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Characterization of an Arabidopsis Mutant That Is Nonresponsive to Inducers of Systemic Acquired Resistance

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Systemic acquired resistance (SAR) is a general defense response in plants that is characterized by the expression of pathogenesis-related (PR) genes. SAR can be induced after a hypersensitive response to an avirulent pathogen or by treatment with either salicylic acid (SA) or 2,6-dichloroisonicotinic acid (INA). To dissect the signal transduction pathway of SAR, we isolated an Arabidopsis mutant that lacks the expression of an SA-, INA-, and pathogen-responsive chimeric reporter gene composed of the 5′ untranslated region of an Arabidopsis PR gene, β-1,3-glucanase (*BGL2*), and the coding region of β-glucuronidase (*GUS*). This mutant, *npr1* (nonexpresser of PR genes), carries a single recessive mutation that abolishes the SAR-responsive expression of other PR genes as well. While SA-, INA-, or avirulent pathogen-induced SAR protects wild-type plants from *Pseudomonas syringae* infection, the mutant cannot be protected by pretreatment with these inducers. The insensitivity of *npr1* to SA, INA, and avirulent pathogens in SAR induction indicates that these inducers share a common signal transduction pathway. Moreover, in *npr1*, the localized expression of PR genes induced by a virulent *Pseudomonas* pathogen is disrupted, and the lesion formed is less confined. These results suggest a role for PR genes in preventing the proximal spread of pathogens in addition to their suggested role in SAR.

INTRODUCTION

Acquired resistance in plants was first reported in the early 1900s (reviewed by Chester, 1933). Systemic acquired resistance (SAR), which develops in distal, uninfected parts of the plant, was experimentally defined by Ross (1961), who demonstrated that tobacco becomes resistant to infection by a number of viruses after a hypersensitive response (HR) to an avirulent strain of tobacco mosaic virus. Subsequently, it has been demonstrated that SAR is induced after an HR to other viruses, bacteria, and fungi and that the resistance induced by any one pathogen is effective against a broad spectrum of viral, bacterial, and fungal diseases (Cruikshank and Mandryk, 1960; Hecht and Bateman, 1964; Lovrekovich et al., 1968; Kuc, 1982; Dempsey et al., 1993; Uknes et al., 1993; Cameron et al., 1994; Mauch-Mani and Slusarenko, 1994). In addition, chemicals, such as salicylic acid (SA) and 2,6-dichloroisonicotinic acid (INA), have been found to induce resistance when applied exogenously to plants (White, 1979; Métraux et al., 1991).

Several lines of evidence indicate that endogenously produced SA is involved in the signal transduction pathway(s) coupling the perception of pathogen infection with the onset of SAR. In tobacco and cucumber, an increase in SA concentration has been observed after pathogen infection when accompanied by the establishment of SAR (Malamy et al., 1990; Métraux et al., 1990; Rasmussen et al., 1991). The accumulation of SA is also associated with the subsequent induction of genes including those encoding pathogenesis-related (PR) proteins (Van Loon and Van Kammen, 1970; Ward et al., 1991; Yalpani et al., 1991). In tobacco and Arabidopsis, exogenously applied SA can induce the accumulation of PR mRNAs, which is a characteristic of SAR (White, 1979; Ward et al., 1991; Uknes et al., 1992). These results have led to the hypothesis that one of the consequences of pathogen infection is the accumulation of SA in vivo, which induces the expression of a set of proteins that act to limit further infection of the host (Ward et al., 1991). Direct support for this hypothesis has come from the observation that transgenic tobacco plants that express a bacterial salicylate hydroxylase are unable to accumulate SA and, consequently, do not exhibit SAR (Gaffney et al., 1993). However, these plants can still transmit a signal that induces SAR in grafted wild-type scions, indicating that although SA appears to be required as a local signal for SAR, it is not necessary as a systemic signal (Vernooij et al., 1994). In addition, transgenic tobacco lines constitutively producing individual PR proteins are more resistant to some fungal pathogens; chitinase leads to resistance to *Rhizoctonia solani* (Broglie et al., 1991), and PR-1a leads to resistance to *Peronospora tabacina* and *Phytophthora parasitica* (Alexander et al., 1993). Thus, SA is required in vivo for the establishment of SAR, and at least some of the PR genes appear to participate directly in the resulting pathogen resistance.

Although SA has been shown to be a signal molecule in SAR, little is known about the signaling pathway downstream of SA that leads to the induction of SAR. In tobacco, an SA binding

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protein has been identified; this protein has catalase activity. In vitro, the activity of this catalase is inhibited by SA; in vivo, catalase inhibitors, including SA, lead to increased H_2O_2 levels and the induction of PR genes (Chen et al., 1993). Thus, H_2O_2 has been suggested to be a signal molecule acting downstream of SA. The role of other compounds, such as INA, in the induction of SAR has yet to be defined.

To initiate a genetic dissection of the pathway(s) through which the increased level of SA leads to the onset of SAR, we looked for mutants that do not express PR genes in the presence of added SA or INA. Because there is no visible phenotype known to be associated with such mutants, we generated transgenic Arabidopsis plants expressing β-glucuronidase (*GUS*) under control of the Arabidopsis β-1,3-glucanase (*BGL2*) promoter (Dong et al., 1991); the *BGL2* gene is one of the PR genes regulated by SA (Uknes et al., 1992). Seed from the transgenic line (*BGL2–GUS*) were mutagenized with ethyl methanesulfonate (EMS), and the mutants were screened after SA or INA treatment for aberrant expression of *GUS*. Here, we report the isolation and the biological, genetic, and molecular characterization of a novel mutant, *npr1* (nonexpresser of PR genes).

