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Tomato Transcription Factors Pti4, Pti5, and Pti6 Activate Defense Responses When Expressed in Arabidopsis

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The Pti4, Pti5, and Pti6 proteins from tomato were identified based on their interaction with the product of the *Pto* disease resistance gene, a Ser-Thr protein kinase. They belong to the ethylene-response factor (ERF) family of plant-unique transcription factors and bind specifically to the GCC-box *cis* element present in the promoters of many pathogenesis-related (*PR*) genes. Here, we show that these tomato ERFs are localized to the nucleus and function in vivo as transcription activators that regulate the expression of GCC box–containing *PR* genes. Expression of *Pti4*, *Pti5*, or *Pti6* in Arabidopsis activated the expression of the salicylic acid–regulated genes *PR1* and *PR2*. Expression of jasmonic acid– and ethylene-regulated genes, such as *PR3*, *PR4*, *PDF1.2*, and *Thi2.1*, was affected differently by each of the three tomato ERFs, with Arabidopsis-*Pti4* plants having very high levels of *PDF1.2* transcripts. Exogenous application of salicylic acid to Arabidopsis-*Pti4* plants suppressed the increased expression of *PDF1.2* but further stimulated *PR1* expression. Arabidopsis plants expressing Pti4 displayed increased resistance to the fungal pathogen *Erysiphe orontii* and increased tolerance to the bacterial pathogen *Pseudomonas syringae* pv *tomato*. These results indicate that Pti4, Pti5, and Pti6 activate the expression of a wide array of *PR* genes and play important and distinct roles in plant defense.

INTRODUCTION

Plants respond to pathogen attack by activating multiple defense mechanisms to protect themselves from infection. These rapid cellular responses often are triggered by the recognition of specific pathogens and the activation of highly regulated signal transduction pathways. A major target of these pathways is the cell nucleus, where signals lead to the transcriptional activation of a large array of defense genes (Maleck et al., 2000; Schenk et al., 2000). The products of these genes include pathogenesis-related (PR) proteins as well as enzymes involved in the biosynthesis of protective secondary metabolites. Although the functions of many PR proteins remain unknown, some PR proteins, such as β-1,3-glucanase (PR2) and chitinase (PR3), are hydrolytic enzymes that have been shown to degrade fungal cell walls and to inhibit fungal growth both in vivo and in vitro (Broglie et al., 1991; Sela-Buurlage et al., 1993; Zhu et al., 1994). It was shown recently that osmotin (PR5) induces apoptosis in yeast, and it may act similarly toward plant fungal pathogens (Narasimhan et al., 2001).

Several signaling molecules, such as salicylic acid (SA), ethylene (ET), and jasmonic acid (JA), have been shown to be important components of defense response pathways (Dong, 1998; Reymond and Farmer, 1998; Dempsey et al., 1999; Pieterse and van Loon, 1999). Infection by microbial pathogens results in an increase in the levels of these molecules in plants, and many *PR* genes that are induced upon pathogen infection also are upregulated by one or more of these signaling molecules (Malamy et al., 1990; Thomma et al., 1998; Dempsey et al., 1999). The SA-dependent defense signaling pathway regulates the expression of acidic PR proteins such as PR1, PR2, and PR5. The ET/JA-dependent signaling pathway(s) regulates the expression of vacuole-localized basic PR proteins such as PR3, PR4, and PDF1.2. Genetic and biochemical evidence exists for communication between the different pathways (Feys and Parker, 2000), which could be either coregulatory or antagonistic responses (Maleck and Dietrich, 1999). In addition, the nature of this communication appears to be pathogen dependent,

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which is consistent with the finding that activation of the SA- and/or ET/JA-dependent defense pathways also is pathogen specific (Thomma et al., 1998).

The promoters of several *PR* genes have been studied to identify the specific *cis*-acting elements that confer responsiveness to SA, ET, or JA. In some cases, the identification of these *cis*-acting elements has led to the isolation of the cognate transcription factors. Many ET-inducible *PR* genes contain an 11-bp GCC box (TAAGAGCCGCC) in their promoter regions (Eyal et al., 1993). Transcription factors that bind the GCC box specifically, the ET-responsive element binding proteins (EREBPs), were first isolated from tobacco and shown to be ET induced and involved in the expression of GCC box–containing *PR* genes. These proteins were later renamed ET response factors (ERFs) (Ohme-Takagi and Shinshi, 1995; Suzuki et al., 1998). The ERF domain was believed previously to be closely related to the AP2 domain, which is found in AP2 domain transcription factors involved in plant development (Okamuro et al., 1997; Riechmann and Meyerowitz, 1998). It has now been shown that ERFs possess a highly conserved DNA binding domain, and the solution structure of this domain shows that it is novel, with a unique form of DNA recognition (Ohme-Takagi et al., 2000). ERF-encoding genes are present only in higher plants.

ERFs are present in plant species from phylogenetically distinct taxa. These genes have been characterized in Arabidopsis (ERF1, AtEBP, and AtERFs; Büttner and Singh, 1997; Solano et al., 1998; Fujimoto et al., 2000), tomato (Pti4/5/6; Zhou et al., 1997), and soybean (GmEREBP1; M. Mazarei, D.P. Puthoff, J.K. Hart, S.R. Rodermel, and T.J. Baum, unpublished data) in addition to tobacco (EREBPs; see above). They all share common features, such as being induced by biotic and abiotic stresses and mediating the expression of GCC box–containing genes such as *PDF1.2*. ERFs in Arabidopsis have been shown to be both activators and repressors of GCC box–mediated gene expression (Fujimoto et al., 2000). Interestingly, a novel JA- and elicitor-responsive element consisting of a GCC box–like element also is involved in the regulation of secondary metabolite biosynthetic genes in *Catharanthus roseus* (Menke et al., 1999; Memelink et al., 2001). The transcription factors that bind this element, the ORCAs, belong to the AP2 family (Menke et al., 1999; Memelink et al., 2001), suggesting a global role for the AP2/ERFs and related transcription factors in both ET and JA signaling pathways. Therefore, the elucidation of the role of ERFs in the defense response in plants is an emerging, important field of study.

In tomato, resistance to bacterial speck disease is governed by the *Pto* resistance gene, which encodes a Ser/Thr protein kinase (Martin et al., 1993). Previously, we identified three tomato ERF transcription factors, Pti4, Pti5, and Pti6, by virtue of their specific interaction with Pto kinase in a yeast two-hybrid screen (Zhou et al., 1997). Because Pti4/5/6 interact with Pto and also specifically bind the GCC-box *cis* element, a role for these genes in the activation of *PR* genes during the plant defense response was proposed (Zhou et al., 1997; Gu and Martin, 1998; Gu et al., 2000). The expression of *Pti4/5/6* is enhanced in response to infection by *Pseudomonas syringae* pv *tomato* bacterial pathogens and by treatment with different signaling molecules (Thara et al., 1999; Gu et al., 2000). Furthermore, the Pto kinase phosphorylates Pti4 in vitro, and this phosphorylation enhances the GCC box binding activity of Pti4 (Gu et al., 2000). These observations suggested that Pti4, activated by the gene-for-gene *avrPto–Pto* interaction, may regulate the induction of defense-related genes that result in plant disease resistance.

In this study, we first characterized the localization and transactivation activity of Pti4/5/6. Then, we expressed the tomato ERFs in Arabidopsis to take advantage of the many mutants of defense signaling pathways in this species and its well-characterized plant–microbe interactions. Our results indicate that Pti4/5/6 play a direct role in mediating *PR* gene expression in vivo and that Pti4 activity, in particular, activates *PR* gene expression, resulting in enhanced defense against certain bacterial and fungal pathogens.

