistinguishable (Student's *t* test, $P > 0.5$) from growth of challenge bacteria in plants which did not receive any primary inoculation (data not shown).

As evidenced by comparison of the solid lines in Figure 2A, DC3000·*avrRpt2* elicited a strong SAR response on Columbia plants. Inoculation with this bacterial strain led to a 1.5 to 3 order of magnitude suppression of growth of the challenge bacteria. In contrast, inoculation of *ndr1-1* mutant plants with this strain did not lead to SAR (compare dashed lines in Fig. 2A).

Inoculation of *ndr1-1* mutant plants with DC3000·*avrB* elicited a SAR response comparable in magnitude to that shown by Columbia plants at the 2-d time point (Fig. 2B). The *ndr1-1* response appears to be partially impaired relative to Columbia at the 4-d time point. In conclusion, the bacterial strain that does not elicit a HR on *ndr1* mutant plants also does not elicit SAR on *ndr1-1*. However, the bacterial strain that elicited an exaggerated cell death response on *ndr1* mutant plants elicited SAR on *ndr1-1*, albeit to a lesser degree than on Columbia plants. The same qualitative trends are seen if the experiment is performed using bacterial strains of a *P.s.g.* Race 5 back-

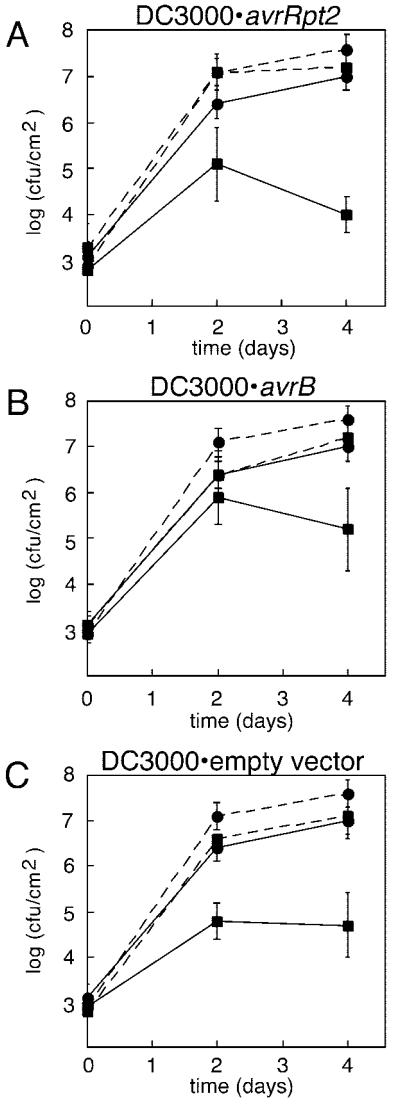

Figure 2. Biological induction of SAR in *ndr1-1* versus Columbia. Columbia plants (solid lines) or *ndr1-1* plants (dashed lines) were inoculated with bacteria (squares for data points) or a $MgCl_2$ blank (circles for data points) 2 d prior to inoculation with *P. syringae* pv tomato DC3000 carrying only the empty pVSP61 vector. Primary inoculated leaves were excised prior to secondary inoculation. Data points represent means of triplicate determinations of in planta bacterial growth at specified time points. A, *P.s.* tomato DC3000 carrying *avrRpt2* used for primary inoculation with bacteria. B, *P.s.* tomato DC3000 carrying *avrB* used for primary inoculation with bacteria. C, *P.s.* tomato DC3000 carrying empty vector used for primary inoculation with bacteria.

ground rather than DC3000-based strains in the primary inoculations (data not shown). All experiments shown in Figure 2 were repeated an additional one to three times with consistent results.

Figure 2C shows results of experiments in which the bacterial strain used in the primary inoculations carried only the empty pVSP61 vector without a cloned *avr* gene. Comparison of the solid lines Figure 2C indicates that the virulent bacterial strain (DC3000·pVSP61) elicited a strong SAR response on Columbia, comparable in magnitude with that elicited by DC3000·*avrRpt2*. A reduced SAR response was shown by *ndr1-1* mutant plants (compare dashed lines). We conclude that SAR elicited by the virulent bacterial strain is partially *NDR1*-dependent.

In all experiments shown in Figure 2, DC3000·pVSP61 multiplies to a greater extent in *ndr1-1* plants than in Columbia, irrespective of whether SAR has been induced. This behavior has been previously documented and is correlated with increased severity of disease symptoms (Century et al., 1995). Similar results have been obtained with *npr1* and *eds* mutants (Cao et al., 1994; Rogers and Ausubel, 1997; Volko et al., 1998).