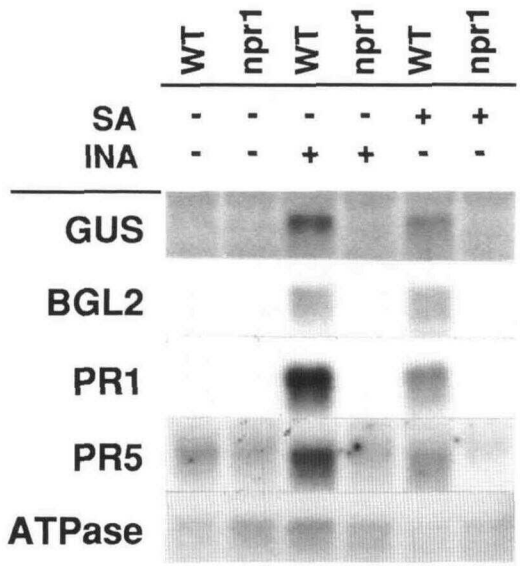

Figure 1. RNA Gel Blot Analysis of Expression of PR Genes in Wild Type and *npr1* in Response to Treatment with SA or INA.

RNA samples were extracted from 15-day-old seedlings grown on MS media in the presence (+) or absence (–) of either 0.5 mM SA or 0.1 mM INA and probed with *GUS*, endogenous *BGL2*, PR-1, PR-5, and β-ATPase gene-specific probes. WT, wild-type *BGL2–GUS* transgenic line.

RESULTS

Identification of the *npr1* Mutant

BGL2–GUS transgenic plants were mutagenized with EMS, and the M_2 plants were screened for SA- or INA-nonresponsive mutants. Individual plants grown in the presence of 0.5 mM SA or 0.1 mM INA were assayed for GUS activity (see Methods). Of 13,468 M_2 plants tested, 181 did not exhibit GUS activity in the presence of either SA or INA. In the M_3 generation, 77 of 139 lines tested maintained a mutant phenotype for GUS activity, with 76 nonresponsive to both SA and INA and one line nonresponsive to SA but responsive to INA.

RNA gel blot analysis was performed with these 77 mutant lines to identify those with modified expression of PR genes. The expression of the Arabidopsis mitochondrial β-ATPase gene served as a control for sample loading. Among the mutant lines tested, 76 expressed PR genes at some level, whereas one mutant (*npr1*) exhibited a dramatic reduction in expression of the *GUS*, *BGL2*, and PR-1 genes compared to the wild type in the presence of SA or INA, as shown in Figure 1. Therefore, this mutant was selected for further study. The *npr1* mutant was tested for the induction of PR-5, another PR gene that has been cloned in Arabidopsis (Uknes et al., 1992), and a similar reduction in expression was observed (Figure 1). The reduction in PR gene expression after SA or INA treatment was quantified for *npr1* relative to the parent *BGL2–GUS* line (representing the wild type). In *npr1*, the expression of both *GUS* and *BGL2* was 10-fold lower than that of the wild type and that of PR-5 was fivefold lower. The most dramatic reduction was observed for PR-1, which was 20-fold lower than the wild type.

Quantitative GUS Assay

To accurately measure the level of GUS activity, a quantitative GUS assay was performed on *npr1* plants and the wild-type *BGL2–GUS* plants grown in the presence of either SA or INA, or in the absence of both. As shown in Figure 2, in the absence of the inducer, the background level of GUS activity was fivefold lower in the *npr1* mutant than in the wild type. Wild-type plants grown in the presence of 0.5 mM SA showed a 52-fold increase in GUS activity compared to the uninduced plants, whereas in the SA-induced *npr1* plants, the increase in GUS activity was only sevenfold. Moreover, the induction by 0.1 mM INA was 48-fold for the wild type versus fivefold for *npr1*. Thus, although GUS activity in the SA- or INA-treated *npr1* plants was somewhat induced, the activity was at most only slightly higher than the background level of the untreated wild type.

Genetic Analysis of the *npr1* Locus

A backcross of *npr1/npr1* with its wild-type parent (*NPR1/NPR1* in the *BGL2–GUS* background) resulted in F_1 progeny

(*NPR1/npr1*, 16 tested) with the same pattern of GUS staining (using 5-bromo-4-chloro-3-indolyl glucuronide [X-gluc] as the substrate) observed in the wild type after SA or INA treatment. GUS staining was never detected in the SA- or INA-treated *npr1/npr1* homozygotic plants even after 2 days of incubation at 26°C. Self-fertilization of the F_1 plants produced F_2 progeny that segregated for GUS activity, intense staining, or complete absence of staining, which were present with a ratio of 219:64 among the 283 F_2 plants examined, demonstrating that the mutant phenotype is recessive and due to a single nuclear mutation ($\chi^2 = 0.86$; $P > 0.1$).

SA-, INA-, and Avirulent Pathogen-Induced Protection against *Pseudomonas syringae* pv *maculicola* ES4326 Infection

To examine whether the lack of SA- or INA-induced PR gene expression would affect SAR protection against a virulent pathogen infection, 15-day-old wild-type and *npr1* plants were treated with either 1 mM SA or 0.65 mM INA and 2 days later were exposed to a *P. s. maculicola* ES4326 bacterial suspension. As shown in Figure 3, 3 days after infection, significant protection was observed in the SA- or INA-treated wild-type plants with less than 10% of plants showing slight yellowing. Chlorotic lesions developed in ~90% of the untreated wild-type control plants. However, such protection was not observed in *npr1* mutant plants; chlorotic lesions were clearly seen in over 90% of untreated and at least 80% of SA- or INA-treated plants. The symptoms on *npr1* were also more severe than on the wild-type plants. Treatment with only 1 mM SA, 0.65 mM INA, or surfactant (0.01% Silwet-77, used for the bacterial infection) had a minimal effect on both the wild-type and the *npr1* plants (data not shown).