RESULTS

Pti4, Pti5, and Pti6 Are Localized to the Nucleus

Inspection of the amino acid sequences of Pti4/5/6 revealed that each one contains typical nuclear localization sequences (NLSs) of the bipartite class, with two clusters of basic residues separated by 4 to 20 amino acids (Figure 1B) (Zhou et al., 1997; Gu et al., 2000). To examine the subcellular localization of Pti4/5/6, their coding regions were fused to the 3′ end of a β-glucuronidase (GUS) reporter gene and expressed under the control of the 35S promoter of *Cauliflower mosaic virus* (CaMV). Each GUS fusion construct was introduced by particle bombardment into W-38 tobacco suspension cells. Transient expression of *Pti4/5/6*::*GUS* fusions showed that GUS activity was localized to the nucleus of transformed tobacco cells (Figure 1A).

Pti4 Nuclear Localization Does Not Require Pto

To determine if the nuclear localization of Pti4 is Pto dependent, a green fluorescent protein (GFP)–Pti4 fusion was developed, and the construct was transfected into protoplasts isolated from Arabidopsis ecotype Columbia (Col-0). We used Arabidopsis Col-0 because, unlike W-38 tobacco, Pto-like activity has not been identified in this ecotype (Thilmony et al., 1995; Y. Gu and G. Martin, unpublished data). Protoplasts transfected with a GFP control construct showed green fluorescence throughout the entire cytoplasm and nucleus. In contrast, the GFP-Pti4 fusion proteins were localized exclusively to the nucleus, indicating that Pti4 contains

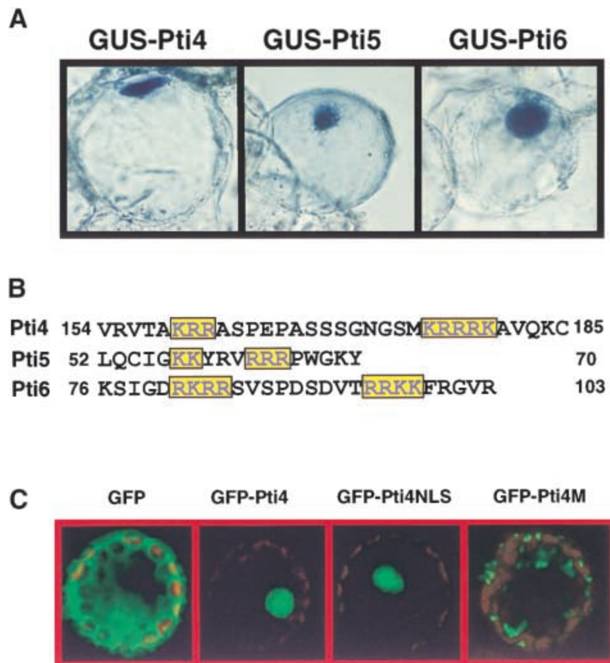

Figure 1. Nuclear Localization of Pti4/5/6.

(A) Transient expression of GUS-Pti4/5/6 fusions in tobacco suspension cells. 35S::GUS-Pti4/5/6 constructs were introduced into W-38 tobacco suspension cells by particle bombardment. Localization of the fusion proteins was visualized after the addition of 5-bromo-4-chloro-3-indolyl-β-D-glucuronide substrate to the cells.

(B) Putative bipartite nuclear localization signals of Pti4/5/6. The two basic clusters are colored and boxed. Residue numbers corresponding to the Pti4/5/6 amino acid sequences in GenBank are given for each peptide.

(C) Arabidopsis protoplasts were transfected with the indicated constructs, as described in the text. GFP fluorescence was visualized using a confocal microscope, as described in Methods.

an NLS that is sufficient to target the fusion proteins into the nucleus (Figure 1C).

To further characterize the bipartite NLS domain of Pti4 (Figure 1B), a DNA segment encoding the 32 amino acids spanning the putative Pti4 NLS was fused to the *GFP* gene, and the construct was expressed transiently in Arabidopsis protoplasts. As shown in Figure 1C, the GFP-Pti4-NLS fusion was localized to the nucleus, indicating that this region spanning 32 amino acids functions as an NLS. To examine this functional domain further, the two clusters of the basic charged residues of the NLS from the full-length Pti4 sequence were deleted, and the resulting mutant (Pti4M) was fused to GFP. This deletion caused the loss of nuclear localization of Pti4, and the green fluorescence was visualized as patches in the protoplasts (Figure 1C). Therefore, this bipartite domain region is the sole NLS necessary to target Pti4 protein into the nucleus.

Pti4/5/6 Are Transcription Activators

To determine if Pti4/5/6 can function as activators of GCC box–mediated transcription, Arabidopsis protoplasts were cotransfected with a GCC box–luciferase (*LUC*) construct and a vector expressing Pti4, Pti5, or Pti6 under the CaMV 35S promoter (Figure 2A). Compared with expression of the control, the expression of Pti5 and Pti6 increased the GCC box–mediated transcription by threefold and fourfold, respectively. To determine whether Pti5 or Pti6 activates the reporter gene via interaction with the GCC-box *cis* element, cotransfection of Pti5/6 with a mutated *m*GCC-*LUC* construct was performed; transactivation activity of Pti5/6 of this construct was not observed (Figure 2B). Surprisingly, cotransfection with Pti4 did not result in the activation of GCC box–mediated transcription. We also observed that the Pti4 protein has lower affinity in binding to a synthetic GCC box in vitro than Pti5 and Pti6 (Y. Gu, unpublished data).

We verified the expression of Pti4/5/6 proteins in Arabidopsis protoplasts by immunoblot analysis using an anti-hemagglutinin (HA) antibody (Figure 2C). Although the expression level varied for each effector protein, Pti4 was detected consistently in the protoplasts from each transfection. To further study the possible transactivation function of Pti4, the promoter of a known ET-regulated gene, *PDF1.2* from Arabidopsis, was isolated. The *PDF1.2* promoter, which contains a GCC box, was fused to a *LUC* reporter gene (Figure 2D). Cotransfection of this reporter construct with the Pti4 effector plasmids resulted in threefold enhanced transcription of the reporter gene (Figure 2E). However, neither Pti5 nor Pti6 significantly increased transcription mediated by the *PDF1.2* promoter. Based on these results, we postulate that Pti4/5/6 bind GCC boxes differently depending on the flanking nucleotide sequences.

To analyze the transactivation function of Pti4/5/6 without the complications caused by the differential binding of the GCC box, the DNA binding domain of the yeast GAL4 protein (GAL4DB) was fused to the full-length Pti4/5/6 (Figure 3A). The reporter construct (UAS_{GAL4}-GUS) contained nine copies of the GAL4 upstream activation sequence fused to the GUS reporter gene. As shown in Figure 3B, GAL4DB-Pti4 gave a 3.4-fold increase in activation of the reporter gene over the control construct, and GAL4DB-Pti5 and GAL4DB-Pti6 increased the expression of the reporter gene by 1.7- and 1.8-fold, respectively.

Expression of Pti4 in Arabidopsis Causes Phenotypic Changes Associated with the Response to ET Treatment

To analyze the role of Pti4/5/6 in the regulation of GCC-box *PR* gene expression, Arabidopsis transgenic plants were developed that constitutively express *Pti4*, *Pti5*, or *Pti6* mRNAs from the 35S promoter. Approximately 30 individual transgenic plants were generated for each construct. RNA

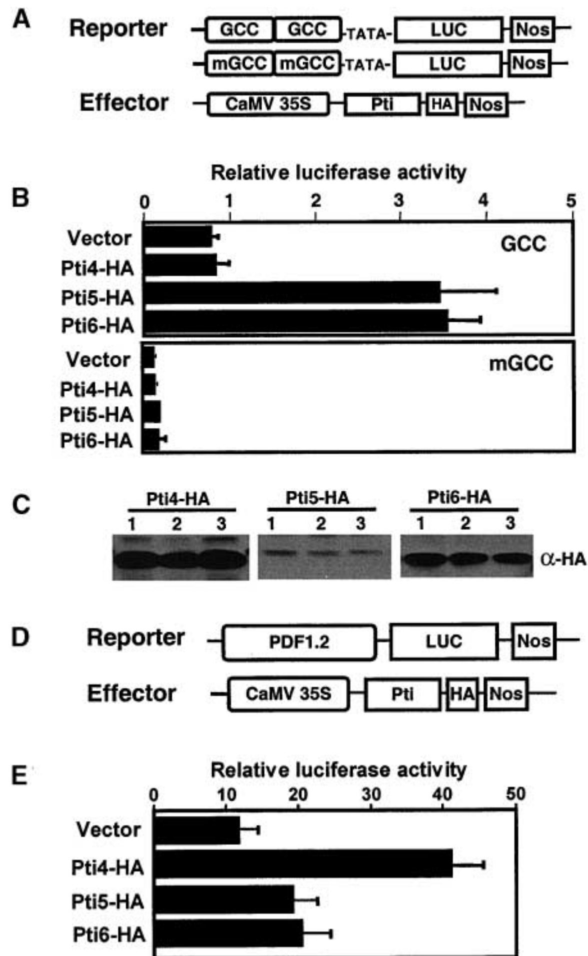

Figure 2. Transactivation of GCC Box–Mediated Transcription by Pti4/5/6.