Bacteria-Induced Hydrogen Peroxide Production in *ndr1-1* versus Columbia

Arabidopsis *ndr1-1* plants did not show the HR, *PR1*-driven transcription, or SAR in response to bacteria carrying *avrRpt2*. These responses were elicited by bacteria carrying *avrB*. Therefore, signaling molecules implicated in eliciting these responses were investigated. Arabidopsis leaf tissue has been reported to release large amounts of ROS upon homogenization (Wolfe et al., 2000). We have confirmed using several published tissue homogenate methods for hydrogen peroxide quantitation that requisite signal-to-noise and reproducibility was not obtainable from these methods (data not shown). Instead, we have employed a recently published in vivo method for hydrogen peroxide quantitation (Wolfe et al., 2000). Leaves are infiltrated with 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), excised, exposed to 365 nm of UV light, and photographed. The use of UV light does not induce artifactual ROS production. The wavelength of UV light used to excite the dichlorofluorescein (DCF) is of much lower energy than that known to induce ROS production, this treatment lasts only 1 min, and photography is performed immediately afterward. This dye fluoresces only upon oxidation to DCF, and is known to be oxidized in Arabidopsis responding to *P. syringae* bacteria primarily by hydrogen peroxide (Bass et al., 1983; Wolfe et al., 2000). DCF fluorescence thus serves as a semiquantitative assay of hydrogen peroxide levels. Evidence has been presented that both DCF and DCFH will leak back into the apoplast following ester hydrolysis in the cytoplasm (Wolfe et al., 2000, and references therein). We have confirmed these observations via confocal microscopy (data not shown). As such, this assay does not distinguish between intracellular and extracellular pools of hydrogen peroxide.

Time courses of increase in DCF fluorescence are presented in Figure 3. The low background fluorescence seen following inoculation with bacteria carrying the empty pVSP61 vector was exclusively red

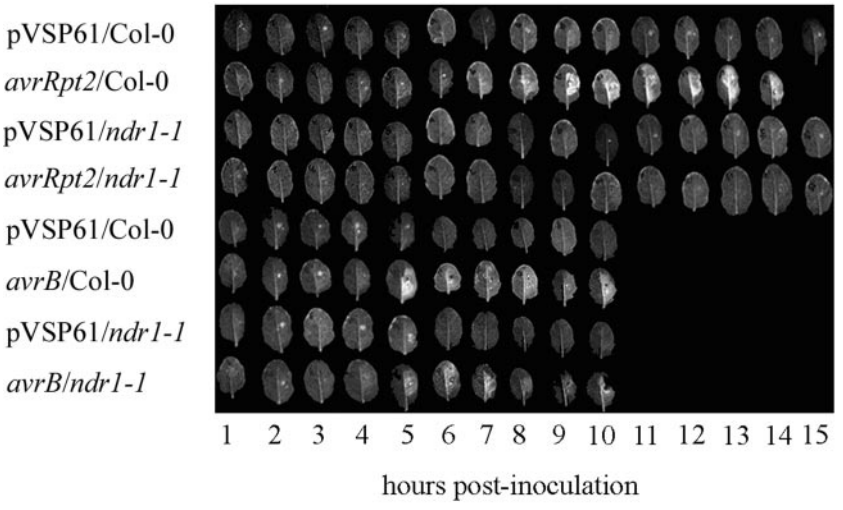

Figure 3. Biological induction of hydrogen peroxide accumulation in *ndr1-1* versus Columbia. Columbia or *ndr1-1* plants were inoculated with *P. syringae* pv tomato DC3000 carrying a plasmid-borne copy of *avrRpt2*, *avrB*, or only the empty pVSP61 vector. DCFH-DA was inoculated 15 to 30 min prior to excising of the leaves at the indicated time points for brief exposure to 365 nm of UV light followed immediately by photography.

chlorophyll fluorescence. A similar background was seen on noninoculated half-leaves. As documented in Table I, green, DCF fluorescence was first seen in Columbia leaves at 4 to 7 h postinoculation, depending on bacterial strain. The first signs of the macroscopic HR were seen 2 to 4 h later with bacteria carrying *avrB*, but as much as 9 h later with bacteria carrying *avrRpt2*. The intensity of DCF signal at time points prior to onset of the macroscopically visible HR was greater in Columbia plants inoculated with bacteria carrying *avrB* than in those inoculated with bacteria carrying *avrRpt2*. This conclusion was confirmed in replicates of the experiment shown in Figure 3 where this comparison was made between leaves photographed at the identical time (data not shown). Differences were very large with the DC3000 or the *P.s.m.* 4326 backgrounds but were somewhat less pronounced with the *P.s.g.* Race 5 background.

By contrast, bacteria carrying *avrRpt2* elicited no detectable signal above background in *ndr1-1* mutant plants. DCF fluorescence of *ndr1-1* mutant plants in response to bacteria carrying *avrB* was similar to that of Columbia in kinetics but of lesser intensity at early time points.

Bacteria-Induced SA Accumulation in *ndr1-1* versus Columbia

Next, we investigated SA levels in pathogen-infected *ndr1-1* versus Columbia plants. Preliminary attempts to use DC3000-based strains allowed measurements of SA levels up to 70 μg of total SA per gram fresh weight at 42 h postinoculation. All data precisely mirrored the HR phenotypes in that the highest levels of SA were seen only when programmed cell death was occurring (data not shown). However, there were obvious pathogen-induced changes in leaf water content during the experiment, even at the low levels of inoculum employed, with both virulent and avirulent strains of bacteria. Normalization based on fresh weight thus would not have allowed accurate comparisons between time points.