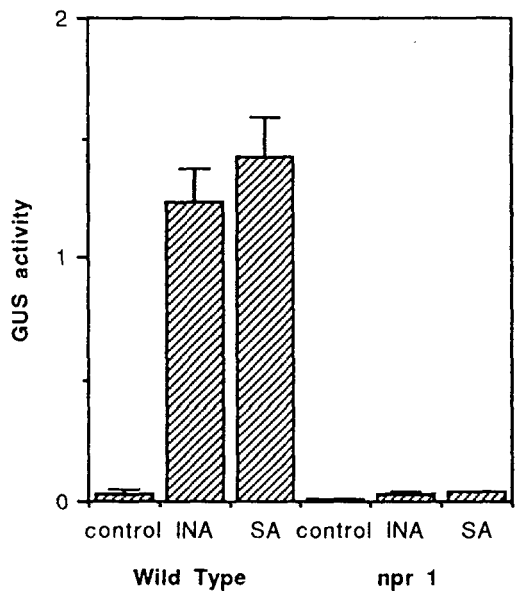

Figure 2. Effect of SA and INA on GUS Activity in *BGL2-GUS* Wild-Type and *npr1* Plants.

GUS activity is given in absolute fluorescence units per minute per microgram of protein. The control is 15-day-old seedlings grown on MS medium. SA indicates seedlings grown on MS medium with 0.5 mM SA, and INA indicates seedlings grown on MS medium with 0.1 mM INA. The values represent the average of three replicate samples ± SE.

The growth of *P. s. maculicola* ES4326 was measured in both wild-type and *npr1* plants that had been treated with water, SA, or INA 2 days before *P. s. maculicola* ES4326 infection. The resulting growth curves are shown in Figure 4. Leaves were collected 0, 0.5, 1.0, 2.0, and 3.0 days after bacterial infiltration. For the untreated wild-type plants, *P. s. maculicola* ES4326 proliferated 10,000-fold during this time period. However, for SA- or INA-treated wild-type plants, the growth of *P. s. maculicola* ES4326 was only ~10-fold, 1000 times lower than the untreated control (Figure 4A). A Student's *t* test of the difference between the means at the 3-day time point clearly shows that growth of the pathogen is greatly inhibited in the wild-type plants treated with SA or INA compared to those sprayed with water ($P < 0.001$). Such a dramatic difference in *P. s. maculicola* ES4326 growth, which resulted from SAR protection, was not observed in the *npr1* plants, where a Student's *t* test shows no statistically discernible difference in growth after 3 days for all conditions ($P > 0.05$); the growth of *P. s. maculicola* ES4326 in *npr1* plants was similar for mock-treated and either SA- or INA-treated plants (Figure 4B). Comparing the untreated *npr1* plants with the untreated wild type, the level of *P. s. maculicola* ES4326 seems to have reached saturation 1 day earlier in the mutant than in the wild type (Figure 4C). Moreover, the difference in *P. s. maculicola* ES4326 growth between the SA- or INA-treated wild type and *npr1* was 500- to 1000-fold (Figures 4A and 4B).

To test the response to an avirulent pathogen, the *npr1* plants were infiltrated with *P. s. maculicola* ES4326 carrying an avirulent gene *avrRpt2* (Dong et al., 1991; Whalen et al., 1991). A typical HR was observed in these *npr1* plants as characterized by the rapid appearance of necrotic lesions, detection of autofluorescence in the cell wall regions of the infected cells, and inhibited growth of *P. s. maculicola* ES4326/*avrRpt2* (data not shown). The ability of this avirulent gene to induce SAR in *npr1* plants was then tested. To distinguish the inducing bacterial strain from the challenging strain, the bean pathogen *Pseudomonas syringae* pv *phaseolicola* strain NPS3121 (*P. s. phaseolicola* NPS3121; Lindgren et al., 1986) containing the *avrRpt2* gene was used to induce SAR in both the *npr1* and wild-type plants. *P. s. phaseolicola* NPS3121 by itself causes no disease symptoms or visible HR on Arabidopsis ecotype Columbia, whereas *P. s. phaseolicola* NPS3121/*avrRpt2* elicits a strong HR (Yu et al., 1993). Three days after the inoculation, uninfected leaves on the same plants were challenged with the virulent pathogen *P. s. maculicola* ES4326, and the growth

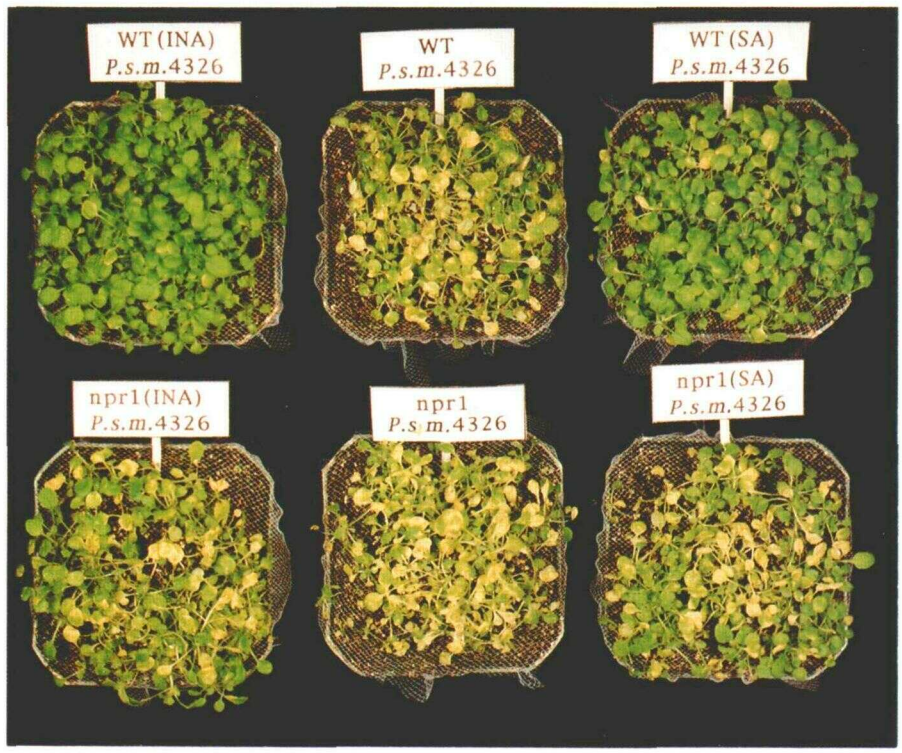

Figure 3. Response of *npr1* and Wild-Type Plants to *P. s. maculicola* ES4326 Infection following SA or INA Pretreatment.