(A) Schemes of the effector and reporter constructs used in the cotransfection experiments. The reporter constructs contain two copies of the GCC box or the mutated mGCC box in tandem that were fused upstream to the CaMV 35S minimal TATA promoter, the coding region from the *LUC* gene, and the nopaline synthase (NOS) terminator. The effector plasmids contain a CaMV 35S promoter fused to HA-tagged Pti4, Pti5, or Pti6 cDNA.

(B) Transactivation of the *GCC-LUC* reporter gene by Pti4/5/6. Arabidopsis protoplasts were cotransfected with a mixture of plasmids containing reporter, effector (empty vector with no insert was used as a control), and internal control constructs (35S-LUC). Dual luciferase activity was measured 20 hr after transfection of the protoplasts, as described in Methods. The data shown are derived from triplicate samples and three independent experiments.

(C) Expression of effector proteins in transfected Arabidopsis protoplasts. Twenty micrograms of total protein from transfected Arabidopsis protoplasts was separated by SDS-PAGE. The expression of effector proteins was detected by protein gel blot analysis using an anti-HA antibody (α-HA). Lanes 1, 2, and 3 contain protein samples from three independent transfections.

(D) Scheme of the *PDF1.2* promoter–LUC construct. A 1.2-kb segment of the *PDF1.2* promoter (Manners et al., 1998) was amplified by PCR from Arabidopsis genomic DNA and fused to the coding region of the *LUC* gene. The effector construct containing the Pti4 coding region is the same as in **(A)**.

(E) Transactivation of the *PDF1.2-LUC* reporter gene by Pti4. Arabidopsis protoplasts were cotransfected with *PDF1.2-LUC*, *35S*::*Pti4-HA*, and the internal control construct. Empty vector was used as a control for the effector plasmid. Dual luciferase activity in protoplasts was measured 20 hr after transfection. The data are derived from triplicate samples and three independent experiments.

gel blot analysis was performed on primary transformants to determine the expression of the transgenes (data not shown). Homozygous lines (from the T3 generation) with a single insertion of the transgene were selected for each construct and used for further analysis. Of six independent homozygous lines expressing *Pti4*, plants from five lines displayed phenotypic changes. These plants were slightly smaller and darker green compared with wild-type plants (Figure 4A). Transgenic plants expressing *Pti5* or *Pti6* appeared no different than wild-type plants.

Because Pti4 is known to be involved in the ET signaling pathway (Gu et al., 2000), we used the triple-response assay (Solano et al., 1998) to determine whether the expression of Pti4/5/6 activated ET responses. Compared with control wild-type seedlings, etiolated *Pti4*-expressing seedlings in the absence of ET showed inhibition of hypocotyl elongation, a phenotype caused by ET treatment (Figure 4B). However, unlike the constitutive ET response mutant *ctr*, the *Pti4*-expressing seedlings did not display severe inhibition of root growth or exaggerated apical hook curvature. Therefore, the expression of *Pti4* appears to activate a subset of ET responses. This partial seedling triple-response phenotype was not observed in transgenic plants expressing *Pti5* or *Pti6* (data not shown).

Expression of Pti4/5/6 in Arabidopsis Upregulates Different Sets of *PR* Genes

Our previous results suggested that Pti4/5/6 play a direct role in the regulation of tomato GCC-box *PR* genes (Thara et al., 1999; Gu et al., 2000). To determine whether Pti4/5/6 play a role in activating *PR* genes in Arabidopsis, the expression of different classes of *PR* genes was examined in transgenic plants expressing *Pti4*, *Pti5*, or *Pti6*. As shown in Figure 5, expression of *Pti4* in Arabidopsis caused an increase in the steady state abundance of *PR3*, *PR4*, and *PDF1.2* transcripts, which are known to be ET inducible and to contain GCC boxes in their promoters. The expression of *PDF1.2* transcripts was induced 20- to 40-fold in the *Pti4*-expressing plants examined. Transgenic plants expressing *Pti5* or *Pti6* showed weak or no increase in the expression of these ET-regulated *PR* genes. This finding is consistent with the results described above that Pti4 (and not Pti5 or Pti6)

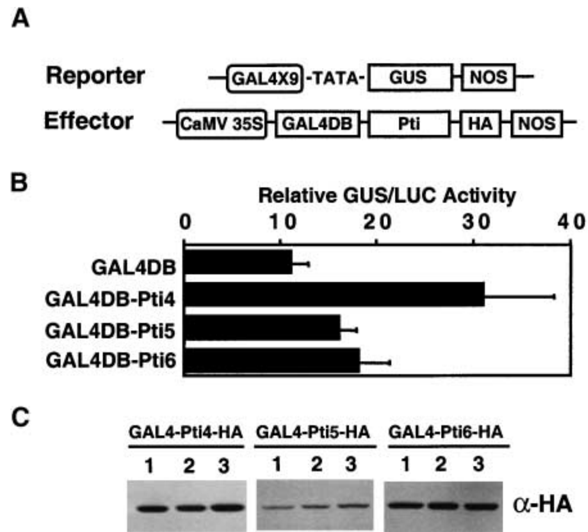

Figure 3. Transactivation of the *GAL4X9-GUS* Reporter Gene by GAL4DB-Pti4/5/6 Fusion Proteins.

(A) Schemes of reporter and effector constructs. The reporter construct contains nine copies of the GAL4 DNA binding site linked to a minimal CaMV 35S promoter, the GUS gene, and the NOS terminator. The effector constructs contain the CaMV 35S promoter fused to the GAL4 DNA binding domain (GAL4DB), Pti4/5/6-HA, and the NOS terminator.

(B) Transactivation of the *GAL4X9-GUS* reporter gene by GAL4DB-Pti4/5/6. Arabidopsis protoplasts were cotransfected with a mixture of plasmids containing GAL4X9-GUS, GAL4DB-Pti4/5/6-HA, and internal control constructs. The internal control plasmid used to normalize for transfection efficiency contains the CaMV 35S promoter fused to the *LUC* gene. Protoplasts were incubated for 20 hr after transfection. GUS and LUC activity in the protoplasts were determined according the method described by Sprenger-Haussels and Weisshaar (2000).

(C) Expression of GAL4DB-Pti4/5/6-HA fusion proteins. Twenty micrograms of total protein from transfected Arabidopsis protoplasts was separated by SDS-PAGE. The expression of the HA-tagged fusion proteins was detected by protein gel blot analysis using an anti-HA antibody (α-HA).

significantly enhanced the transcription of the Arabidopsis *PDF1.2* promoter (Figure 2E).

JA regulates the expression of a subset of *PR* genes, some of which also are ET inducible, such as *PDF1.2* (Thomma et al., 1998; Schenk et al., 2000). The effect of the expression of *Pti4/5/6* on the expression of *Thi2.1*, which encodes the potent fungal defense protein thionin, was examined (Epple et al., 1997; Bohlmann et al., 1998). *Thi2.1* is induced by JA but not by ET (Epple et al., 1995) and requires a functional JA signal transduction pathway (Bohlmann et al., 1998; Xie et al., 1998). The transcript abundance of *Thi2.1* was increased in *Pti4*- and *Pti6*-expressing plants but not in *Pti5*-expressing plants (Figure 5).