To obtain the most informative data possible, we switched to the *Psg* Race 5-based strains. Because these strains are not Arabidopsis pathogens, disturbances to leaf water content were minimized. An inoculum of 1×10^6 bacteria mL^{-1} was used throughout (this is sufficiently low to minimize HR-related loss of water content), and time points were taken at time zero, 12 h postinfiltration, and 42 h postinfiltration. Means of data from three replicate experiments are presented in Figure 4. Columbia plants accumulate both free and total SA in response to all treatments. An unexpectedly large response was seen to treatment with the $MgCl_2$ blank and non-pathogen strain carrying only the empty vector (see below for explanation). Nonetheless, the accumulation of total SA was significantly greater in response to bacteria carrying either *avr* gene as com-

Table I. *Kinetics of bacteria-induced hydrogen peroxide accumulation and the HR in ndr1-1 versus Columbia*

Units are time in hours for all data.

	DC3000 avrB	*DC3000 avrRpt2*	*Psm* 4326 *avrB*	*Psm* 4326 *avrRpt2*	*Psg* Race 5 *avrB*	*Psg* Race 5 *avrRpt2*
Earliest DCF fluorescence in Columbia	5	5	4	7	4–5	4
Initial signs of leaf collapse in Columbia	8	10	8	12	7–8	12–13
Complete leaf collapse in Columbia	10	14	10	15	10	15
Earliest DCF fluorescence in *ndr1-1*	5	None	4	None	4–5	None
Initial signs of leaf collapse in *ndr1-1*	7–8	None	8	None	7–8	None
Complete leaf collapse in *ndr1-1*	9	None	9	None	10	None

pared with the other treatments. Two-way analyses of variance were used with data from the 42-h time point. Differences in means were found to be significant for comparisons of *avrRpt2* versus empty vector ($P < 0.001$), *avrB* versus empty vector ($P < 0.1$), *avrRpt2* versus blank ($P < 0.01$), and *avrB* versus blank ($P < 0.1$).

It is striking that *ndr1-1* mutant plants showed greatly reduced accumulation of both free and total SA at the 12-h time point relative to Columbia in response to all treatments. This impairment in SA accumulation was also seen at the 42-h time point in response to treatment with either the $MgCl_2$ blank or the non-pathogen strain carrying only the empty vector. However, by the 42-h time point, accumulation of free SA in response to bacteria carrying either *avr* gene was comparable in *ndr1-1* mutant plants with that in Columbia plants. Accumulation of total SA at 42 h in response to avirulent bacteria was less in *ndr1-1* mutant plants than in Columbia. However, impairment in the response to avirulent bacteria was not seen to the same extent as impairment in the response to the $MgCl_2$ blank or to the non-pathogen strain carrying only the empty vector. The differences seen at this time point in *ndr1-1* mutant plants between the responses to bacteria carrying *avrRpt2* versus *avrB* were not statistically significant (ANOVA, $P > 0.2$).

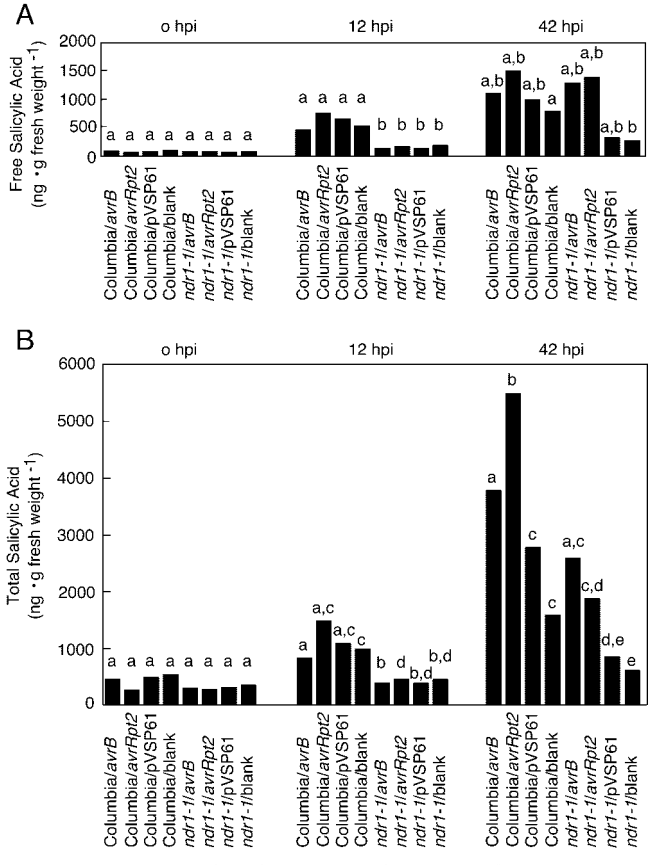

Figure 4. Bacteria-induced SA accumulation in *ndr1-1* versus Columbia. 1×10^6 bacteria mL^{-1} of *P.s.g.* Race 5 carrying the specified avirulence gene were inoculated into leaves of Columbia or *ndr1-1* plants. Means of free (A) and total (B) SA determinations for identical time points from three separate experiments are presented. Lowercase letters indicate statistically significant differences between these means (ANOVA, $P < 0.1$ or in some cases greater significance). These comparisons were made separately for each time point.