Both the wild-type (WT) *BGL2–GUS* and *npr1* plants were treated with H_2O, 1 mM SA, or 0.65 mM INA 48 hr prior to *P. s. maculicola* ES4326 infection. The *P. s. maculicola* ES4326 bacteria used for infection were suspended in 10 mM $MgCl_2$ and 0.01% surfactant at an OD_{600} reading of 0.2. The photograph was taken 3 days after infection.

of *P. s. maculicola* ES4326 in the plants was measured. As shown in Figure 5, a significant reduction in bacterial growth was observed in the wild-type plants preinoculated with *P. s. phaseolicola* NPS3121/*avrRpt2* compared to the mock-treated samples (300-fold); however, no difference in *P. s. maculicola* ES4326 growth was detected in *npr1* plants.

Disease Symptoms and *BGL2–GUS* Expression Induced by *P. s. maculicola* ES4326 Infection

P. s. maculicola ES4326 was able to establish infection in SA-, INA-, and avirulent pathogen–treated *npr1* plants as well as in the untreated plants. The lesions formed on the untreated mutant plants and the untreated wild type were compared further. For this purpose, the *P. s. maculicola* ES4326 suspension was infiltrated into 4-week-old wild-type and *npr1* leaves. The injection was controlled so that only half of the leaf was infiltrated with the bacteria; this could be monitored by the soaking appearance of the half-leaf. As shown in Figure 6A, 48 hr after infiltration, chlorotic lesions were visible on the wild-type leaves; these lesions were normally confined to the infiltrated halves of the leaves as defined by the midrib vein. Strikingly different lesions were observed on the *npr1* leaves, where the lesions were much more diffuse and often "spread" into the uninfected halves of the leaves. Sampling of 12 leaves for both wild type and *npr1* revealed significant growth of the bacteria in the uninoculated half of 11 *npr1* leaves compared to none of the wild-type leaves.

For these leaves infected with *P. s. maculicola* ES4326, the pattern of *BGL2–GUS* expression was examined by X-gluc staining. As shown in Figure 6B, in a wild-type leaf on which the lesion was confined, a high level of GUS staining was detected in the peripheral region of the lesion. In contrast, no significant GUS activity was detected on the *npr1* leaf, where the lesion was more extensive than on the wild type.

DISCUSSION

In the absence of a known phenotype for an SAR-related mutant, we used SA- or INA-inducible PR gene expression to screen for SAR-impaired mutants by employing an SA- and

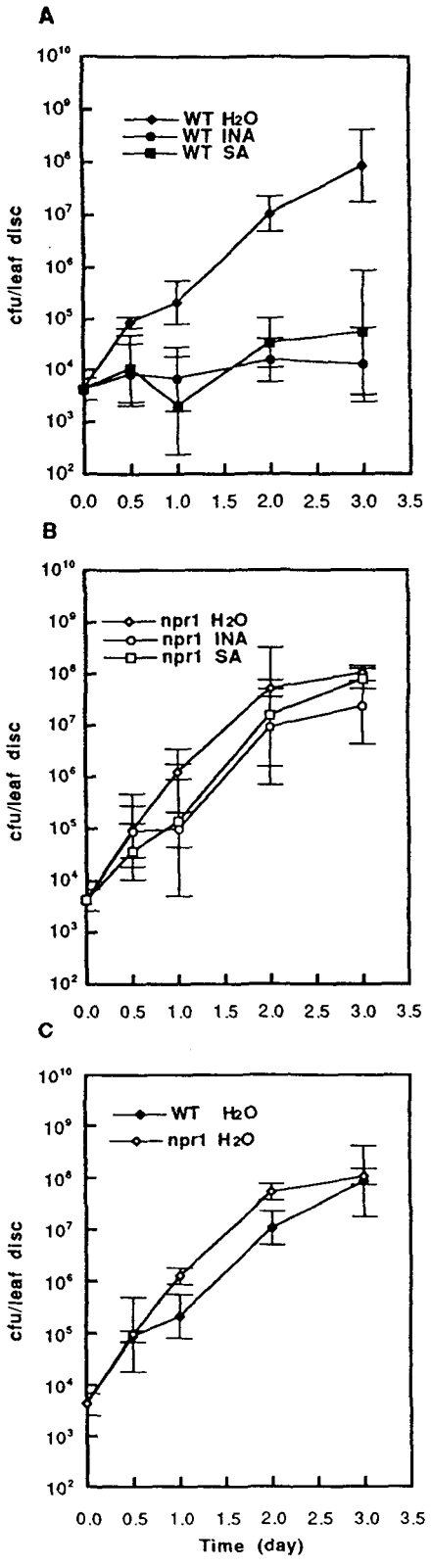

Figure 4. Growth of *P. s. maculicola* ES4326 in Wild-Type and *npr1* Plants Pretreated with H_2O, SA, or INA.

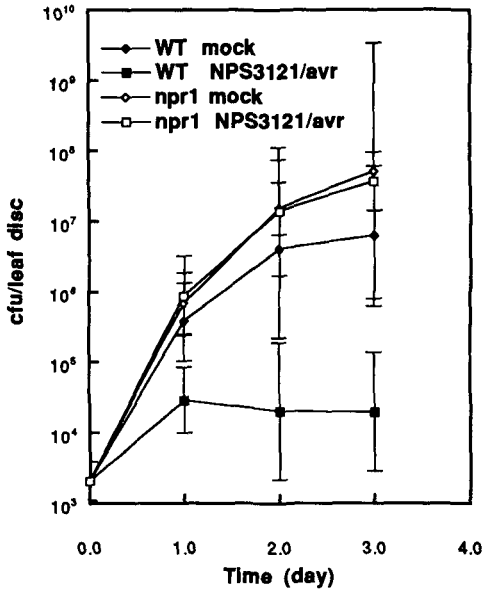

Figure 5. Growth of *P. s. maculicola* ES4326 in Wild-Type and *npr1* Plants Preinoculated with *P. s. phaseolicola* NPS3121/*avrRpt2*.

