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Nuclear Localization of NPR1 Is Required for Activation of *PR* Gene Expression

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Systemic acquired resistance (SAR) is a broad-spectrum resistance in plants that involves the upregulation of a battery of pathogenesis-related (*PR*) genes. NPR1 is a key regulator in the signal transduction pathway that leads to SAR. Mutations in *NPR1* result in a failure to induce *PR* genes in systemic tissues and a heightened susceptibility to pathogen infection, whereas overexpression of the NPR1 protein leads to increased induction of the *PR* genes and enhanced disease resistance. We analyzed the subcellular localization of NPR1 to gain insight into the mechanism by which this protein regulates SAR. An NPR1–green fluorescent protein fusion protein, which functions the same as the endogenous NPR1 protein, was shown to accumulate in the nucleus in response to activators of SAR. To control the nuclear transport of NPR1, we made a fusion of NPR1 with the glucocorticoid receptor hormone binding domain. Using this steroid-inducible system, we clearly demonstrate that nuclear localization of NPR1 is essential for its activity in inducing *PR* genes.

INTRODUCTION

Plants, like animals, are capable of mounting an immune response after a primary pathogen infection. One such response is known as systemic acquired resistance (SAR). SAR, which is often triggered by a local infection, can provide long-term resistance throughout the plant to subsequent infections by a broad range of pathogens (Ross, 1961; Kuc, 1982; Ryals et al., 1996). The activation of SAR correlates with the expression of the pathogenesis-related (*PR*) genes. Even though the functions of most *PR* gene products are unknown, some of these proteins have been shown to confer various degrees of pathogen resistance (Schlumbaum et al., 1986; Mauch et al., 1988; Broglie et al., 1991; Woloshuk et al., 1991; Terras et al., 1992, 1995; Alexander et al., 1993; Liu et al., 1994; Ponstein et al., 1994; Zhu et al., 1994).

Activation of *PR* gene expression and the establishment of SAR require the signal molecule salicylic acid (SA). Concentrations of SA have been shown to increase in both infected and uninfected tissues after pathogen infection (Malamy et al., 1990; Métraux et al., 1990, 1991; Rasmussen et al., 1991). The exogenous application of SA or its synthetic analogs, such as 2,6-dichloroisonicotinic acid (INA) and benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester, results in expression of the *PR* genes and activation of SAR (White, 1979; Ward et al., 1991; Görlach et al., 1996; Lawton et al., 1996). The essential role of SA in SAR has been demonstrated in transgenic tobacco and Arabidopsis plants that express the bacterial salicylate hydroxylase (*nahG*) gene. In these plants, SA is converted to the inactive compound catechol, and the induction of *PR* gene expression and SAR is inhibited (Gaffney et al., 1993; Delaney et al., 1994; Lawton et al., 1995).

Transduction of the SA signal requires the function of NPR1, a protein first identified in Arabidopsis through a mutant screen (Cao et al., 1994). The *npr1* (nonexpressor of *PR* genes) mutant fails to respond to various SAR-inducing agents (SA, INA, and avirulent pathogens), displaying little expression of *PR* genes and exhibiting increased susceptibility to bacterial and fungal infections. Other mutant alleles of *npr1* (also known as *nim1* and *sai1*) have been isolated by various genetic screening strategies (Delaney et al., 1995; Glazebrook et al., 1996; Shah et al., 1997). The *NPR1* gene encodes a novel protein containing an ankyrin repeat domain and a BTB/POZ (*broad-complex*, *tramtrack*, and *bric-à-brac*/poxvirus, zinc finger) domain (Cao et al., 1997; Aravind and Koonin, 1999), both of which are involved in protein–protein interactions (Michaely and Bennet, 1992; Bork, 1993; Li et al., 1997; Aravind and Koonin, 1999). The importance of these domains in NPR1 is verified by the isolation of loss-of-function point mutations in the highly conserved amino acids within them.

The absence of any known DNA binding domains in NPR1 suggests that it may either play an indirect role in regulating

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the *PR* genes or serve as a regulator of the transcription factor or factors that control *PR* gene expression. Recently, we and other researchers showed that NPR1 interacts with several members of the TGA subclass of basic domain/leucine zipper transcription factors (Zhang et al., 1999; Després et al., 2000; Zhou et al., 2000). These TGA factors can bind to the SA-responsive *as-1* element found in the *PR-1* gene promoter (Lebel et al., 1998). In an in vitro gel mobility shift assay, Després et al. (2000) showed that the DNA binding activity of TGA2 is enhanced by NPR1. However, the mechanism by which this enhancement is achieved has not been determined, because NPR1 does not appear to be part of the TGA2/DNA complex. Therefore, the biological significance of NPR1–TGA interactions remains to be determined.

One piece of information that is required to better understand the function of NPR1 is the subcellular localization of the protein during the activation of SAR. To observe the subcellular localization of NPR1 in living plant cells, we fused the NPR1 cDNA with the coding region of green fluorescent protein (GFP) from *Aequorea victoria* (Chiu et al., 1996). We found that this biologically active fusion protein accumulates in the nucleus in response to both chemical and biological inducers of plant defense responses. This nuclear accumulation of NPR1-GFP correlates with the expression of *PR* genes. Using a fusion between NPR1 and the glucocorticoid receptor hormone binding domain (HBD), the nucleocytoplasmic localization of which can be controlled by the steroid dexamethasone (DEX; Beato, 1989), we demonstrate that nuclear localization of NPR1 is required for *PR* gene activation.

RESULTS

NPR1-GFP Is Functional in Planta

To use GFP as a reporter for NPR1 localization, the *GFP* coding region was fused to the 3′ end of the *NPR1* cDNA. The expression of this NPR1-GFP fusion protein is under the control of a modified, constitutive cauliflower mosaic virus (CaMV) 35S promoter (Mindrinos et al., 1994). Previously, constitutive expression of the *NPR1* cDNA alone was shown to complement all of the *npr1* mutant phenotypes, namely, lack of inducible *PR* gene expression, reduced tolerance to high concentrations of exogenous SA, and enhanced susceptibility to pathogen infections (Cao et al., 1997). To determine whether fusing GFP to the C-terminal end of the protein affected the activity of NPR1, we transformed the *35S*::*NPR1-GFP* construct into *npr1-1* and *npr1-2* mutant plants. The *npr1-1* and *npr1-2* plants carry point mutations in the ankyrin repeat and BTB/POZ domains, respectively (Cao et al., 1997). The *npr1-1* plant also carries the *BGL2*::*GUS* reporter, the SAR-responsive expression of which is markedly reduced because of the mutation (Cao et al., 1994).