The transcript abundance of two known SA-regulated *PR* genes, *PR1* and *PR2*, in transgenic plants also was assessed. Expression of *Pti4/5/6* in Arabidopsis increased the expression of the *PR2* gene substantially, with the transcript abundance being similar to that in plants treated with SA (Figure 5). The abundance of *PR1* transcripts was induced only minimally in most of the transgenic plants compared with wild-type plants.

Pti4/5/6 Enhance the Expression of SA-Induced *PR* Genes, and Pti4 May Mediate SA Antagonism of ET-Regulated *PR* Genes

To further study the effect of *Pti4/5/6* on the expression of SA-regulated *PR* genes, RNA isolated from plants treated with SA was hybridized with the *PR1* gene probe. An increase in the level of expression of *PR1* was observed in Pti4/5/6 transgenic plants compared with SA treated wild-type plants, and this increase became clear at higher (150

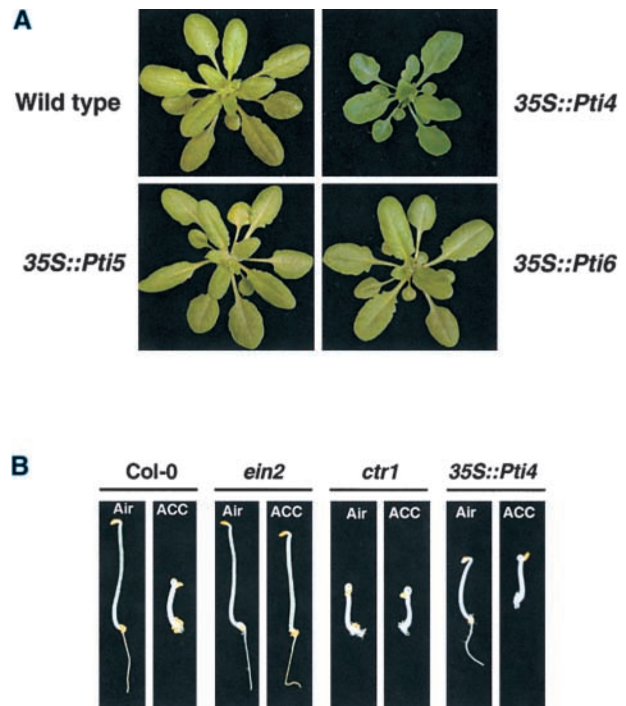

Figure 4. Ectopic Expression of Pti4/5/6 in Arabidopsis and Triple-Response Assay of the Transgenic Plants.

(A) Phenotypes of Arabidopsis transgenic plants carrying the *35S*::*Pti4*, *35S*::*Pti5*, or *35S*::*Pti6* transgene. An untransformed wild-type Col-0 plant is shown for comparison.

(B) Overexpression of *Pti4* caused constitutive activation of the ET response phenotype. Three-day-old seedlings overexpressing *Pti4/5/6* were germinated on agar plates in the dark with or without 20 μM 1-aminocyclopropane-D-carboxylic acid (ACC). Untransformed wild type, *ein2*, and *ctr1* mutants are shown as controls.

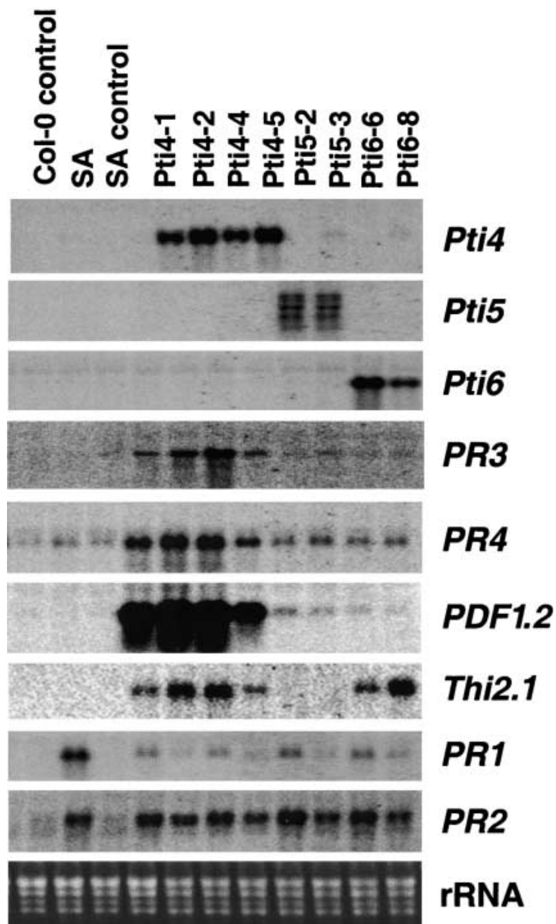

Figure 5. Overexpression of *Pti4/5/6* in Arabidopsis Causes Constitutive Upregulation of *PR* Genes.

Two to four individual transgenic lines (homozygous, from the T3 generation) were chosen for analysis for each construct expressing *Pti4/5/6*. Total RNA was isolated from leaves of 4-week-old Arabidopsis plants. Duplicated RNA gel blots were hybridized with the probes indicated. Equal loading was verified by visualizing rRNA on a gel stained with ethidium bromide.

and 500 μM) concentrations of SA (Figure 6). *PR1* transcripts were approximately threefold more abundant in Pti4/5/6 plants compared with wild-type plants when treated with 500 μM SA.

We reported previously that SA suppresses the ET induction of GCC box–containing *PR* genes in tomato (Gu et al., 2000). To further elucidate this suppression mechanism, Arabidopsis plants expressing *Pti4*, *Pti5*, or *Pti6* were treated with different concentrations of SA. As shown in Figure 6, the abundance of *PDF1.2* transcripts decreased in *Pti4*-expressing plants upon SA treatment. This suppression was observed even at a low concentration of SA (50 μM), whereas at a high SA concentration (500 μM), the accumulation of *PDF1.2* transcripts was abolished completely. In Pti5, Pti6, and wild-type plants, the low levels of *PDF1.2* transcripts present also were suppressed by treatment with higher concentrations of SA.

Transcriptional Regulation of *PDF1.2* by Overexpression of Pti4 Is Not Affected by the *jar1* or *ein2* Mutation

In Arabidopsis, the expression of the *PDF1.2* gene is dependent on functional ET and JA signaling pathways (Penninckx et al., 1998). As shown in Figure 5, the expression of Pti4 caused an increase of *PDF1.2* transcripts. To study the role of Pti4 in the ET/JA induction of defense gene expression, we treated wild-type plants, the ET-insensitive mutant *ein2*, the JA-insensitive mutant *jar1*, and *Pti4*-expressing plants with ET and JA. As shown in Figure 7A, both ET and JA induced the expression of *PDF1.2* in wild-type plants, and ET failed to induce the expression of *PDF1.2* in *ein2* or *jar1* mutant plants. JA did not induce the expression of the *PDF1.2* gene in the *ein2* mutant but weakly induced its expression in the *jar1* mutant. Plants expressing *Pti4* showed an additional marked increase of *PDF1.2* transcripts when treated with either ET or JA compared with wild-type control plants.

To further elucidate the transcriptional activation mechanism of *PDF1.2* by Pti4, Arabidopsis plants that express Pti4 in *ein2* and *jar1* mutant backgrounds were generated. As shown in Figure 7B, of seven primary transformants in each mutant background, five plants of *ein2* and six plants of *jar1* expressed the *Pti4* transgene, and these transformants also accumulated *PDF1.2* transcripts constitutively. Homozygous T3 progeny overexpressing *Pti4* were derived from two primary transformants in each mutant background (i.e., lines *jar1-1/35S*::*Pti4* and *jar1-4/35S*::*Pti4* and lines *ein2-2/35S*::*Pti4* and *ein2-5/35S*::*Pti4*). The T3 homozygous lines also showed constitutive expression of the *PDF1.2* gene (data not shown).