UV-C Induction of SA Accumulation in *ndr1-1* versus Columbia

The production of SA in response to vacuum infiltration with the $MgCl_2$ blank would be explained if the treatment, which involves exposure to anoxic conditions followed by rapid air reperfusion, generated oxygen radicals. The impairment of *ndr1-1* plants in this response would then suggest that the *ndr1* block lies downstream of ROS production and upstream of SA production. However, we could not detect hydrogen peroxide production induced by vacuum infiltration using the DCF-DA method (data not shown), most likely because it was low-level. The DCF-DA method can detect integral accumulation of hydrogen peroxide over time. It would be of greater sensitivity in experiments using a continuous inducer of signaling such as bacteria than in this experiment.

Therefore, to test this hypothesis and validate these conclusions, we used 254 nm of UV-C light as a noninvasive way to generate ROS in planta (Yalpani et al., 1994; Green and Fluhr, 1995). Plants were exposed to UV-C light for 10 min. Leaf samples were taken and processed for SA quantitation. To maximize precision with respect to time of sampling to facilitate comparisons, only single tissue samples were taken for each data point. The sampling process for a single time point was completed in less than 10 min.

The results of the experiment are presented in Figure 5. Columbia plants showed increases in SA levels at the 6-h time point. Slight increases in total SA beyond that attributable to free SA were also seen at this time point. SA levels continued to increase with time. In contrast, SA levels in *ndr1-1* mutant plants showed only a minor increase. Replicates of this experiment yielded similar results. These results support the placement of the *ndr1-1* block between ROS generation and SA production. Obtaining the same qualitative results with both UV-C and anoxia/rapid air reperfusion supports the contention that it is ROS and not some other consequence of these treatments that is responsible for these effects.

UV-C Induction of SAR in *ndr1-1* versus Columbia

If *NDR1* indeed acts to link ROS production to SA production, *ndr1-1* plants should also be impaired in UV-C induction of SAR. We tested this prediction by treating *ndr1-1* and Columbia plants with UV-C under identical conditions to those described above.

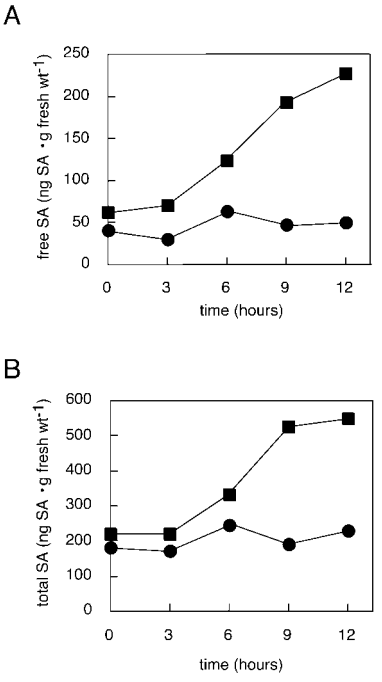

Figure 5. UV-C-elicited SA production in *ndr1-1* versus Columbia. Tissue was harvested from *ndr1-1* (circles for data points) or Columbia (squares for data points) plants at the indicated times following a 10 min of exposure to UV-C light. Free (A) and total (B) SA content of the samples were quantitated. To achieve maximum precision with respect to time of sampling, only one sample was taken for each time point. As such, no error bars are shown. Similar results were obtained in replicate experiments.

Two days post-treatment, these plants and control *ndr1-1* and Columbia plants that did not receive any treatment were inoculated with DC3000·pVSP61, and bacterial growth curves were performed. The data are presented in Figure 6. SAR was clearly induced by the UV-C treatment in Columbia plants (compare solid lines). In contrast, SAR was not induced by UV-C in *ndr1-1* mutant plants (compare dashed lines). The slightly greater growth of bacteria in untreated *ndr1-1* relative to untreated Columbia is consistent with previously documented results (Century et al., 1995) as discussed above.

Benzo(1,2,3)-Thiadiazole-7-Carbothioic Acid *S*-Methyl Ester (BTH) Induction of SAR in *ndr1-1* versus Columbia

If the *ndr1-1* block is upstream of SA production, exogenous application of a SA analog should elicit SAR regardless of whether the plants are mutated in the *NDR1* gene. BTH is metabolized in planta into a structural analog of SA and elicits effects similar to application of exogenous SA (Lawton et al., 1996). BTH treatment of plants does not induce SA production in either Columbia or *ndr1-1* (data not shown).

The response of *ndr1-1* and Columbia plants to BTH was compared. Plants were vacuum infiltrated with either 0.12 mM BTH or water. Two days later, these plants and control plants that did not receive any treatment were inoculated with DC3000·pVSP61, and bacterial growth curves were performed. The data are presented in Figure 7. Columbia (solid lines) and *ndr1-1* (dashed lines) plants both displayed BTH-induced SAR (compare curves where data points are diamonds or squares with curves where data points are circles). Three replicate experiments gave consistent results (data not shown). These results are consistent with the *ndr1* block being upstream of the action of BTH.

Columbia plants also displayed SAR in response to vacuum infiltration with a water blank (compare solid line with squares for data points with solid line with diamonds). The *ndr1-1* plants were completely impaired in infiltration-induced SAR (compare dashed line with squares for data points with dashed line with diamonds). This result is consistent with the results presented in Figure 6 showing impairment of UV-C-induced SAR in *ndr1-1* mutant plants. These results are also consistent with the impairment of vacuum infiltration-induced SA production in *ndr1-1* mutant plants (Fig. 4).