Two leaves of each *npr1* and wild-type (WT) *BGL2-GUS* plant were infiltrated with 10 mM of $MgCl_2$ solution without bacteria (mock) or *P. s. phaseolicola* NPS3121/*avrRpt2* (OD_{600} = 0.02; NPS3121/*avr*) 3 days prior to *P. s. maculicola* ES4326 infection (OD_{600} = 0.001). Samples were taken 0, 1, 2, and 3 days after *P. s. maculicola* ES4326 infection. Error bars represent 95% confidence limits of log-transformed data (Sokal and Rohlf, 1981). Ten samples were taken for each treatment and genotype at each time point. cfu, colony-forming units.

INA-inducible reporter gene. It had been shown that this reporter gene, *BGL2-GUS*, can be induced by exogenously applied SA or INA. Thus, we screened for "nonexpresser of PR gene" mutants by their failure to show GUS activity after treatment with SA or INA. The *BGL2-GUS* expression in individual plants was easily measured by a modified GUS assay with no tissue processing.

npr1 Harbors a Recessive Mutation That Affects SA- and INA-Responsive Expression of PR Genes

Three classes of mutations were predicted to be carried by the mutants that were nonresponsive to SA or INA treatment:

(A) Wild type (WT) *BGL2-GUS* treated with H_2O, SA, or INA.
(B) Mutant *npr1* treated with H_2O, SA, or INA.
(C) Wild type and *npr1* treated with H_2O.
Both the wild-type and *npr1* plants were treated with H_2O, 2 mM SA, or 0.65 mM INA 48 hr prior to *P. s. maculicola* ES4326 infection (OD_{600} = 0.001). Samples were taken 0, 0.5, 1.0, 2.0, and 3.0 days after infection. Error bars represent 95% confidence limits of log-transformed data (Sokal and Rohlf, 1981). Five to eight samples were taken for each treatment and genotype at each time point. cfu, colony-forming units.

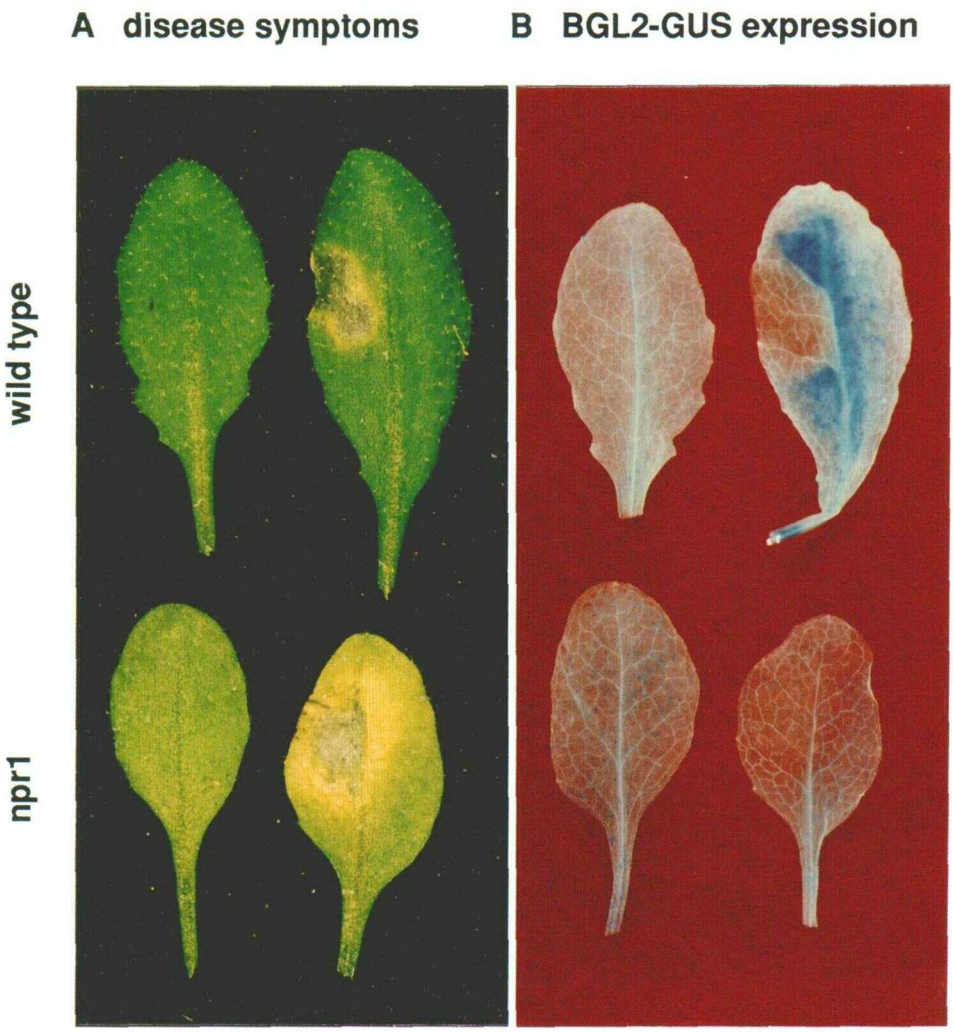

Figure 6. Disease Symptoms and *BGL2–GUS* Expression Induced by *P. s. maculicola* ES4326 on Wild-Type and *npr1* Leaves.

(A) Disease symptoms on wild-type and *npr1* leaves 48 hr after infiltration with 10 mM of $MgCl_2$ (the leaf samples on the left) or *P. s. maculicola* ES4326 (the leaf samples on the right).

(B) Histochemical staining for GUS activity of the same leaves shown in **(A)** treated with 10 mM of $MgCl_2$ or *P. s. maculicola* ES4326.