The *35S*::*NPR1-GFP* transformants (in *npr1-1* and *npr1-2*) were analyzed for restoration of inducible *PR* gene expression, tolerance to a high concentration (0.5 mM) of SA in the growth medium, and resistance to the virulent oomycete pathogen *Peronospora parasitica Noco2* and the bacterial pathogen *Pseudomonas syringae* pv *maculicola* (*Psm*) *ES4326*. First, the amounts of *NPR1-GFP* transcript and protein were examined in the transgenic plants before and after INA induction. As shown in Figures 1A and 1B, INA induction has little effect on the constitutive expression of *NPR1-GFP* in the lines analyzed. Therefore, any differences in NPR1-GFP fluorescence observed after INA induction will not be the result of a change in protein concentration. In contrast, the expression of the endogenous *NPR1* gene approximately doubles in response to induction (Figure 1A), as described previously (Cao et al., 1997). In the *35S*::*NPR1-GFP* lines analyzed, the amounts of NPR1-GFP transcript and protein were only approximately two- to threefold greater than those of the endogenous NPR1.

We next examined the expression of *PR-1* in these *35S*::*NPR1-GFP* transgenic plants. As shown in Figure 1A, NPR1-GFP, but not GFP alone, restored inducible *PR-1* expression to *npr1* seedlings grown on Murashige and Skoog (1962) (MS) medium containing INA (MS-INA). Similar results were obtained when the seedlings were grown on MS medium containing SA (MS-SA; data not shown). The fact that *PR* gene expression in the transgenic lines is inducible rather than constitutive indicates that, even though it is expressed at a higher level in the mutant, the NPR1-GFP protein still requires activation, as does the endogenous NPR1. NPR1-GFP also restored inducible *BGL2*::*GUS* expression to the *npr1-1* seedlings grown on MS-INA (Figure 1C) or MS-SA (data not shown). *BGL2*::*GUS* expression in these transgenic seedlings is present primarily in the cotyledons and older leaves but is absent in the roots. This pattern of expression is identical to that observed in the wild-type background (Figure 1C). Expression of *NPR1-GFP* also enabled the *npr1* seedlings to grow on plates containing a high concentration (0.5 mM) of SA (Figure 1D). These seedlings developed green cotyledons and leaves and were indistinguishable from wild type. The *npr1* seedlings expressing *GFP* alone, however, developed chlorotic cotyledons and were developmentally arrested at the cotyledon stage when grown on MS-SA, indicating that GFP alone does not restore SA tolerance to *npr1* (Figure 1D). Finally, expression of *NPR1-GFP* also restored resistance to pathogen infection in the *npr1* mutant. As shown in Figure 1E, wild-type plants infected with a low dose (OD_{600} = 0.0001) of the virulent bacterial strain *Psm ES4326* did not display any visible disease symptoms, whereas the *npr1* mutant plants developed chlorotic lesions at the site of infection. Expression of *NPR1-GFP*, but not *GFP* alone, in *npr1* rendered the plant resistant to *Psm ES4326* (Figure 1E). Similar results were obtained with the oomycete pathogen *P. parasitica Noco2* (data not shown).

Because the NPR1-GFP fusion protein complemented all

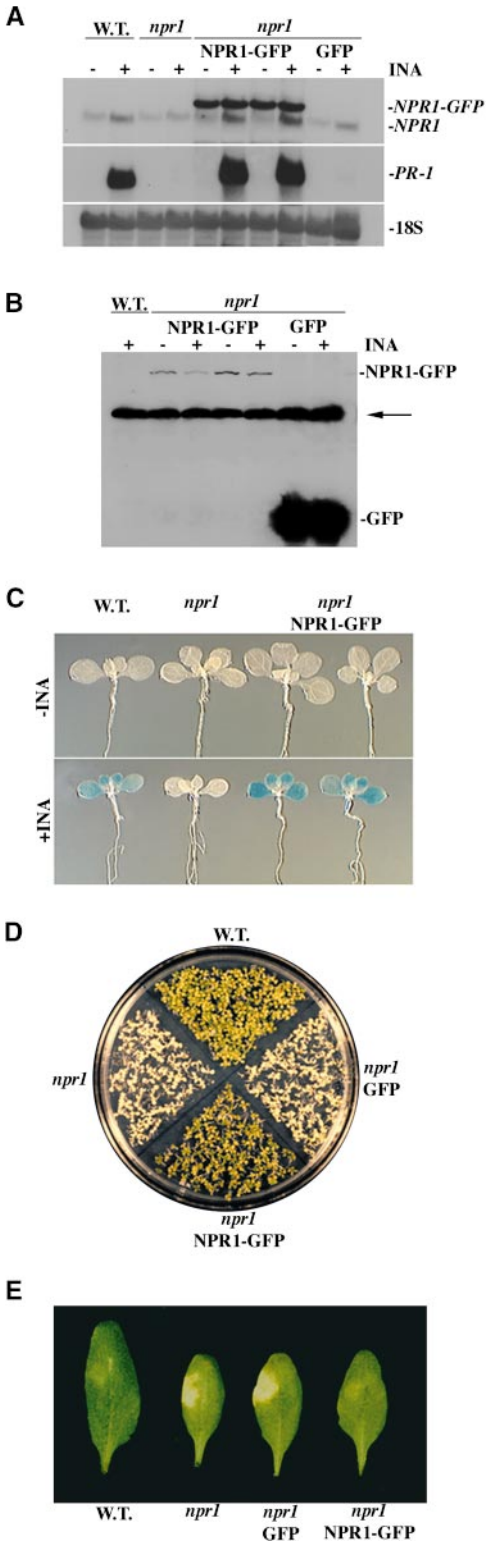

Figure 1. Complementation of the *npr1* Mutant Phenotypes by the NPR1-GFP Fusion Protein.

of the mutant phenotypes of *npr1*, we conclude that this protein is biologically active and could be used as a marker to examine the subcellular localization of NPR1 in living plant cells during SAR.