DISCUSSION

A model for disease resistance signal transduction is shown in Figure 8. Bacteria carrying *avrRpt2* do not elicit *PR* gene transcription or SAR on *ndr1-1* mutant plants, whereas bacteria carrying *avrB* can elicit both, albeit not as strongly as on wild-type plants. Yet, SA levels induced by these bacteria were shown to be reduced to similar levels in the *ndr1-1* mutant. These results imply that at least two pathways contribute to *PR* gene transcription and SAR. One branch shown in Figure 8 is the well-characterized SA-dependent pathway that depends on the *NPR1* gene (Cao et al.,

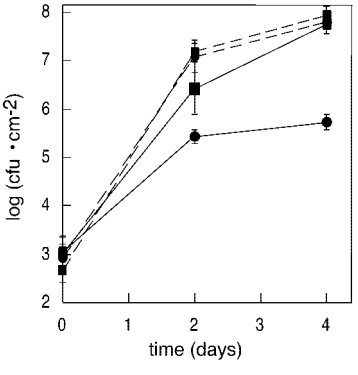

Figure 6. UV-C induction of SAR in *ndr1-1* versus Columbia. Columbia plants (solid lines) or *ndr1-1* plants (dashed lines) were exposed to UV-C light for 10 min (circles for data points) or left untreated (squares for data points) 2 d prior to inoculation with *P. syringae* pv tomato DC3000 carrying only the empty pVSP61 vector. Data points represent means of triplicate determinations of in planta bacterial growth at specified times. This experiment was repeated three times with similar results.

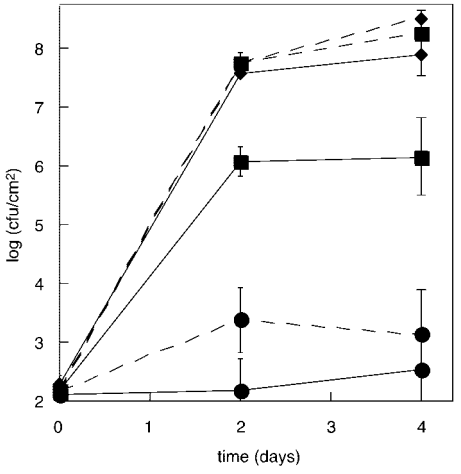

Figure 7. BTH induction of SAR in *ndr1-1* versus Columbia. Columbia plants (solid lines) or *ndr1-1* plants (dashed lines) were infiltrated with BTH (circles for data points) or a water blank (squares for data points) or were not treated (diamonds for data points). Two days later, plants were inoculated with *P. syringae* pv tomato DC3000 carrying only the empty pVSP61 vector. Data points represent means of triplicate determinations of in planta bacterial growth at specified times. This experiment was performed three times with similar results.

1994; Delaney et al., 1995; Glazebrook et al., 1996; Shah et al., 1997). We have shown a correlation between which bacterial strains elicit cell death and which elicit *PR* gene transcription and SAR on *ndr1-1* plants. This correlation suggests that the other branch is directly dependent either on cell death or on something closely correlated with the cell death response. The two branches can make independent contributions to *PR* gene transcription as shown by the induction of *PR* gene transcription in *npr1* mutant plants by bacteria carrying *avrRpt2* (Glazebrook et al., 1996; Shah et al., 1997). The two branches make a synergistic contribution to SAR as shown by the inability of *npr1-1* plants to manifest SAR in response to avirulent, HR-inducing bacteria (Cao et al., 1994). However, SAR and *PR* gene expression can be induced by stimulation of only the *NDR1/NPR1* branch (e.g. by exogenous SA or BTH).

Multiple lines of evidence indicate that *NDR1* functions downstream of ROS production and upstream of SA production. Mutant *ndr1-1* plants are impaired in SA production elicited by bacteria or by producing ROS in situ in response to either UV-C light or anoxia/rapid air reperfusion. Mutant *ndr1-1* plants are also impaired in induction of SAR by bacteria or ROS but not in induction of SAR by BTH. However, *ndr1-1* plants can still make SA as evidenced by comparable uninduced SA levels to wild-type plants and by the delayed rise in SA levels seen 42 h post-inoculation with *P.s.g.* Race 5 carrying *avrB* or *avrRpt2*. Because *ndr1-1* is a null allele, *NDR1*-independent pathways for SA synthesis must exist.

Bacteria carrying *avrRpt2* did not elicit hydrogen peroxide production on *ndr1-1* mutant plants as assayed by DCF fluorescence. In contrast, bacteria carrying *avrB* did elicit DCF fluorescence in *ndr1-1* mutant plants, albeit at levels lower than in Columbia plants at early time points. SA levels paradoxically were comparable on *ndr1-1* mutant plants regardless of which *avr* gene was carried by the bacteria. Because ROS increases are known to lead to SA production, these results taken together argue strongly that the DCF fluorescence assay is not measuring an early oxidative burst.

We propose instead that most of the DCF fluorescence is due to hydrogen peroxide produced close to the time of cell death in primary responding cells or in cells surrounding the dying cells. We suspect that the first cell death events occur prior to the first macroscopically visible signs of the HR. The first cell death events probably occur either at the time of onset of detectable DCF fluorescence or just after this time. Wolfe et al. (2000) have documented low-level cell death assayed as decreases in chlorophyll fluorescence beginning at times comparable with those for the onset of DCF fluorescence in Columbia plants inoculated with DC3000·*avrRpt2*. Most plant cells, however, did not die until several hours later. The first detectable cell death events in the HR elicited by *P. syringae* pv. *phaseolicola* on an incompatible lettuce cultivar preceded macroscopic evidence of tissue collapse by 5 h (Bestwick, 1995).