Arabidopsis Plants Expressing *Pti4* Are Resistant to *Erysiphe orontii* and Show Increased Tolerance to Pseudomonas

As shown in Figure 5, the expression of *Pti4/5/6* in Arabidopsis led to the constitutive expression of several *PR* genes. This raised the possibility that defense responses are activated in these plants and that resistance to certain pathogens might be increased. The *Pti4/5/6* plants were first tested with *Erysiphe orontii*, a biotrophic fungal pathogen (recently renamed *Golovinomyces orontii*; Braun, 1999) that infects wild-type Arabidopsis Col-0 and typically does not induce the ET/JA-dependent PR genes *PDF1.2* and *Thi2.1* (Plotnikova et al., 1998). As shown in Figure 8A and Table 1, when two independent lines of Arabidopsis expressing *Pti4*

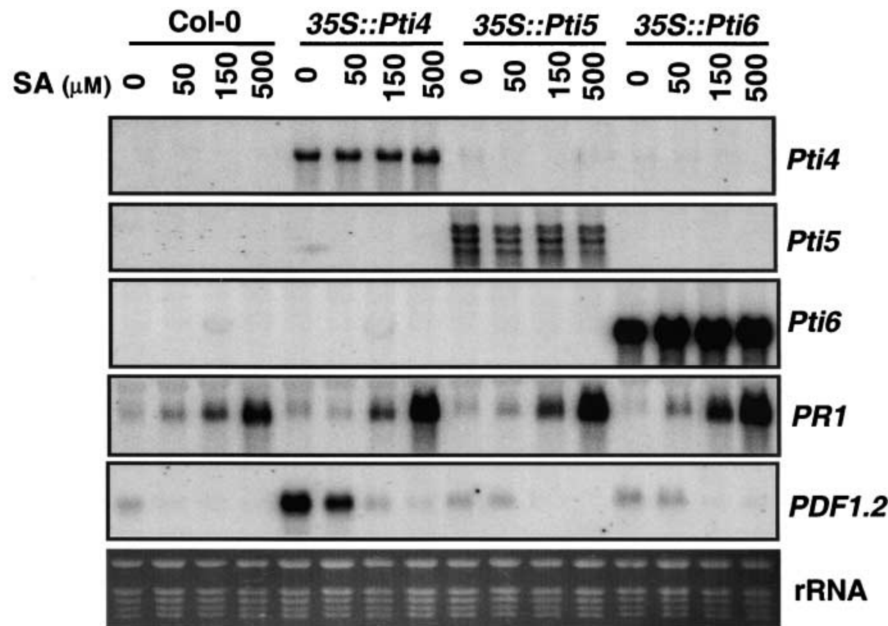

Figure 6. Overexpression of Pti4/5/6 Sensitized the SA Signaling Pathway, and SA Suppressed the Expression of *PDF1.2* Transcripts in the *Pti4*-Overexpressing Line.

Three-week-old seedlings of wild-type plants or Pti4/5/6-overexpressing lines were treated with different concentrations of SA as indicated for 16 hr. Total RNA was extracted from treated leaf tissues, and duplicated blots were hybridized with the probes indicated. Equal loading was verified by visualizing rRNA on a gel stained with ethidium bromide.

were infected with Erysiphe, greatly enhanced resistance was observed in the inoculated leaves at 14 days after inoculation. In contrast, in wild-type plants, Erysiphe growth was observed as powdery mildew covering 50% or more of the infected leaf area. The stronger resistance observed in line Pti4-2 compared with Pti4-5 is consistent with the greater abundance of *PR3*, *PR4*, *PDF1.2*, and *Thi2.1* transcripts in the former line. Transgenic plants expressing *Pti5* or *Pti6* did not show enhanced resistance against the Erysiphe pathogen compared with wild-type plants (Table 1).

Pti4/5/6 plants also were inoculated with a bacterial pathogen, Pseudomonas strain DC3000, that is known to induce both the SA-dependent genes *PR1* and *PR2* and the ET/JA-dependent *PR* genes *PDF1.2* and *Thi2.1*. Pseudomonas strain DC3000 is a virulent pathogen that causes lesions and chlorosis on many Arabidopsis ecotypes, including Col-0. In three independent experiments, bacterial growth in leaves of Pti4-2 and Pti4-5 plants was not significantly different from that in wild-type plants 4 days after infection (data not shown). However, leaves of both independent transgenic lines showed markedly less chlorosis compared with wild-type plants (Figure 8B). *NahG* plants also were infected to serve as another susceptible control to evaluate response to the pathogen. These plants showed the most serious disease symptoms among all of the plants studied (Figure 8B). No enhanced tolerance to Pseudomonas-induced chlorosis was observed for the *Pti5* and *Pti6* overexpressers (data not shown).

DISCUSSION

In an effort to identify signaling components of the Pto disease resistance pathway, we previously discovered three transcription factors, Pti4/5/6, which interact physically with Pto kinase and bind the GCC-box *cis* element present in the promoters of many *PR* genes. The discovery of Pti4/5/6 established a direct molecular link between pathogen recognition and the activation of *PR* gene expression involved in host defense responses (Zhou et al., 1997; Gu and Martin, 1998). In this study, we have demonstrated an in vivo function for Pti4/5/6 in defense by expressing them in Arabidopsis plants. We found that Pti4/5/6 mediate the expression of both SA- and ET/JA-regulated *PR* genes and that Pti4 may play a role in the communication between these pathways. Pti4 expression also leads to enhanced resistance to a fungal pathogen and to increased tolerance to a bacterial pathogen. This is an example of the expression of ERF genes in a heterologous background, and it suggests that these genes might be useful generally in engineering diverse plant species for increased disease resistance.

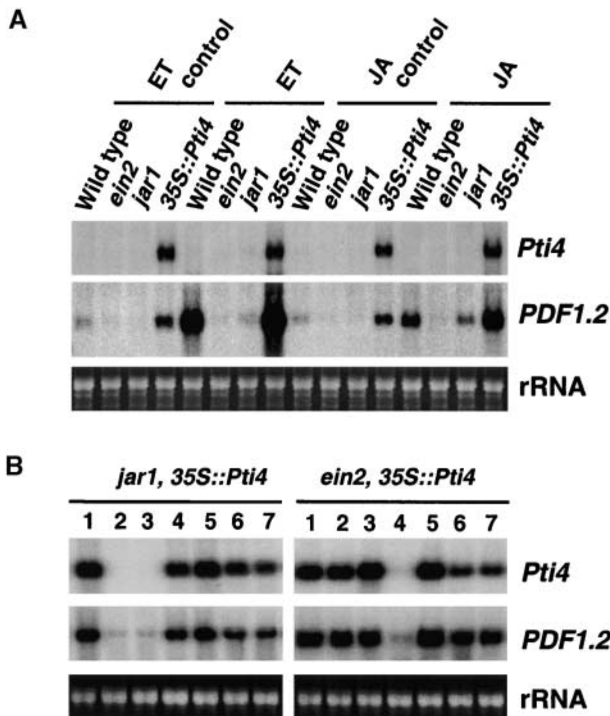

Figure 7. Activation of *PDF1.2* by the Overexpression of *Pti4* Is Independent of the ET and JA Signaling Pathways.

(A) Induction of *PDF1.2* by JA and ET in Arabidopsis wild-type plants, *jar1* and *ein2* mutants, and Pti4-expressing plants. Treatment of plants with ET and JA was performed as described in Methods. Total RNA was extracted from treated leaf tissues, and the RNA gel blot was hybridized with a *PDF1.2* probe. Equal loading was verified by visualizing the rRNA on a gel stained with ethidium bromide.

(B) Activation of *PDF1.2* by overexpression of *Pti4* in the *jar1* and *ein2* backgrounds. Total RNAs were extracted from seven putative T1 transgenic lines for each mutant background. Duplicated blots were hybridized with the *Pti4* and *PDF1.2* probes. Equal loading was verified by visualizing the rRNA on a gel stained with ethidium bromide.