NPR1-GFP Accumulates in the Nucleus in Response to Activators of SAR

SA is a signal molecule required for the activation of SAR. The exogenous application of SA or its chemical analog INA has been shown to activate the expression of *PR* genes and SAR. However, these chemicals fail to activate *PR* gene expression or SAR in *npr1* mutants, suggesting that SA and INA signaling requires the function of the NPR1 protein. To determine whether SA or INA affects the subcellular localization of NPR1, we grew seedlings expressing NPR1-GFP on noninducing MS or SAR-inducing medium (MS-SA or MS-INA) and analyzed for GFP fluorescence. As shown in Figure 2A, NPR1-GFP was detected primarily in the cytoplasm and nuclei of guard cells when seedlings were grown on MS medium. A small amount of nuclear NPR1-GFP fluorescence also was detected in a few mesophyll cells (Figure 2A). However, when seedlings were grown on either MS-INA (Figure 2A) or MS-SA (data not shown), strong NPR1-GFP fluorescence was detected exclusively in the nuclei of both guard cells and mesophyll cells. Such striking nuclear fluorescence was not detected in seedlings expressing GFP alone. Instead, GFP was localized primarily in the cytoplasm and to a lesser extent in the nuclei when seedlings were grown under either noninducing or SAR-inducing conditions

(A) Gel blot of RNA (20 μg) from wild type (W.T.), *npr1*, two independent *35S*::*NPR1-GFP* transformants (NPR1-GFP; in *npr1*), and a *35S*::*GFP* transformant (GFP; in *npr1*). Seedlings were grown for 8 days on MS medium with (+) or without (−) 0.1 mM INA. The blot was probed for *NPR1*, *PR-1*, and the 18S rRNA.

(B) Gel blot of protein (100 μg) from wild type, two independent *35S*::*NPR1-GFP* transformants (in *npr1*), and a *35S*::*GFP* transformant (in *npr1*). Seedlings were grown for 8 days on MS medium with or without 0.1 mM INA. The blot was probed with antibodies against GFP. The arrow indicates a cross-reacting nonspecific protein.

(C) *BGL2*::*GUS* expression in wild type, *npr1*, and two independent *35S*::*NPR1-GFP* transformants (in *npr1*) grown for 13 days on MS medium with or without 0.1 mM INA.

(D) Growth of wild type, *npr1*, a *35S*::*NPR1-GFP* transformant (in *npr1*), and a *35S*::*GFP* transformant (in *npr1*) on MS medium containing 0.5 mM SA. The image was made after 11 days.

(E) Symptoms on leaves infected with *Psm ES4326*. The left halves of leaves from 4-week-old plants were infected with *Psm ES4326* (OD_{600} = 0.0001). Representative images from wild type, *npr1*, a *35S*::*GFP* transformant (in *npr1*), and a *35S*::*NPR1-GFP* transformant (in *npr1*) were made 4 days after infection.

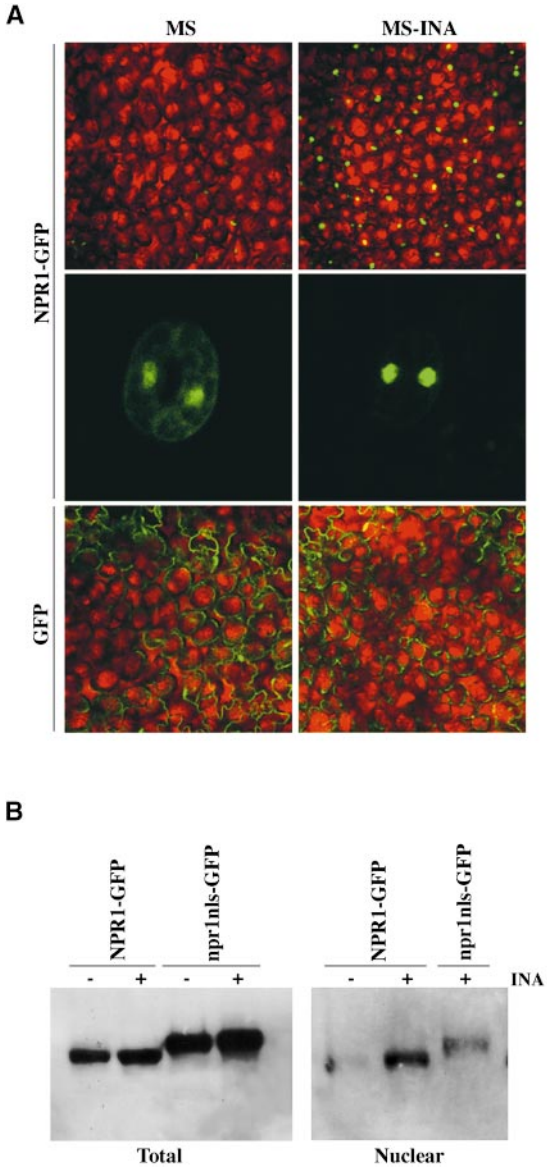

Figure 2. Nuclear Localization of NPR1-GFP in Response to SAR Induction.

(A) Confocal images of GFP fluorescence in mesophyll cells (top and bottom pairs of images) and in guard cells (middle pair of images) of cotyledons from 7-day-old seedlings grown on MS or MS-INA. For mesophyll cells, GFP fluorescence is shown in the green channel and differential interference contrast images are shown in the red channel.

(B) Gel blot of total protein (100 μg) and the nuclear-fractionated protein (12 μg). Protein was isolated from transgenic seedlings grown for 10 days on MS medium with (+) or without (−) 0.1 mM INA. The blot was probed with antibodies against GFP. The cytoplasmic npr1nls-GFP protein serving as a control indicates that the nuclear fraction contains relatively little cytoplasmic contamination.

(Figure 2A). This pattern is consistent with previous observations of GFP localization (Haseloff and Amos, 1995; Chiu et al., 1996).

To determine whether the increased nuclear fluorescence observed in SAR-induced seedlings reflects nuclear accumulation of NPR1-GFP, we analyzed localization of the fusion protein by subcellular fractionation and protein gel blot analysis. As shown in Figure 2B, although the overall amounts of NPR1-GFP were similar in the uninduced and induced plants, the amount of NPR1-GFP protein was approximately threefold greater in the nuclear extract of SAR-induced seedlings than in that of the uninduced seedlings. Therefore, we conclude that the increase in nuclear fluorescence observed in the SAR-induced plants was due to a redistribution of NPR1-GFP to the nucleus.