(1) mutations in regulatory genes that not only affect expression of the transgene but also the endogenous PR genes, (2) mutations in the promoter of the transgene that affect the responsiveness of *BGL2–GUS* but not that of the endogenous PR genes to SA and INA, and (3) mutations in the coding region of the *GUS* gene that abolish the enzymatic activity of GUS but not the transcription of *GUS* mRNA. To distinguish among these classes, the expression of endogenous PR genes was analyzed in the M_3 generation. Regulatory gene mutants were readily distinguished in the M_3 generation by an aberrant level of expression of other SAR-related PR genes.

Among the 77 mutant lines, six were found to have reduced expression of the endogenous PR genes to some degree (class 1); three showed aberrant expression only in *BGL2–GUS* (class 2); and 14 were found to have reduced GUS activity but normal transcription of *BGL2–GUS* (class 3). More examination is underway to complete the characterization of all 77 mutants. The *npr1* mutant was studied in detail because it has the most dramatic reduction in expression of endogenous *BGL2*, PR-1, and PR-5 after either SA or INA treatment (Figure 1). The data indicated that *npr1* harbors a *trans*-acting mutation(s) affecting the response to SA and INA. The possibility of *npr1* being a mutant affecting the uptake of exogenously applied SA or INA was also ruled out by a subsequent experiment in which it was shown that the expression of *BGL2–GUS* induced by *P. s. maculicola* ES4326, instead of by exogenously applied SA or INA, is also abolished in the *npr1* mutant (Figure 6B).

The effect of the *npr1* mutation on PR gene expression was

further examined by a more precise measurement of GUS activity in SA- or INA-treated wild-type and mutant plants (Figure 2). The fold induction by SA and INA is much lower for the *npr1* plants (five- to eightfold) than the wild type (48- to 52-fold). Moveover, the *npr1* mutation also reduces the basal level of expression of *BGL2–GUS* (fivefold). The effect of the *npr1* mutation suggests that the function of the wild-type allele(s) is to qualitatively and quantitatively regulate the expression of SA- and INA-responsive PR genes.

Genetic analysis of the progeny of *npr1/npr1* × *NPR1/NPR1* backcross indicated that a single recessive nuclear mutation determines the "nonexpresser of PR genes" phenotype of the *npr1* mutant. This also suggests that the *NPR1* gene acts as a positive regulator of SAR-responsive gene induction, because one copy of the wild-type allele is sufficient to restore SAR responsiveness. While the gene could be a negative regulator that is inactivated by SAR induction, a mutation abolishing such regulation would likely be dominant. Furthermore, the fact that a single mutation in *npr1* affects the responsiveness of this mutant to SA, INA, and pathogen induction indicates that SA, INA, and pathogens share a common pathway that leads to the expression of PR genes.

The *npr1* Mutation Prevents the Onset of SAR

The SA- or INA-induced protection of wild-type *BGL2–GUS* plants against the virulent pathogen *P. s. maculicola* ES4326 was evident from the dramatic difference in symptoms and growth of the pathogen observed in SA- and INA-treated plants versus the untreated control (Figures 3 and 4). The inducer-treated wild-type plants showed few disease symptoms and 1000-fold less *P. s. maculicola* ES4326 growth. However, such differences were not observed in *npr1* plants; the symptoms observed on the SA- or INA-treated *npr1* plants were as severe as on the untreated control, and the bacterial growth was not statistically different in *npr1* between the SA- or INA-treated samples and the water-treated samples. Thus, the *npr1* mutation blocks SA or INA induction of resistance.

Even though the HR elicited in the *npr1* mutant by bacteria carrying the avirulent gene *avrRpt2* was similar to that described previously in wild-type plants (Dong et al., 1991; Whalen et al., 1991; Yu et al., 1993), the HR-induced SAR protection against infection by the virulent pathogen *P. s. maculicola* ES4326 was absent in the *npr1* plants (Figure 5). This indicates that *npr1* is a mutation that prevents the onset of SAR.

It is known that the onset of SAR is accompanied by the expression of PR genes. However, only a few reported experiments have indicated that PR gene expression determines SAR. Transgenic tobacco plants constitutively expressing a single chitinase gene have been shown to be more resistant to the fungal pathogen *R. solani* (Broglie et al., 1991), and another transgenic line expressing the tobacco PR-1a gene displays increased resistance to two oomycete fungi, *Peronospora tabacina* and *Phytophthora parasitica* (Alexander et al., 1993). *npr1* is a mutant that shows an apparent lack of expression of the PR genes tested. Our studies of *npr1* strongly suggest that the lack of PR gene expression is associated with the loss of SAR, which provides additional support for the hypothesis that PR gene expression is required for SAR.

PR Genes Participate in the Process of Local Defense during Infection by a Virulent Pathogen

Studies of PR gene expression have focused mainly on their involvement with the onset of SAR. When an HR occurs in a plant after encountering an avirulent pathogen, local and systemic signals are produced, which turn on the expression of PR genes throughout the plant. When a plant encounters a virulent pathogen, PR genes are not induced in the uninfected tissues, and no systemic resistance is acquired. However, the pattern of *BGL2–GUS* gene expression in wild-type plants infected with *P. s. maculicola* ES4326 suggests that PR genes are expressed in the marginal regions of a lesion formed after infection by a virulent pathogen (Figure 6B). In addition, our results indicate that there is a local response that limits the spread of the virulent pathogen; this response was absent in the *npr1* plants. In *npr1*, the chlorotic lesions were diffuse and spread into the uninfected halves of the leaves compared to the well-confined lesions on the wild-type leaves (Figure 6A). High levels of bacterial growth were detected more frequently in the uninoculated halves of the leaves of *npr1* plants than in the wild-type plants. Also, no significant GUS activity was observed in *npr1* plants around the extensive lesions (Figure 6B). Moreover, the growth of *P. s. maculicola* ES4326 in the untreated *npr1* plants reached a saturation level 1 day earlier than in the untreated wild-type plants (Figure 4C). These results imply that PR genes participate in the process of local defense during infection by a virulent pathogen.