Nuclear Localization and Transcription Activation by Pti4/5/6

As expected for transcription factors, Pti4/5/6 were localized to the nucleus in both tobacco and Arabidopsis cells. Pti4/5/6 all contain typical bipartite NLSs, and this NLS is sufficient to target Pti4 to the nucleus. This finding raises two interesting questions. (1) Is the phosphorylation of Pti4 (by Pto in tomato or by a functional Pto homolog in Arabidopsis) required for the nuclear localization of Pti4? (2) Where does the physical interaction between Pto (or a homolog) and Pti4 occur? Although experiments in both tobacco and Arabidopsis protoplasts indicate that Pto itself is not required for Pti4 nuclear localization, we cannot exclude a role for phosphorylation in Pti4 localization.

Tobacco is known to express a Pto-like activity that is effective in recognizing AvrPto (Thilmony et al., 1995), and this activity may play a role in Pti4 localization in this species. This possibility is consistent with the observation that *PR* genes often are expressed more rapidly and to a higher degree during incompatible plant–pathogen interactions involving specific resistance genes and their cognate avirulence proteins (Voisey and Slusarenko, 1989; Jia and Martin, 1999). As for the physical interaction between Pti4 and Pto (or a related kinase in Arabidopsis), this could occur either in the cytoplasm or in the nucleus. The ethylene-responsive MAP (ERM) kinase in parsley provides one precedent for the latter possibility. This kinase is activated upon recognition of an elicitor, leading to its translocation into the nucleus, where it interacts with transcription factors that induce the expression of defense genes (Ligterink et al., 1997). At present, we are using GFP fusions and cellular fractionation studies to investigate the subcellular localization of Pto kinase.

By using Arabidopsis protoplasts and reporter constructs carrying a GCC box or a GAL4 DNA binding sequence, transactivation activity for Pti4/5/6 was demonstrated. As with nuclear localization, it is possible that the phosphorylation of Pti4 by Pto (in tomato) or by a functionally analogous kinase (in Arabidopsis and tobacco) facilitates its transactivation activity. A similar mechanism is known for other transcription factors (Hunter and Karin, 1992). In fact, we have shown previously that specific phosphorylation of Pti4 by Pto kinase enhances its DNA binding activity in vitro (Gu et al., 2000). We are mapping the phosphorylation sites of Pti4 to further investigate the role of phosphorylation activity in Pti4 activity.

Activation of ET/JA-Regulated Genes by Pti4/5/6

The expression of Pti4 in Arabidopsis caused the activation of ET-regulated *PR* genes, such as *PDF1.2*, and phenotypic changes associated with the plant response to ET, suggesting that Pti4 can play a role in regulating the expression of genes in the ET signaling pathway. It was observed that Pti4 causes an additive increase of *PDF1.2* expression in transgenic plants exposed to ET and JA compared with wild-type plants. We also observed that the expression of Pti4 in *ein2* and *jar1* mutant plants still led to the activation of *PDF1.2*. These results suggest that Pti4 acts either independent of or downstream of the *EIN2* and *JAR1* genes.

Pti4 is known to play a role in tomato in regulating the expression of GCC-box *PR* genes in defense responses against Pseudomonas (Thara et al., 1999; Gu et al., 2000). Such regulatory functions for Pti4 appear not to require the ET signaling pathway (Thara et al., 1999). Taking into consideration the observations in the current study, we propose that the disease resistance and ET signaling pathways converge at transcription factors such as Pti4. Available experimental evidence on the function of different Arabidopsis ERFs (Solano et al., 1998; Fujimoto et al., 2000) does not in-

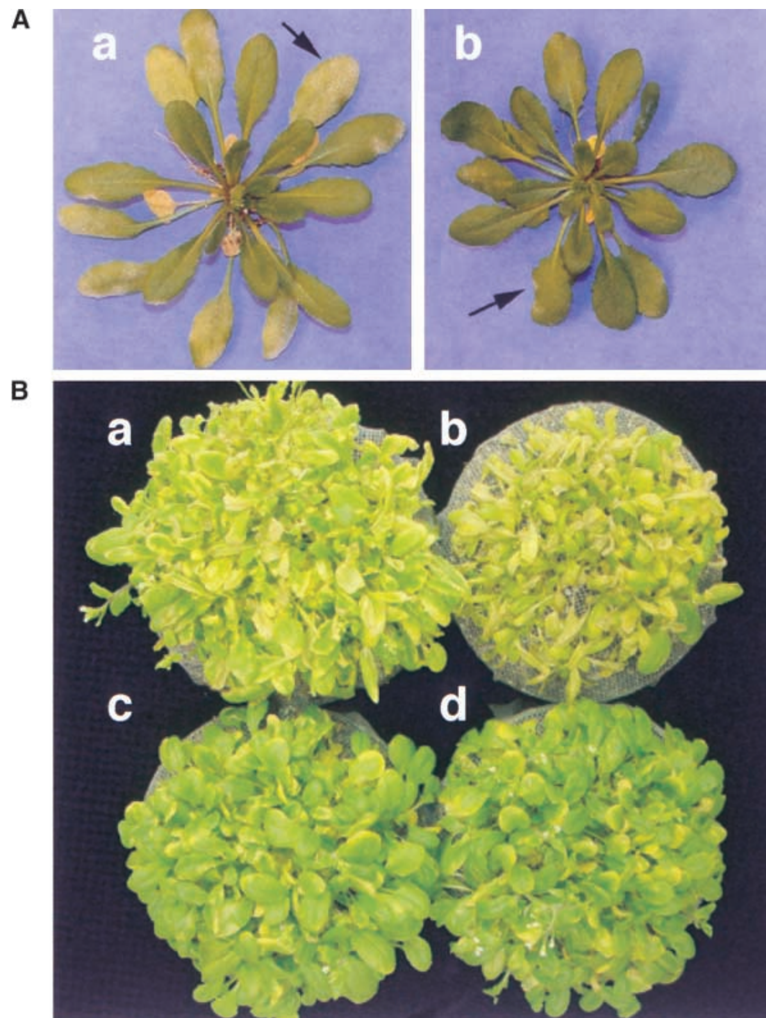

Figure 8. Expression of *Pti4* in Arabidopsis Confers Increased Resistance to Erysiphe and Tolerance to Pseudomonas.

(A) Increased resistance to the biotrophic fungal pathogen Erysiphe. Representative wild-type Arabidopsis Col-0 **(a)** and Pti4-2 **(b)** transgenic plants from the same inoculation box are shown at 14 days after inoculation with Erysiphe. Arrows highlight areas of powdery mildew infection. Three boxes each containing nine transgenic plants and three wild-type plants were assessed for each transgenic line. Experiments on each line were repeated once with similar results. Average Erysiphe disease scores for wild-type and Pti4-2 transgenic plants were +3 and +1, respectively, based on the scoring system of Reuber et al. (1998) as follows: 0, no growth; +1, isolated spots of infection; +2, ~20% coverage of leaves; +3, ~50% coverage of leaves; and +4, nearly 100% coverage of leaves.

(B) Increased tolerance to infection by Pseudomonas strain DC3000. Four-week-old plants were inoculated by dipping them into a suspension of virulent Pseudomonas strain DC3000 (10^6 colony-forming units/mL). Four days after inoculation, differences in the development of disease symptoms on the plants were observed. Arabidopsis wild-type Col-0 **(a)** and *NahG* plants **(b)** showed extensive and complete chlorosis, respectively, whereas Pti4-2 **(c)** and Pti4-5 **(d)** plants showed mild chlorosis.

dicate clearly which gene might be the functional homolog of Pti4. Moreover, the possible role of Arabidopsis ERFs in defense response pathways has yet to be reported.

The expression of *Pti5* or *Pti6* did not cause strong constitutive expression of ET/JA-regulated *PR* genes in Arabidopsis. However, both Pti5 and Pti6 transactivated GCC box–mediated transcription in transient assays conducted with Arabidopsis protoplasts. It is possible that the synthetic GCC-box *cis* element used in our transient assay is present in a different nucleotide context than those in the promoters of most ET-regulated *PR* genes in Arabidopsis. Upon examination of the promoter sequence of the *PDF1.2* gene, it was found that the nucleotides flanking the GCCGCC sequence in this promoter are not the same as those in the synthetic

GCC box (Y. Gu and G. Martin, unpublished data). It is well known that flanking nucleotides can contribute strongly to the binding affinity of transcription factors to their respective target sequences and can serve to discriminate among closely related factors. For example, several Arabidopsis ERFs (AtERFs) have been shown to have distinct DNA binding preferences (Fujimoto et al., 2000). Therefore, Pti5 and Pti6 may upregulate other, as yet unidentified, GCC box–containing defense-related genes.