Nuclear Targeting of NPR1 Requires a Bipartite Nuclear Localization Signal

In both plants and animals, proteins are targeted to the nucleus by specific nuclear localization signals (NLSs). The best characterized NLSs consist of short stretches of basic amino acids (Dingwall and Laskey, 1991; Raikhel, 1992; Nigg, 1997). Computer analysis of the NPR1 protein sequence identified three potential NLSs (NLS1, amino acids 252 to 265; NLS2, amino acids 541 to 554; and NLS3, amino acids 582 to 593). To facilitate identification of the functional NLS or NLSs, we used a transient assay involving the biolistic bombardment of various NPR1-GFP fusion constructs into onion epidermal cells. This assay is a quick and effective means of identifying the NLSs in a variety of plant proteins (Varagona et al., 1992; Meisel and Lam, 1996; van den Ackerveken et al., 1996). On the basis of the constitutive expression of the *BGL2*::*GUS* reporter gene detected in this assay, the onion cells appear to represent the SAR-induced state (data not shown). It is possible, however, that the bombardment procedure causes activation of the reporter gene. As shown in Figure 3A, NPR1-GFP localized predominantly to the nucleus of onion cells, whereas GFP alone was distributed in both the cytoplasm and the nucleus. Deletion of the C-terminal 57 amino acids of NPR1 resulted in exclusive cytoplasmic localization of the fusion protein (npr1Δ57-GFP; Figure 3A), indicating that the C terminus is required for nuclear targeting of NPR1. The results observed in onion cells were reproduced in transgenic plants when the same constructs were transformed into Arabidopsis (Figure 3A).

As shown in Figure 3B, the C-terminal 57 amino acids of NPR1 contain the second and third potential NLSs in NPR1. Mutations in the first possible NLS (residues 252 to 265) did not affect the nuclear localization of NPR1-GFP in the onion cell assay (data not shown). Therefore, a systematic site-directed mutagenesis was performed to identify which NLS in the C-terminal 57 amino acids was required for nuclear import. As shown in Figure 3C, mutagenesis of five basic

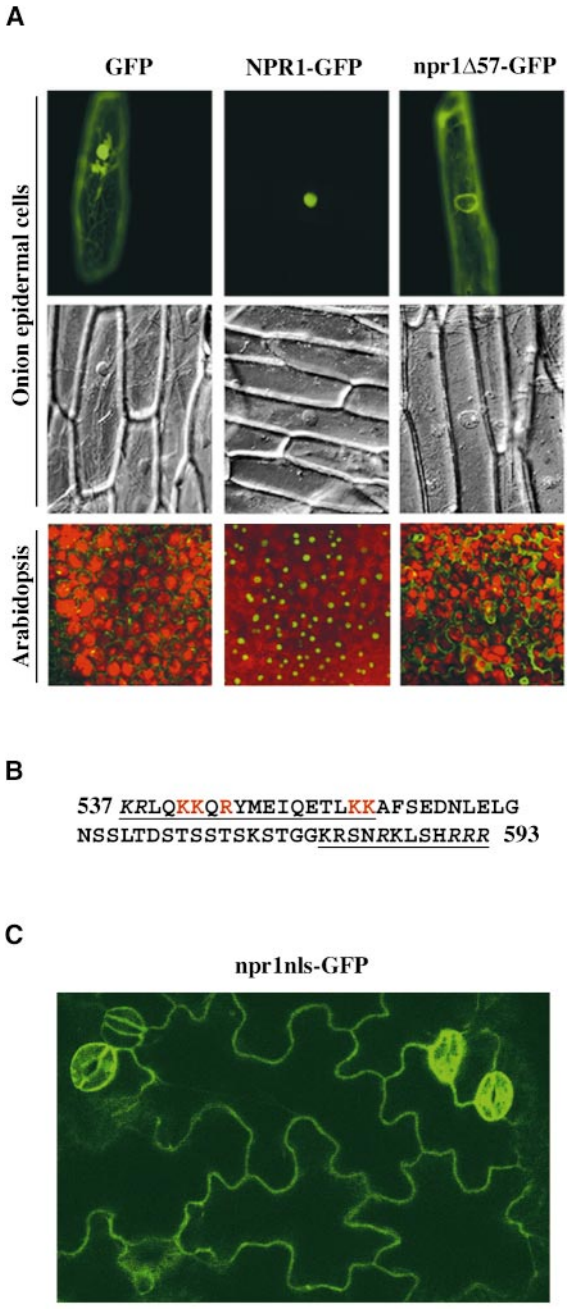

Figure 3. Identification of the NLS in NPR1 by Mutagenesis.

(A) Subcellular localization of GFP, NPR1-GFP, and npr1Δ57-GFP (a mutant lacking the C-terminal 57 amino acids of NPR1). GFP fluorescence (top images) and differential interference contrast images (middle images) of onion epidermal cells were compared to show the subcellular localization of GFP (cytoplasmic and nuclear), NPR1-GFP (nuclear), and npr1Δ57-GFP (cytoplasmic). Confocal GFP images (bottom images) were captured from 7-day-old transgenic Arabidopsis seedlings expressing GFP, NPR1-GFP, or npr1Δ57-GFP grown on MS-INA. GFP fluorescence is shown in the green channel; differential interference contrast images are shown in the red channel.

amino acids together in the second NLS (shown in red in Figure 3B) resulted in localizing the fusion protein exclusively in the cytoplasm (npr1nls-GFP) in transgenic Arabidopsis. Mutations in each amino acid separately reduced, but did not abolish, the nuclear import of the fusion proteins (data not shown). Mutations in six additional basic amino acids (shown in italics in Figure 3B) in this C-terminal region had no effect on nuclear localization of the fusion protein, as determined by the onion cell assay (data not shown).

Increased Nuclear Accumulation of NPR1-GFP Is Associated with Increased *PR* Gene Expression

Nuclear accumulation of NPR1-GFP in response to SAR induction suggests that NPR1 probably functions in the nucleus to regulate *PR* gene expression. We were able to establish a correlation between NPR1 nuclear localization and *PR* gene expression in the NPR1-GFP transgenic plants by growing the plants on media containing different concentrations of SA. As shown in Figure 4, even though the amounts of *NPR1-GFP* transcript and protein were not affected by varying the concentration of SA, the nuclear accumulation of NPR1-GFP was noticeably altered. Seedlings grown on medium containing 0.3 mM SA displayed substantially more nuclear fluorescence of NPR1-GFP than did those grown on medium containing 0.1 mM SA (Figure 4A). Subcellular fractionation of the protein from these seedlings confirmed that the accumulation of NPR1-GFP in the nuclear extract of seedlings grown on 0.3 mM SA was approximately threefold greater than that in seedlings grown on 0.1 mM SA (Figure 4B). To analyze whether the increased NPR1 in the nucleus resulted in increased expression of *PR* genes, we examined the amounts of *PR-1* expression in these seedlings. As shown in Figure 4C, the increase in nuclear NPR1-GFP correlates with noticeably greater expression of the *PR-1* gene. Although previous data indicate that 0.1 mM SA is sufficient for full induction of *PR-1* in *npr1* mutant plants transformed with the wild-type *NPR1* gene (Cao et al., 1997), apparently a greater concentration of SA is required for full induction of *PR-1* by the NPR1-GFP fusion protein. The correlation between NPR1-GFP nuclear fluorescence and *PR* gene expression also was observed during pathogen infection. When Arabidopsis leaves were infected

(B) Sequence of the C-terminal 57 amino acids of NPR1. The two potential NLSs are underlined. Point mutations in the amino acids shown in red had a marked effect on NPR1-GFP nuclear localization, whereas mutations in the amino acids shown in italics had no detectable effect on nuclear localization. Mutations in all five amino acids shown in red resulted in the exclusive cytoplasmic localization of the fusion protein npr1nls-GFP.