PR Genes Are Induced Locally and Systemically through a Common Signal Transduction Pathway

We believe that PR genes are not only involved in determining SAR but also function in restricting further spread of a pathogen during an infection. An increase in the endogenous SA level is required for induction of SAR (Malamy et al., 1990; Métraux et al., 1990; Rasmussen et al., 1991; Gaffney et al., 1993); similarly, the SA level is elevated 1 day after infection with the virulent pathogen *P. s. maculicola* ES4326 (10-fold; A. Guo, S.A. Bowling, D.F. Klessig, and X. Dong, unpublished data). In *npr1* plants, where the SA-, INA-, and pathogen-responsive signaling pathway is blocked, both systemic and local resistance responses are disrupted, suggesting that the onset of both systemic and local resistance employs a common signal transduction mechanism that includes an increase in SA level followed by the induction of PR gene expression. The difference between the HR-induced SAR response and an infection-triggered local response is the production of a systemic signal. To induce SAR, a systemic signal must be

produced at the site of the necrotic lesion (HR), which then quickly turns on PR genes in distal parts of the plant and "immunizes" the plant. Such a signal is not produced during an infection mounted by a virulent pathogen; instead, the PR genes are only expressed locally in the areas surrounding the lesions. The production of systemic and local signals may determine where and how quickly the PR genes are expressed. It is not known whether a subset of PR genes is specific to each response or if the remainder of the PR genes, like *BGL2*, are shared by both responses.

The results obtained from the analysis of the *npr1* mutation illustrate the power of a genetic approach in dissecting the signal transduction pathway of SAR. Discovery and characterization of more such mutants will outline a genetic pathway that will complement biochemical studies. Furthermore, the identification of these mutants in Arabidopsis will help lead to the cloning of the regulatory genes in the SAR signal transduction pathway.

METHODS

Plant Growth Conditions

Arabidopsis thaliana ecotype Columbia was grown either in pots on Metro-Mix 200 soil (Grace-Sierra, Malpitas, CA) or on plates with MS media (Murashige and Skoog, 1962). Plants on soil were kept in a growth room at high humidity with a 14-hr photoperiod under fluorescent lamps at a light intensity of 100 to 200 $\mu E\ m^{-2}\ sec^{-1}$, while those on MS plates were kept in a growth chamber at 22°C and 70% humidity with a 14-hr photoperiod at a light intensity of 100 $\mu E\ m^{-2}\ sec^{-1}$. All seed were vernalized at 4°C for 2 days before placement in a growth environment.

Mutant Screen

An XbaI-SphI fragment (2025 bp) containing 1746 bp of noncoding sequence upstream of the start codon of β-1,3-glucanase (*BGL2*) was fused at the ATG site to the coding region of β-glucuronidase (*GUS*) and transferred into the vector pBI101, which was then used to transform *A. thaliana* ecotype Columbia (Valvekens et al., 1988). Plants homozygous for this *BGL2–GUS* construct were identified by resistance to kanamycin. Mutagenesis was performed in the *BGL2–GUS*/*BGL2–GUS* transgenic line by exposing ~36,000 seeds to 0.3% ethyl methanesulfonate (EMS) for 11 hr. Seed were sown, and the plants were allowed to self-fertilize to produce M_2 seed, which were collected in 12 independent pools. The M_2 seed were germinated on MS medium with the addition of 0.8% agar, 0.5 mg/mL 2-(*N*-morpholino)ethanesulfonic acid, pH 5.7, 2% sucrose, 50 μg/mL kanamycin, and 100 μg/mL ampicillin. Either 0.5 mM salicylic acid (SA) or 0.1 mM 2,6-dichloroisonicotinic acid (INA; obtained from Ciba-Geigy LTD, Basle, Switzerland) was added to induce systemic acquired resistance (SAR). After incubation for 15 days, each seedling to be assayed was numbered and a single leaf was then removed from each seedling and put into the corresponding sample well of a 96-well microtiter plate that contained 100 μL of GUS substrate solution (50 mM Na_2HPO_4, pH 7.0, 10 mM Na_2EDTA, 0.1% Triton X-100, 0.1% sarkosyl, 0.7 μL/mL β-mercaptoethanol, and 0.7 mg/mL 4-methylumbelliferyl β-D-glucuronide). After all of the samples were collected, the microtiter plate was placed under vacuum for 2 min to infiltrate the samples and then incubated at 37°C overnight. Samples were then examined for the fluorescent product of GUS activity (4-methylumbelliferone) under a long-wavelength UV light. Those seedlings that showed no GUS activity were identified on the MS plate and transplanted to soil for seed setting. The procedure was repeated in the progeny of these putative mutants to ensure that the mutant phenotype was heritable and to identify the homozygous mutants.

RNA Analysis

Seedlings (15 days old) grown on MS medium, MS medium with 0.5 mM SA, and MS medium with 0.1 mM INA were collected and frozen in liquid nitrogen. RNA was extracted as follows. Tissues were ground using a chilled mortar and pestle. Extraction mixtures were formed by adding 500 μL of 80°C phenol and 500 μL of 80°C RNA extraction buffer (100 mM LiCl, 100 mM Tris, pH 8.0, 10 mM Na_2EDTA, and 1.0% SDS) to the tissue in a microcentrifuge tube, and each extraction mixture was vortexed vigorously for at least 30 sec, then centrifuged at 14,000 rpm in a microcentrifuge for 5 min. The aqueous phases were then transferred into tubes containing 500 μL of chloroform/isoamyl alcohol (24:1). These mixtures were vortexed for at least 30 sec and centrifuged at 14,000 rpm for 5 min. The chloroform extractions were repeated; the aqueous phases were then transferred to new tubes, in which 0.1 volume of 3 M NaOAc, pH 5.2, and 2.5 volumes of chilled 100% ethanol were added. The samples were kept at −80°C for 30 min or more, then centrifuged at 14,000 rpm at 4°C for 10 min. The pellets were vacuum-dried and resuspended in 30 to 50 μL of water. The RNA concentrations were determined by UV absorbance.