The kinetics presented in Table I are consistent with this interpretation. The initial time of onset of

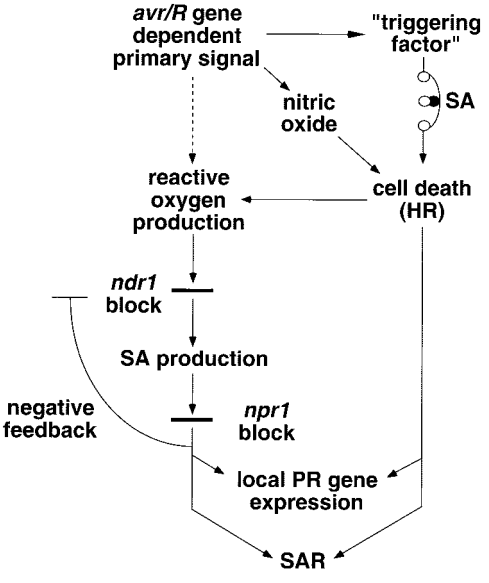

Figure 8. Model for disease resistance signal transduction. The dashed line leading from *avr/R* gene-dependent primary signal to reactive oxygen production is meant to indicate that this pathway is quantitatively much less significant than the cell death-induced reactive oxygen production. SA potentiation of cell death is represented with a symbol for a rheostat. Although pictured as being distinct, "triggering factor" could be nitric oxide, reactive oxygen, both, or something different. The SA-dependent negative feedback loop's precise target is not known. *NDR1*-independent pathways for SA production discussed in the text are not pictured.

DCF fluorescence is similar in most cases. However, bacteria eliciting slower increases in DCF fluorescence also elicit more delayed HRs. We suggest that the lack of DCF fluorescence in *ndr1-1* mutant plants inoculated with bacteria carrying *avrRpt2* is a consequence rather than a cause of the absence of programmed cell death.

If this interpretation of the data is correct, then the effects of the *ndr1-1* mutation are explicable as a manifestation of agonist-dependent gain control. SA would function in gain control by lowering the threshold of a "triggering factor" required to see programmed cell death. Gain control might occur both in primary responding cells and in more distal cells because there is evidence that hydrogen peroxide, SA, and perhaps other signals can be transported intercellularly (Levine et al., 1994; Mölders et al., 1996).

We conclude based on data presented herein that this HR-inducing "triggering factor" accumulates to a higher level in response to bacteria carrying *avrB* than in response to those carrying *avrRpt2*. We have demonstrated a similar degree of impairment of SA production by *ndr1-1* plants in response to bacteria carrying *avrB* or *avrRpt2*. This similar level of impairment could set the threshold value for the triggering factor at a level that the HR is seen in response to bacteria carrying *avrB* but not those carrying *avrRpt2*. This mechanism could also explain the ability of the *nahG* transgene to prevent the HR caused by bacteria carrying *avrRpt2* but not that caused by bacteria carrying *avrRpm1* (Rate et al., 1999). SA levels would be irrelevant to the plant's "decision" to mount a HR if the primary signal is sufficiently strong.

We cannot rule out the possibility that there are differences in very low-level, early production of hydrogen peroxide that would not be detectable with the DCF assay. It is formally possible that it is a signal produced as a consequence of very low-level ROS accumulation that is potentiated by SA to lead to programmed cell death. Nonetheless, we favor an alternative model that something other than hydrogen peroxide is the "triggering factor" that must reach a critical threshold value. One possibility for what this factor might be is nitric oxide. It is also possible that both ROS and nitric oxide (and perhaps other signals) contribute to the plant cell's decision to undergo programmed cell death (McDowell and Dangl, 2000).

An alternative explanation for the differences we have documented between signaling induced by bacteria carrying *avrRpt2* as opposed to *avrB* is suggested by recent results concerning the virulence function of *avrRpt2* (Chen et al., 2000). It has been suggested that *avrRpt2* in its role as a virulence factor can suppress defense responses. Could the signaling impairments seen with *ndr1-1* plants in response to bacteria carrying *avrRpt2* be because of exaggeration of suppression of defense responses?

A strong prediction of this alternative explanation would be that defense responses of *ndr1-1* plants elicited by bacteria carrying *avrRpt2* would be impaired to a greater extent at higher levels of bacterial inoculum. In fact, the opposite is seen. When *ndr1-1* plants are inoculated with 1×10^9 cfu mL^{-1} *P.s.g.* Race 5·*avrRpt2*, an HR is seen (Shapiro, 2000). Inoculation of *ndr1-1* plants with this concentration *P.s.g.* Race 5 carrying only the empty vector elicited no response, validating the use of this assay (Shapiro, 2000). We feel these results are consistent with *ndr1-1* phenotypes in response to bacteria carrying *avrRpt2* being caused by limited production of a "triggering factor" and inconsistent with an explanation based on more efficacious suppression of defense responses.