(C) Cytoplasmic localization of npr1nls-GFP in leaf epidermal cells of transgenic seedlings.

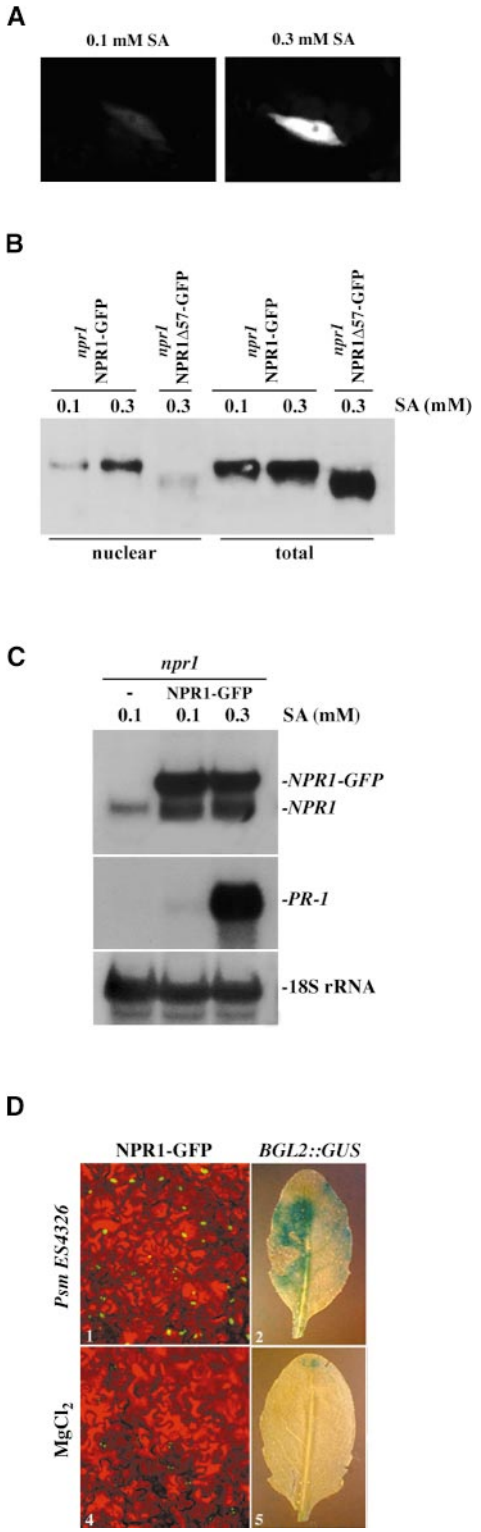

Figure 4. Increased Nuclear Accumulation of NPR1-GFP Correlates with Increased Expression of *PR* Genes.

by the bacterial pathogen *Psm ES4326* or *Psm ES4326/avrRpt2* (data not shown), strong *BGL2*::*GUS* expression and nuclear fluorescence of NPR1-GFP were observed in the cells surrounding the lesions (Figure 4D). In the systemic tissues, in which the amounts of SA and *PR* gene expression are much lower, the nuclear fluorescence of NPR1-GFP was more sporadic (data not shown).

Nuclear Localization of NPR1 Is Required for Activation of *PR* Gene Expression

To demonstrate the cause-and-effect relationship between the nuclear localization of NPR1 and its activity in inducing *PR* gene expression, we sought to regulate the subcellular localization of NPR1 by generating a fusion with the rat glucocorticoid receptor HBD (Picard et al., 1988). The HBD contains two NLSs (Savory et al., 1999), and this system has been used to control the nuclear transport of various transcriptional regulators in Arabidopsis and other plants (Schena et al., 1991a; Aoyama et al., 1995; Simon et al., 1996; Aoyama and Chua, 1997; Sablowski and Meyerowitz, 1998; Wagner et al., 1999). As shown in Figure 5A, proteins fused to the HBD are retained in the cytoplasm through an association with the heat shock protein hsp90. In cells treated with the steroid hormone DEX, hsp90 is released and the HBD fusion protein is translocated into the nucleus.

The NPR1-HBD fusion protein was constitutively expressed in *npr1* mutant plants under the control of the CaMV 35S promoter, and the resulting transgenic plants were analyzed for restoration of *PR-1* gene expression. In the control *35S*::*NPR1* plants (Cao et al., 1998), *PR-1* ex-

(A) Representative images of nuclear NPR1-GFP fluorescence in leaf mesophyll cells of seedlings grown for 12 days on MS medium containing either 0.1 or 0.3 mM SA.

(B) Gel blot of total protein (40 μg) and the nuclear-fractionated protein (20 μg). Protein was isolated from transgenic seedlings *35S*::*NPR1-GFP* or *35S*::*npr1*Δ57-GFP (NPR1-GFP or npr1Δ57-GFP) grown for 12 days on MS medium containing either 0.1 or 0.3 mM SA. The blot was probed with antibodies against GFP. The cytoplasmic npr1Δ57-GFP protein served as a control, indicating that the nuclear fraction contains relatively little cytoplasmic contamination.

(C) Gel blot of RNA from *npr1* and an *npr1* line expressing NPR1-GFP. Seedlings were grown for 12 days on MS medium containing either 0.1 or 0.3 mM SA. The blot was probed for *NPR1*, *PR-1*, and 18S rRNA.

(D) GFP fluorescence and *BGL2*::*GUS* expression in a *35S*::*NPR1-GFP* transformant (in *npr1*) after infiltration with either *Psm ES4326* (OD_{600} = 0.001 in 10 mM $MgCl_2$) or 10 mM $MgCl_2$. Leaves were infected on their left halves, and representative GFP fluorescence images from the infected halves of the leaves are shown. The expression of *BGL2*::*GUS* then was examined as described previously (Cao et al., 1994). All images were made 3 days after treatment.