RNA samples (5 μg) were separated by electrophoresis through formaldehyde-agarose gels and transferred to a hybridization membrane (GeneScreen; Du Pont–New England Nuclear), as described by Ausubel et al. (1994). After the completion of transfer, RNA samples were UV cross-linked to the membrane. Prehybridization and hybridization were performed at 55°C in 0.5 M Na_2HPO_4, pH 7.2, 7% SDS, and 10 mg/mL BSA (Church and Gilbert, 1984). Radioactive probes were synthesized by random priming using ^{32}P-labeled α-dCTP and isolated DNA fragments. A SalI-EcoRI fragment that contained the entire coding region of *GUS* was used to detect *BGL2–GUS* expression. A 246-bp HinfI fragment that contained the Arabidopsis *BGL2* gene-specific coding region (Dong et al., 1991) was used to detect endogenous *BGL2*. EcoRI-XhoI fragments from clones of Arabidopsis pathogenesis-related protein-1 (PR-1) and PR-5 cDNAs (Uknes et al., 1992) were used to detect PR-1 and PR-5 genes, and a 1.3-kb EcoRI-HindIII fragment of the tobacco mitochondrial β-ATPase gene (Boutry and Chua, 1985) was used to identify the corresponding Arabidopsis β-ATPase mRNA. For hybridization of *BGL2–GUS* and β-ATPase genes, the blot was washed in a solution of 2 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate) and 1% SDS twice at room temperature and then twice at 55°C. For blots hybridized with the PR-1, PR-5, and *BGL2* genes, the final two washes were performed at 65°C rather than 55°C. The blots were exposed to x-ray film and analyzed using PhosphorImager and ImageQuant (Molecular Dynamics, Sunnyvale, CA) to quantitate the radioactivity of each RNA band.

Quantitative GUS Assay

Fifteen-day-old seedlings grown on MS medium, MS medium with 0.5 mM SA, and MS medium with 0.1 mM INA were collected and frozen

in liquid nitrogen. Three replicate samples were taken for each genotype and treatment. The frozen samples were ground with chilled pestles in microcentrifuge tubes. The fluorogenic assay of GUS activity was conducted according to the procedure of Jefferson (1987). Protein concentrations were measured using Bio-Rad dye reagent, and the specific GUS activity was determined by the rate of increase of fluorescence over protein concentration.

Histochemical GUS Assay

For the histochemical GUS assay, fresh samples were put into staining solution containing 0.5 mg/mL 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc; first dissolved in dimethyl formamide at 25 mg/mL) in 0.1 M Na_2HPO_4, pH 7.0, 10 mM Na_2EDTA, 0.5 mM K^+ ferricyanide/ferrocyanide, and 0.06% Triton X-100 (Jefferson et al., 1987). The samples were placed under vacuum for 2 min to infiltrate the samples and then incubated at 26°C. The staining buffer was then removed, and the samples were dehydrated by sequential changes of 30, 75, and 95% ethanol.

Infection with Bacterial Pathogen *P. s. maculicola* ES4326

For both the wild type and *npr1* (nonexpresser of PR genes), 40 to 50 seeds suspended in 0.1% agar were spread onto each pot of soil and incubated under 14 hr light for 15 days. Plants of each genotype were used in three different treatments: one group was sprayed with water, one group was sprayed with 1 mM SA, and the third group was treated with 0.65 mM INA (25 mL per pot). Two days later, half of the samples in each treatment were further treated with 10 mM $MgCl_2$ containing 0.01% surfactant Silwet L-77 (Union Carbide, Danbury, CT) as the control, and the other half were exposed to *P. s. maculicola* ES4326 bacterial suspension (OD_{600} = 0.2; grown in King's B medium [King et al., 1954]) in 10 mM $MgCl_2$, 0.01% surfactant (30 mL per pot). Symptoms were examined 3 days after the infection.

Growth of *P. s. maculicola* ES4326 in the Plants

Four-week-old wild-type and *npr1* plants were sprayed with water (control), 2 mM SA, or 0.65 mM INA (25 mL per three plants), and 2 days later, leaves were infected with *P. s. maculicola* ES4326 suspension (OD_{600} = 0.001; mid-log growth) in 10 mM $MgCl_2$ by infiltrating half of the leaf (Dong et al., 1991). The avirulent pathogen pretreatment was performed 3 days prior to the secondary challenge, where *P. s. phaseolicola* NPS3121/*avrRpt2* (OD_{600} = 0.02) grown in King's B medium containing 50 μg/mL rifampicin and 15 μg/mL tetracycline was infiltrated into two leaves in each plant and *P. s. maculicola* ES4326 was later infiltrated into the remaining uninfected leaves. Five to 10 leaves were excised per treatment per genotype at 0-, 0.5-, 1.0-, 2.0-, and 3.0-day time points for measurement of bacterial growth. Leaf discs of the same size (0.28 cm^2) were made from these samples using a hole puncher and ground in 1 mL of 10 mM $MgCl_2$. The bacteria in the leaf tissue were extracted by macerating the discs with a plastic pestle followed by vigorous vortexing. Serial dilutions were made from the resulting bacterial suspensions, and 50 μL of each dilution was spread onto King's B medium agar plates containing 100 μg/mL streptomycin. The plates were incubated at 28°C for 2 days, and the number of colonies for each sample was then recorded. Statistical analyses were performed by Student's *t* tests of the differences between two means of log-transformed data (Sokal and Rohlf, 1981).

Genetic Analysis

Mutant *npr1* pollen was crossed into wild-type *BGL2–GUS* female. F_1 and F_2 plants were grown on MS medium with 0.5 mM SA or 0.1 mM INA and were tested for *GUS* expression. Segregation in the F_2 generation was analyzed with a χ^2 test for goodness of fit (Sokal and Rohlf, 1981).

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