Activation of SA-Regulated Genes by Pti4/5/6

The expression of Pti4/5/6 in Arabidopsis enhanced the expression level of the SA-regulated genes *PR1* and *PR2*. In addition, upon SA treatment, the transcript levels of *PR1* were induced to higher levels in Pti4/5/6 plants than in wild-type SA-treated plants. Although Pti4/5/6 are not known to bind *cis* elements of SA-regulated *PR* genes directly, they may act indirectly by interacting with protein factors that are involved in SA-regulated *PR* gene expression. In animals, cross-coupling of transcription factors is known to play an important role in mediating responses to various signaling events (Schule and Evans, 1991). Interestingly, AtEBP, an AtERF transcription factor, was identified because of its interaction with OBF4, the *ocs* element binding factor that belongs to the class of bZIP proteins that includes Arabidopsis TGA transcription factors (Büttner and Singh, 1997). Several TGA transcription factors have been shown to bind specifically to the SA-responsive elements in the promoters of *PR1* genes (Zhang et al., 1999; Després et al., 2000). Thus, it is possible that TGA transcription factors interact with Pti4/5/6 directly or indirectly and thereby enhance SA-regulated *PR* gene expression.

We found that with increasing concentrations of SA, the increase in *PR1* expression was accompanied by a decrease in *PDF1.2* transcripts. This effect was most noticeable in Pti4 plants, in which *PDF1.2* transcripts were most abundant. There are two possible explanations for this result. First, SA may act independently of Pti4 to suppress *PDF1.2* expression. There are previous reports of SA-mediated suppression of *PDF1.2* expression. For example, in Arabidopsis, the expression of the *PDF1.2* gene is higher in *NahG* plants in which a bacterial SA-degrading enzyme is overexpressed (Penninckx et al., 1998). We have demonstrated previously that SA also suppresses the ET induction of GCC-box *PR* gene expression in tomato (Gu et al., 2000). A second possibility, as discussed below (see model), is that SA might play a role in inhibiting Pti4 activity toward the *PDF1.2* promoter.

Table 1. Quantitative Analysis of Plant Responses to Erysiphe

	No. of Plants with Each Disease Score				
Arabidopsis Line	0.0	1.0	2.0	3.0	4.0
Pti4-2		27			
Wild type				9	
Pti4-5		9	14		
Wild type				9	
Pti5-2				27	
Wild type				9	
Pti6-6				27	
Wild type				9	

Wild-type Arabidopsis and Arabidopsis-*Pt4/5/6* plants grown for 4.5 weeks were infected with an inoculum of Erysiphe using a settling tower. Three inoculation boxes were used for each line to account for variability in the inoculum within and between boxes. Each box contained three wild-type and nine transgenic plants. Plants were scored at 14 days after inoculation using the scale described in the legend to Figure 8A. Experiments with each line were repeated once with similar results.

Pti4 Enhances Host Responses to Pathogens

Plants expressing Pti4 supported bacterial growth comparable to wild-type plants but showed significantly less chlorosis. Such decreased symptom development after bacterial infection is referred to as tolerance and has been observed previously in Arabidopsis disease signaling mutants and certain ecotypes (Bent et al., 1992; Buell and Somerville, 1995). Chlorosis caused by Pseudomonas is attributable primarily to the bacterial toxin coronatine, because strains unable to produce this toxin cause much decreased disease symptoms (Mittal and Davis, 1995). There is evidence that both the Arabidopsis EIN2 protein and the coronatine-insensitive COI1 protein are involved in the development of bacterial disease symptoms (Bent et al., 1992; Feys et al., 1994). The increased tolerance in Pti4 plants, therefore, may reflect interference in the activity of EIN2 or COI1 or another component of the coronatine perception response. Increases in bacterial resistance have been found in plants that overexpress other ERF genes, including *Pti5* and a close tobacco homolog of *Pti6* (He et al., 2001; Park et al., 2001). No studies have been reported in which multiple ERF-like genes are overexpressed together in a single plant, although this would appear to be a reasonable strategy to increase levels of resistance even more.

Plants expressing *Pti4* showed significant resistance to Erysiphe, a fungal biotroph that is virulent on Arabidopsis Col-0. Infection of Arabidopsis by Erysiphe typically results in the induction of the SA-dependent *PR* genes *PR1*, *PR2*, *PR5*, and *GST1* (Reuber et al., 1998). Interestingly, a few studies also show that genes belonging to the ET/JA pathway may contribute to resistance against Erysiphe (Dewdney et al., 2000; Ellis and Turner, 2001). For example, the Arabidopsis *cev1* mutant, which appears to have constitutively active ET and JA signaling pathways and which constitutively expresses *PDF1.2* and *Thi2.1*, exhibits increased re-

sistance to Erysiphe and other powdery mildews (Ellis and Turner, 2001). The observation that the *Pti4*-expressing Arabidopsis lines displayed enhanced resistance to Erysiphe suggests that there is an additive effect of defense genes belonging to both the SA and ET/JA signaling pathways.

A Model for the Role of Pti4 in *PR* Gene Expression

Our current and previous results suggest the following model for the role of Pti4/5/6 (and possibly the functionally analogous AtERFs) in defense response (Figure 9). First, pathogen attack and/or the associated increase in ET activates the expression of the *Pti4* gene (or the *AtERF* genes) (Fujimoto et al., 2000; Gu et al., 2000) (Figures 3 and 6). In the Arabidopsis *Pti4* line, this expression is constitutive, so the level of Pti4 protein already is increased and induction by pathogen or ET is not needed. When Pti4 (or AtERF) becomes available, Pto kinase (or an analogous Arabidopsis kinase) phosphorylates the protein, which might facilitate its localization, DNA binding, and/or interaction with other transcription factors. The putative kinase phosphorylating Pti4 likely is not dependent on ET or JA, because the Pti4-mediated activation of *PDF1.2* is not affected in the *ein2* and *jar1* mutants. The specificity of Pti4/5/6 promoter binding may be conferred by nucleotides that flank the GCC box. This context-specific DNA binding would account for the different subsets of *PR* genes that are regulated by Pti4/5/6 and might provide the plant with additional control of the defense responses it deploys.

The stimulation of *PR1* gene expression by SA is accompanied by a concomitant inhibition of expression of *PDF1.2*. As mentioned above, these changes might be independent of Pti4. However, based on the observed involvement of Pti4 in the expression of both of these genes, it is possible that Pti4 plays a role in the communication between the SA and ET signaling pathways. If that is the case, it seems unlikely that SA affects Pti4 activity directly; rather, it might play an indirect role (e.g., by attenuating Pti4 phosphorylation). What are the possible mechanisms of this attenuation? One possibility is that unphosphorylated Pti4 plays a role in SA-regulated PR gene expression, whereas phosphorylated Pti4 is more effective in the activation of JA/ET-regulated PR gene expression. This notion is supported by our observation that the phosphorylation of Pti4 enhances its binding to the GCC box in vitro. SA then might inhibit a protein kinase that phosphorylates Pti4, which could act as a "switch" to divert Pti4 toward the activation of SA-regulated PR gene expression (e.g., by interaction with TGA factors, as discussed above).