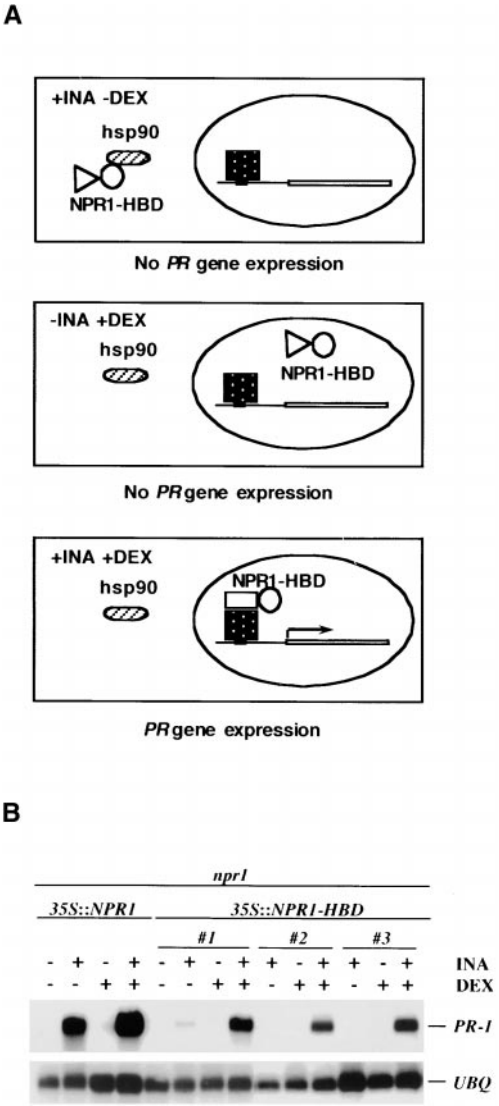

Figure 5. Nuclear Localization of NPR1 Is Essential for Its Function in Activating *PR-1* Gene Expression.

(A) Strategy used to control the nuclear localization of NPR1.
(B) Gel blot of RNA (10 μg) from *35S*::*NPR1* (in *npr1*) and three independent *35S*::*NPR1-HBD* transformants (in *npr1*). Seedlings were grown for 14 days on MS medium with (+) or without (−) DEX (5 μM) and with or without INA (20 μM). The blot was probed for *PR-1* and ubiquitin (*UBQ*) mRNA.

pression was induced by INA but not by DEX, indicating that hormone treatment does not affect the wild-type NPR1 protein or *PR-1* expression (Figure 5B). In the *35S*::*NPR1-HBD* plants, inducible expression of *PR-1* was restored only when the plants were treated with both INA and DEX (Figure 5B). This result indicates that the HBD can regulate the nuclear localization of NPR1 and that nuclear localization of NPR1 is required for *PR* gene expression (Figure 5B). DEX alone was not sufficient to activate *PR* genes in the *35S*::*NPR1-HBD* plants, which suggests that additional regulatory mechanisms involving SA or INA also must be required (Figure 5). One of the *35S*::*NPR1-HBD* lines showed a little *PR-1* expression after INA treatment alone, suggesting that cytoplasmic retention of the HBD fusion protein may be incomplete when the protein is expressed at a high level (Figure 5).

DISCUSSION

NPR1 is a key regulator of SAR-related *PR* gene expression. Plants overexpressing *NPR1* show enhanced resistance to various pathogens without constitutively expressing the *PR* genes (Cao et al., 1998). This indicates that the NPR1 protein requires activation to be functional (Cao et al., 1998; Figures 1A to 1C). The mechanism of activation could involve the translocation of NPR1 to another cellular compartment and/or a chemical or structural modification of the protein. To better understand the regulation of NPR1 and, more specifically, to determine the requirements for NPR1 protein activation, we examined the subcellular localization of NPR1 in living plant cells by expressing an NPR1-GFP fusion protein in transgenic plants.

Constitutive expression of *NPR1-GFP* complemented all of the known phenotypes associated with the *npr1* mutants (Figure 1). This finding indicates that the fusion protein is biologically functional and therefore is correctly localized. The quantities of NPR1-GFP protein remained constant before and after induction; therefore, the enhanced nuclear fluorescence observed after SAR induction (Figure 2A) must have been caused by an accumulation of NPR1-GFP in the nucleus. This conclusion was further confirmed by the detection of increased amounts of NPR1-GFP in a nucleus-enriched fraction from SAR-induced plants relative to that in uninduced plants (Figure 2B). The nuclear localization of NPR1-GFP must be directed by an NLS in NPR1 because the predicted size of NPR1-GFP (92 kD) well exceeds the size exclusion limit (40 to 60 kD) for passive diffusion of proteins through the nuclear pores (Raikhel, 1992). On the other hand, GFP alone (26 kD) is distributed in both the cytoplasm and the nucleus. Identification of a bipartite NLS in NPR1 (Figure 3) further verifies that NPR1 is targeted specifically to the nucleus.

It is still unclear where NPR1 is localized before induction. In guard cells, NPR1-GFP is localized in both the cytoplasm and the nuclei in the absence of an SAR inducer. Treatment with an SAR inducer causes NPR1-GFP to accumulate exclusively in the nuclei, possibly because of an increased retention of the protein in the nuclei as a result of chemical modification or physical association with other proteins. One likely explanation for the lack of cytoplasmic

fluorescence in the larger mesophyll cells is that the fusion protein is too diffuse to be detected. Indeed, compared with the amounts of GFP protein detected in *35S*::*GFP* transgenic lines, the amounts of NPR1-GFP in the *35S*::*NPR1-GFP* transgenic plants are markedly lower (Figure 1B). Analyses of transgenic plants expressing the cytoplasmically localized npr1nls-GFP mutant protein revealed that, for many lines, the cytoplasmic fluorescence was visible only in the guard cells, because of their smaller size. Cytoplasmic fluorescence in the larger cells was detectable only in lines that expressed the fusion protein in greater amounts (Figure 3).