HRs seen in response to bacteria carrying *avrB*, *avrRpm1*, *avrPphB*, or *avrRps4* are more severe on *ndr1-1* mutant plants than on Columbia plants (Century et al., 1995; Shapiro, 2000). Yet, we have shown that *ndr1-1* is a loss-of-function mutation with respect to ROS-induced SA production. This more vigorous cell death response might be explained if the *ndr1* mutation delayed or prevented induction of a feedback loop that negatively regulated the HR. This delay might be a consequence of the documented delay in SA accumulation (Fig. 4). Evidence has been presented for SA-dependent negative feedback (Cao et al., 1994). Whether the negative feedback loop identified in this study is identical to that which is responsible for regulating cell death is not certain.

The model in Figure 8 presents a framework for generating testable hypotheses concerning the action of these and other disease resistance signaling genes for which mutants are available. We expect that further work will clarify the uncertain aspects of this model and that the model will be a useful guide to using these signaling genes in attempts to engineer plant disease resistance.

MATERIALS AND METHODS

Bacteria and Plant Growth

Pseudomonas syringae, *Agrobacterium tumefaciens*, and *Escherichia coli* strains were cultured according to published methods (Whalen et al., 1991). Arabidopsis was grown in an MTR-30 growth chamber (Conviron, Winnipeg, MN) set for 8 h of light, 16 h of darkness, at 22°C, with 70% relative humidity for experiments involving bacterial growth curves or SA quantitation. For all other purposes, Arabidopsis growth was as described by Whalen et al. (1991). Columbia seed used in experiments was obtained from self-fertilized plants grown from seeds from the same lot used as the parental in the mutagenesis from which *ndr1-1* was isolated.

Construction of *Col-0:PR1/GUS*

The PR1 cDNA (Uknes et al., 1992) was used as a probe to isolate the hybridizing yeast artificial chromosome

yUP12F7. A sub-library was made in the *A. tumefaciens* cosmid vector pCLD04541 (Jones et al., 1992) using published methods (Bent et al., 1994). The PR1 cDNA was used again as a probe of colony lifts to identify a cosmid that included 3.2 kb of DNA 5′ to the *PR1* coding region. This DNA was amplified with *Pyrococcus furiosus* polymerase using the T7 forward primer, which annealed to the vector immediately adjacent to the insert DNA, and a reverse primer designed from the known PR1 sequence (5′-CGAGAATAGCCAGTAGAATTCCTTTTTCTAAG-3′). The reverse primer introduced an *Eco*RI site and removed the *PR1* start codon. The PCR product was cut with *Xba*I and *Eco*RI and ligated into the polylinker of pBluescript KS+ between the *Xba*I and *Eco*RI sites. The entire upstream region except the proximal 104 bp just upstream of the original start codon was then replaced with DNA from the cosmid (which had never been subject to PCR) to make pADS3.

The 3.2 kb of DNA 5′ to the *PR1* coding region (hereafter, the *PR1* promoter) was next subcloned upstream of the *E. coli GUS* gene and the nopaline synthase terminator (*NOS*). An *Eco*RI linker was blunt-end ligated into the *Cla*I site of pSLJ4K1 (Jones et al., 1992) to make pADS5. GUS and NOS were then excised together from pADS5 as an *Eco*RI/*Hin*dIII fragment and ligated between the *Eco*RI and *Hin*dIII sites of pCLD04541 (Jones et al., 1992) to make pADS6. The *Sac*I/*Eco*RI fragment of pADS3 was then cloned between the *Sac*I and *Eco*RI sites of pADS6 to make pADS7.

pADS7 was conjugated into *A. tumefaciens* strain GV3101 (Schell and Koncz, 1986) via triparental mating using the helper plasmid pRK2013 (Ditta et al., 1980). Plant transformation was performed using vacuum infiltration (Bechtold et al., 1993; Bent et al., 1994). Forty-three independent transgenic lines were isolated. A line homozygous for a single locus (two copies of the transgene integrated head-to-head as determined by Southern blotting) insertion, which showed strong GUS induction under all conditions predicted by northern analysis of *PR1* expression (Uknes et al., 1992) and in all tissues, was chosen. This line was designated *Col-0:PR1/GUS*.

Construction of *ndr1-1:PR1/GUS*

Col-0:PR1/GUS was used as the male parent in a cross to *ndr1-1*. A line homozygous for both the *ndr1-1* mutation and the transgene was identified in the F2. This line displayed lack of an HR in response to DC3000·*avrRpt2* and an exaggerated HR in response to DC3000·*avrRpm1* (Century et al., 1995). Southern-blot analysis showed the presence of only the *ndr1-1* allele at the *NDR1* locus [*ndr1-1* contains a 1.2-kb deletion (Century et al., 1997)]. This line also showed nonsegregation of kanamycin resistance (the selectable marker associated with the transgene) in the F3.

Assay of GUS Activity

GUS activity was assessed by standard procedures (Jefferson, 1987). Single 0.125-cm^2 punches of leaf tissue were taken using a number one cork borer and used for GUS assay with the fluorogenic substrate 4-methyl umbelliferyl β-D-glucuronide (Rose Scientific, Edmonton, AB). Data is shown from leaves harvested either 1 or 2 d postinoculation. Reactions were incubated precisely 2 h at 37°C and then quenched. The data is presented as the raw fluorescence units obtained via excitation at 365 nm and measuring emission at 450 nm using a fluorescence microplate reader (Dynex, Chantilly, Virginia). No attempt was made to derive rates of GUS production because preliminary experiments showed increases with time to be nonlinear. All values were within the linear range of the instrumentation and corresponded to the range within which fluorescence increases in direct proportion to concentration of pure methyl-umbelliferone.