Previous characterization of the *npr1* mutant revealed that NPR1 functions downstream of the signal molecule SA (Cao et al., 1994). The data presented here suggest that cell SA levels may in fact regulate the amount of NPR1 that accumulates in the nucleus. Transgenic plants grown on medium containing 0.3 mM SA accumulated more NPR1-GFP in the nucleus than did plants grown on medium containing 0.1 mM SA (Figure 4). In addition, after pathogen infection, the nuclear accumulation of NPR1-GFP was much greater in cells surrounding the infection site (Figure 4), which have been shown to have greater amounts of endogenous SA (Malamy et al., 1990). We also detected an increase in NPR1-GFP nuclear accumulation in systemic tissues after a local infection by an avirulent pathogen. However, the systemic induction of NPR1-GFP nuclear localization is not as consistent as the local response observed after pathogen infection. A likely explanation for this finding is that the relatively small amounts of SA in uninfected tissues are not sufficient to induce consistent, detectable nuclear accumulation, although they are adequate to influence *PR* gene expression. We cannot rule out, however, the possibility that nuclear accumulation of NPR1 is required only locally to induce resistance and to produce the systemic signal.

Fusion of HBD to NPR1 allowed us to control the nucleocytoplasmic localization of NPR1 by using the hormone DEX. In the absence of steroid, NPR1-HBD is sequestered in the cytoplasm by hsp90. As a result, no *PR-1* expression was detected in *35S*::*NPR1-HBD* plants after treatment with INA (Figure 5). These results show that nuclear localization of NPR1 is essential for its function in activating the *PR* genes. Interestingly, SA or INA is still required, in addition to DEX, to induce *PR-1* expression in *35S*::*NPR1-HBD* plants. SA or INA may be necessary not only for the nuclear accumulation of NPR1 but also for a chemical or structural modification of the protein. SA or INA also might be involved in activation of regulatory components other than NPR1. Genetic characterization of an *npr1*-suppressor mutant, *sni1*, indicates that this may be the case (Li et al., 1999). In the *sni1 npr1* double mutant, *PR* gene expression is restored. However, this NPR1-independent *PR* gene expression still requires the presence of SA or INA.

How does NPR1 localized to the nucleus regulate the expression of *PR* genes? Recently, NPR1 was found to interact with members of the TGA subclass of basic domain/leucine zipper transcription factors in several yeast two-hybrid screens and in vitro (Zhang et al., 1999; Després et al., 2000; Zhou et al., 2000). This finding indicates that NPR1 may regulate gene expression through a direct physical interaction with the transcription factors. This is consistent with previous promoter studies that showed the binding motif of TGA transcription factors (known as the *as-1* element) to be required for SA-induced gene expression (Lebel et al., 1998). The biological significance of the NPR1–TGA interaction has not been determined. NPR1 is unlikely to be involved in the nuclear transport of the TGA transcription factors. One of the TGA factors (AHBP-1b) has been shown to be localized to the nucleus even in a plant carrying an *npr1* mutation that disrupts the NPR1–TGA interaction (M. Kinkema and X. Dong, unpublished data). Alternatively, NPR1 localized to the nucleus could be part of a transcription factor complex, enhancing DNA binding, as suggested by Després et al. (2000), or modulating the transactivation activity of the complex. Recent studies suggest that induction of the *PR* genes involves not only the activation of positive regulators but also the inhibition of negative regulators (Lebel et al., 1998; Li et al., 1999). A genetic study showed that NPR1 may be required to inactivate the nucleus-localized repressor of SAR, SNI1 (Li et al., 1999). Even though more experiments are required to determine the molecular mechanism by which NPR1 regulates *PR* gene expression and SAR, the present study, together with previous results, strongly suggests that NPR1 regulates *PR* gene expression by forming a nuclear protein complex with other transcriptional regulators.

METHODS

Construction of the *NPR1-GFP* and *NPR1-HBD* Fusions

For construction of the *35S*::*NPR1-GFP* reporter plasmid, the *NPR1* cDNA was amplified by polymerase chain reaction (PCR) with the 5′ primer 5′-GGAATTCTCGATCTTTAACCAAATCC-3′ and the 3′ primer 5′-CATGCCATGGACCGACGACGATGAGAGAG-3′. The *NPR1* cDNA PCR fragment was digested with EcoRI and NcoI and cloned into the corresponding sites of pRTL2ΔN-mGFPS65T (kindly provided by Dr. A. von Arnim, University of Tennessee, Knoxville, TN). The *35S*::*NPR1-GFP* fusion, including the cauliflower mosaic virus (CaMV) 35S promoter and the nopaline synthase polyadenylation sequence, was excised with PstI and cloned into pBluescript KS+. After identifying the correctly oriented clones, the NPR1-GFP and nopaline synthase sequences were excised by using EcoRI and SacI and were cloned behind a modified CaMV 35S promoter in the plant transformation vector pBI1.4T (Mindrinos et al., 1994).

For construction of the *35S*::*NPR1-HBD* plasmid, the NPR1 cDNA was cut from the *35S*::*NPR1-GFP* plasmid by using EcoRI and NcoI. The hormone binding domain (HBD) fragment was amplified by PCR from the plasmid pG795 (Schena et al., 1991b) using the 5′ primer 5′-CGGGATCCATGGGTAAAGGGATTCAGCAAGCC-3′ and the 3′ primer 5′-CCGCGCGCTCTCATTTTTGATGAAACAG-3′. The PCR product was digested with NcoI and SacI and purified after gel electrophoresis. The pBI1.4T vector was cut with EcoRI and SacI and

gel-purified. Next, a three-fragment ligation was performed by mixing the EcoRI-NcoI NPR1 fragment, the NcoI-SacI HBD fragment, and the EcoRI-SacI–digested pBI1.4T vector. The construct was verified by DNA sequencing.

Mutagenesis of *NPR1*

The *35S*::*npr1Δ*57-GFP mutant lacking the sequence encoding the C-terminal 57 amino acids was constructed by amplifying the *NPR1* cDNA with the 5′ primer 5′-GGAATTCTCGATCTTTAACCAAATCC-3′ and the 3′ primer 5′-CATGCCATGGACTCAGCAGTGTCGTCTTC-3′. This truncated *NPR1* cDNA fragment (*npr1Δ*57; nucleotides 1 to 1700) was digested with EcoRI and NcoI and cloned into the corresponding sites of pRTL2ΔN-mGFPS65T to generate *35S*::*npr1Δ*57-GFP. The *35S*::*npr1Δ*57-GFP fusion was cloned subsequently into the plant transformation vector pBI1.4T as described above for *35S*::*NPR1-GFP*. Site-directed mutagenesis of the potential nuclear localization signals (NLSs) in NPR1 was performed in the *35S*::*NPR1-GFP* construct by using a PCR-based QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Point mutations were introduced into all three putative NLSs found in NPR1 to replace arginine and lysine residues with glutamine. The presence of the expected mutations in the *35S*::*npr1nls-GFP* constructs was verified by DNA sequencing.