Inoculation of Plants and in Planta Bacterial Growth Curves

Hand inoculation of plants utilized 1-mL tuberculin syringes to inject approximately 10 μL per cm^2 into leaf tissue through wounds made at one site per half-leaf with a 22-G needle. Vacuum infiltration was according to published methods (Whalen et al., 1991). In planta bacterial growth curves were performed according to published methods (Whalen et al., 1991), except that the initial inoculum was 5×10^4 bacteria mL^{-1} and the plating of bacteria was done on nutrient yeast growth agar plates supplemented with rifampicin, kanamycin, and cycloheximide. Independent replicates of growth curves gave differences of equivalent statistical significance to those pictured.

Hydrogen Peroxide Quantitation

Hydrogen peroxide quantitation was according to a published procedure (Wolfe et al., 2000) except that hand infiltration rather than vacuum infiltration was used to inject the dye into plant leaves 15 to 30 min prior to excising the leaves. 2×10^7 bacteria mL^{-1} was used in inoculations of DC3000- or *P.s.m.* 4326-based strains. 1×10^8 bacteria mL^{-1} was used in inoculations of *P.s.g.* Race 5-based strains. These concentrations had previously been established as optimal for assay of the HR (Century et al., 1995, 1997). These levels of inoculum were also used in primary inoculations for SAR experiments.

Following bacterial inoculations, plants were transferred to a Percival Ar-75 growth chamber set for continuous light prior to assay of DCF fluorescence or observation of the HR. Leaves appeared totally dry 15 min postinoculation. Leaf excision did not result in a DCF signal; however, fluorescence was occasionally seen surrounding the inoculation site needle hole. Severely wilted or dead areas of leaves in the process of mounting a HR often could not be infiltrated with dye and appeared dark. Leaves displaying a confluent HR (from late time points in experiments using bacteria carrying *avrB*) could not be infiltrated with dye and thus are not pictured. Treatment with 0.12 mM SA or 0.12 mM BTH did not elicit DCF fluorescence (data not shown). All treatments were performed on triplicate

leaves, and each entire experiment was repeated three to five times.

Confocal microscopy was used to establish that DCFH-DA entered the plant cytoplasm and that increases in fluorescence seen postinoculation were at least partially intracellular. Use of DCFH-DA diacetoxy methyl ester, a derivative of DCFH-DA that did not enter the cytoplasm and remained in the apoplast, led to kinetics of increase in fluorescence that were indistinguishable from those using DCFH-DA (data not shown). These results are consistent with hydrogen peroxide being generated initially in the apoplast with subsequent rapid movement into the cytoplasm.

SA Quantitation

Vacuum infiltration of bacteria was as described for growth curves except that the level of inoculum was 1×10^6 bacteria mL^{-1}. Control experiments were performed to rule out the possibility that vacuum infiltration-induced SA production was caused by inadvertent inoculation with bacteria or fungi normally present on the leaf surface. In these experiments, benomyl was first dissolved in dimethyl sulfoxide (at 10 mg mL^{-1}) and subsequently diluted 100-fold into 10 mM $MgCl_2$. Rifampicin (100 μg mL^{-1}) was dissolved directly in 10 mM $MgCl_2$, and the solution was 0.2 μm filtered. Inclusion of either benomyl or rifampicin did not affect the level of vacuum infiltration-induced SA production (data not shown).

SA extraction was according to standard procedures (Gaffney et al., 1993). Ortho-anisic acid was used as an internal standard (Meuwly and Métraux, 1993). Chromatography was performed essentially according to published procedures (Gaffney et al., 1993). Two injections were made per sample, one to quantitate SA and one to quantitate ortho-anisic acid. SA detection was by excitation at 303 nm and monitoring emission at 437 nm. Detection of ortho-anisic acid was by excitation at 298 nm and monitoring emission at 350 nm. Detection used an LS30 fluorimeter (Perkin-Elmer, Norwalk, CT) connected in-line to the HPLC (Rainin, Woburn, MA).

UV-C and BTH/SA Treatment of Plants

UV-C (254 nm) treatments were for 10 min using a model UVGL-58 lamp (UV Products, Upland, CA) immobilized 24 cm above the plants in a closed, dark cabinet. BTH was introduced using vacuum infiltration for SAR induction experiments. BTH was introduced by hand infiltration in the control experiments documenting the lack of induction of hydrogen peroxide or SA production. SA was introduced by hand infiltration. BTH (0.12 mM; or SA, introduced as the sodium salt) in double-distilled water was used in all cases.

Statistics

Significance of differences between means in both SAR experiments and *PR1*-driven GUS activity determinations was determined using Student's *t* tests. Values of *P* were read from a *t* table. Differences between SA levels were assessed for significance using a two-way analysis of variance where either *NDR1* allele (*ndr1-1* or *NDR1*) or bacterial strain used was considered a fixed effect and experiment number was treated as a random factor.

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