Plant Transformation and Growth Conditions

The pBI1.4T plasmids carrying *35S*::*NPR1-GFP*, *35S*::*npr1Δ*57-GFP, *35S*::*npr1nls-GFP*, *35S*::*GFP* (Haseloff et al., 1997), and *35S*::*NPR1-HBD* were electroporated into *Agrobacterium tumefaciens* strain GV3101 (pMP90), and the resulting bacteria were used to transform various *npr1* mutants (*npr1-1*, *npr1-2*, and *npr1-3*; Bechtold and Pelletier, 1998). Because the *npr1-1* line contains the *BGL2*::*GUS* reporter gene and therefore is resistant to kanamycin, the *35S*::*NPR1-GFP* transformants were selected on plates of Murashige and Skoog (MS) (1962) medium containing 0.5 mM salicylic acid (SA). This selection strategy takes advantage of the fact that *npr1* mutants have less tolerance than wild type to high concentrations of SA. Nontransformants develop chlorotic cotyledons and arrest at this developmental stage, whereas transformants containing a functional NPR1 develop normally, with green cotyledons and leaves. All *npr1-2* and *npr1-3* transformants were selected on MS medium containing 50 μg/mL kanamycin.

Arabidopsis thaliana (ecotype Columbia) plants were grown either in soil (Metro Mix 200; Grace-Sierra, Milpitas, CA) or on plates with MS medium. For induction, 2,6-dichloroisonicotinic acid (INA; 0.02 to 0.1 mM), SA (0.1 to 0.5 mM), and dexamethasone (DEX; 5 μM; Sigma) were added to the MS medium, and seedlings were grown in the medium for 1 to 2 weeks before analysis.

Infection with Bacterial and Oomycete Pathogens

The left halves of leaves from 4-week-old Arabidopsis plants were infected with the virulent bacterial pathogen *Pseudomonas syringae* pv *maculicola ES4326*, as described previously (Cao et al., 1994). For *Peronospora parasitica Noco2* infections, 3-week-old plants were infected as described previously (Bowling et al., 1994).

RNA Extraction and RNA Gel Blot Analysis

RNA was extracted as described by Cao et al. (1994). Samples were separated on a 1% formaldehyde–agarose gel and transferred to a Genescreen nylon membrane (DuPont–New England Nuclear). Prehybridization and hybridization were performed in 7% SDS, 0.25 M Na_2HPO_4, pH 7.4, 1 mM EDTA, and 1% casein at 65°C. Probes were labeled by asymmetric PCR with ^{32}P-dCTP (Schowalter and Sommer, 1989).

Protein Extraction and Protein Gel Blot Analysis

Proteins were extracted from 6-day-old seedlings by grinding in liquid nitrogen and resuspending the powder in extraction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 50 mM NaF, 1 mM DTT, and a proteinase inhibitor cocktail). The extract was left at 4°C for 30 min with gentle mixing and then centrifuged at 14,000*g* for 10 min. The protein concentration of the supernatant was determined with the Bio-Rad protein assay. Protein samples were loaded onto a 10% SDS-PAGE gel and transferred to nitrocellulose. The blot was probed by using a green fluorescent protein (GFP) monoclonal antibody (Clontech, Palo Alto, CA) that had been preabsorbed against a protein gel blot containing proteins from tomato leaves. The antibody-bound proteins were detected by using a horseradish peroxidase–conjugated anti-mouse secondary antibody (Bio-Rad) followed by chemiluminescence.

Nuclear fractionation was performed based on the protocol described by Xia et al. (1997). Briefly, tissue was homogenized in Honda buffer (2.5% Ficoll 400, 5% dextran T40, 0.4 M sucrose, 25 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, and a proteinase inhibitor cocktail) by using a mortar and pestle and then filtered through 62-μm (pore-size) nylon mesh. Triton X-100 was added to a final concentration of 0.5%, and the mixture was incubated on ice for 15 min. The solution was centrifuged at 1500*g* for 5 min, and the pellet was washed with Honda buffer containing 0.1% Triton X-100. The pellet was resuspended gently in 1 mL of Honda buffer and transferred to a microcentrifuge tube. This nucleus-enriched preparation was centrifuged at 100*g* for 1 min to pellet starch and cell debris. The supernatant was centrifuged subsequently at 1800*g* for 5 min to pellet the nuclei. Transgenic plants expressing the cytoplasmically localized npr1nls-GFP or npr1Δ57-GFP were used as controls to monitor the amount of cytoplasmic contamination in the nuclear extracts. Only a small amount of npr1nls-GFP or npr1Δ57-GFP was present in the nucleus-enriched preparations, as shown in Figures 2B and 4B.

Transient Expression of *NPR1-GFP* in Onion Epidermal Cells

Onion (*Allium cepa*) transformation was performed essentially as described previously (Varagona et al., 1992). Inner epidermal peels of white onions were placed inside-up on modified MS medium (1 × MS salts, 1 × Gamborg's B5 vitamins [Sigma], 30 g/L sucrose, and 2% agar, pH 5.7) containing 100 μg/mL ampicillin. Onion peels were bombarded by using the PDS-1000/He system (DuPont) at 1350 p.s.i. with DNA-coated M-25 tungsten particles (Bio-Rad; Sanford et al., 1993). The particles were coated by precipitating 2 μg of DNA purified on Qiagen (Valencia, CA) columns onto 3 mg of water-washed tungsten particles with 50 μL of 2.5 M $CaCl_2$ and 20 μL of 0.1 M spermidine, followed by washing with 70% ethanol and resuspending in 36 μL of 100% ethanol. Approximately 10 μL of particles

then was placed on each delivery disc. After bombardment, the Petri dishes were sealed with Parafilm and placed in a 22°C incubator for ~18 hr before observation.

Microscopy

Arabidopsis seedlings and leaf tissues were mounted in water and viewed with a Zeiss (Jena, Germany) LSM 410 inverted confocal microscope. GFP was visualized by using an excitation wavelength of 488 nm and a bandpass 510- to 525-nm emission filter. Under the conditions used, only small amounts of chlorophyll autofluorescence were visualized in untransformed plant tissue. Nuclei were stained by vacuum infiltration of seedlings or leaf tissues with 1 μg/mL 4′,6-diamidino-2-phenylindole. Nuclear localization of NPR1-GFP was confirmed by the colocalization of GFP and 4′,6-diamidino-2-phenylindole fluorescence. Onion peels were mounted in water, viewed with a Leica (Wetzlar, Germany) DMRB inverted microscope, and imaged by using MetaMorph imaging software.

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