In this model, lower levels of SA would not inhibit this kinase; instead, they would allow Pti4 to exist in both the phosphorylated and unphosphorylated forms, leading to the activation of both SA- and JA/ET-regulated gene expression, as seen in the *Pti4*-expressing line. A possible candidate for a protein kinase fulfilling this role is the recently

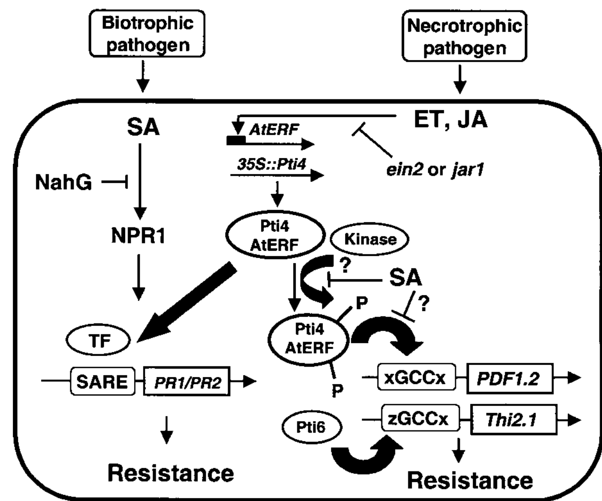

Figure 9. Model for the Proposed Role of Pti4 and AtERFs in Mediating Cross-Talk between the SA and ET/JA Pathways.

NahG refers to the salicylate hydroxylase protein that degrades SA. NPR1 refers to the "non-expresser of PR" protein. *ein2* and *jar1* refer to mutations in Arabidopsis that affect ET perception (*ein2*) and JA biosynthesis (*jar1*). Also shown is a hypothetical transcription factor (TF) that might interact with the Pti4/5/6 or AtERF proteins and play a role in binding an SA-responsive element (SARE). The SA-inhibited protein kinase refers to either Pto (in tomato) or an analogous kinase in Arabidopsis. The possibility that Pti4/AtERF and Pti6 differentially recognize the GCC box when it is flanked by different nucleotides is shown as xGCCx and zGCCx. The possible roles of other signaling components shown are discussed in the text.

described MPK4. The loss of MPK4 activity in an *mpk4* mutant leads to the constitutive activation of SA-regulated PR genes and the inability to induce *PDF1.2* and *Thi2.1* (Petersen et al., 2000). We are testing this model at present by determining the phosphorylation state of Pti4 in plant cells with and without the application of SA and by examining whether the activity of these transcription factors is affected in the *mpk4* mutant.

In conclusion, the tomato ERFs Pti4, Pti5, and Pti6 each plays a distinct role in the activation of defense responses in tomato and in Arabidopsis (Zhou et al., 1997; Thara et al., 1999; Gu et al., 2000). This study demonstrates the expression of ERFs in a heterologous background and indicates that ERFs can play a role in the expression of SA-regulated genes. Based on these results and our previous report (Gu et al., 2000), we propose that the phosphorylation of Pti4/5/6 proteins may facilitate their nuclear localization and/or transactivation properties for GCC-box promoters. We also hypothesize that Pti4 may play a role in mediating the communication between the SA and ET/JA signaling pathways and that the phosphorylation of Pti4 might act as a switch for this communication. Finally, the demonstration that Pti4 expression in Arabidopsis confers enhanced resistance to

Erysiphe and tolerance to Pseudomonas suggests that ERFs from diverse species might be useful for engineering increased disease resistance in plants.

METHODS

Plant Materials, Growth Conditions, and Treatments with Salicylic Acid, Jasmonic Acid, and Ethylene

Arabidopsis thaliana (ecotype Columbia) plants were grown at 22°C with a daylength of 16 hr. For salicylic acid treatments, 4-week-old plants were sprayed with different concentrations of salicylic acid in water, as indicated in Results. Water was used as a control. Jasmonic acid treatment was performed by spraying the plants with 50 μM jasmonic acid dissolved in 0.01% ethanol (control was 0.01% ethanol alone). The control and treated plants were placed in a sealed plexiglass chamber for 24 hr before leaf tissue was harvested. Treatment of plants with ethylene was performed in a gas-tight plexiglass chamber by injecting a volume of ethylene gas to give a final concentration of 20 μL/L. Control plants were handled in an identical manner but without the injection of ethylene. Leaf tissues were harvested 24 hr after treatment.

Plasmid Constructions

All of the plasmid constructs generated in this study were made using standard recombinant DNA techniques and verified by DNA sequencing.

Constructs Used in the Nuclear Localization Assay

The coding regions of *Pti4*, *Pti5*, and *Pti6* were amplified by polymerase chain reaction (PCR) to introduce BglII at the 5′ end and BamHI at the 3′ end. The resulting fragments were digested with BglII and BamHI and subcloned into the expression vector pRTL2-GUS-NIa (Restrepo et al., 1990) by replacing the NIa sequence to yield the in-frame fusion plasmids pRTL2-GUS-Pti4, pRTL2-GUS-Pti5, and pRTL2-GUS-Pti6. To generate green fluorescent protein (GFP) and Pti4 fusions, the coding region of GFP was amplified by PCR to introduce a NcoI site at the 5′ end and a BglII site at the 3′ end. The PCR products were digested with NcoI and BglII and subcloned into pRTL2-GUS-Pti4 plasmid to replace the β-glucuronidase (GUS) coding region. The resulting plasmid, pRTL2-GFP-Pti4, was engineered further to delete the two clusters of basic residues in the nuclear localization sequence domain of Pti4 (Figure 1) using the Quick Exchange Kit (Stratagene) to generate the pRTL2-GFP-Pti4M construct.

Constructs Used in the Protoplast Transient Assay

The reporter construct (*GCC-LUC*) contains two GCC-box repeats that were placed upstream of the minimal −42 to +8 TATA box from the 35S promoter of *Cauliflower mosaic virus* (CaMV) and then joined as a transcriptional fusion to the coding region of the firefly luciferase gene (*LUC*). The construct (*mGCC-LUC*) with the replacement of the GCC box by a mutated GCC box (mGCC box) was used as a control. To generate the effector constructs, the coding regions of *Pti4*, *Pti5*, and *Pti6* were amplified by PCR, tagged with the double hemagglutinin (HA) epitope, and inserted into a plant expression vector containing the CaMV 35S promoter and the nopaline synthase terminator (Kovtun et al., 2000). For the reporter construct with GAL4 *cis* elements (GAL4-GUS), the sequence containing nine tandem repeats of the 17-mer yeast GAL4 DNA binding site (Ma and Ptashne, 1988) was placed upstream of the CaMV minimal 35S promoter and then fused to the coding region of the *GUS* gene. The corresponding effector constructs contain the GAL4 DNA binding domain (amino acids 1 to 94) fused in frame to *Pti4-HA*, *Pti5-HA*, or *Pti6-HA*, and expression of these fusion genes was driven by the 35S promoter.

Constructs Used in the Generation of Transgenic Arabidopsis

The coding regions of *Pti4*, *Pti5*, and *Pti6* were amplified by PCR to introduce a BamHI site at both the 5′ and 3′ ends. The fragments were digested with BamHI and ligated into BamHI-digested pBTEX binary vector (Frederick et al., 1998) to yield plasmid constructs pBTEX35S-Pti4, pBTEX35S-Pti5, and pBTEX35S-Pti6.

Subcellular Localization of Pti4/5/6

Particle bombardment was performed using a Bio-Rad Biolistic PDS1000/He system to transiently express the *GUS* constructs in tobacco W-38 suspension cells. Plasmid DNA (0.66 μg) was coated onto tungsten particles as described by Varagona et al. (1992). DNA-coated particles were bombarded at 1100 p.s.i. into 200 mg of W-38 suspension cells laid on filter paper at a target distance of 9 cm. After bombardment, the cells were incubated in Murashige and Skoog (1962) medium containing 0.5 mg/L 2,4-D, 0.5 mg/L kinetin, and 0.3 mg/L indoleacetic acid for 24 hr at 25°C in the light. GUS activity was determined by histochemical staining. Cells were viewed with a light microscope, and micrographs taken 2 to 4 hr after the addition of substrate.

Subcellular localization of GFP fusions was performed by transiently expressing the GFP constructs in Arabidopsis protoplasts as described below and monitoring the localization of GFP with a confocal laser scanning microscope (Bio-Rad MRC-600). Excitation light at 488 and 514 nm was attenuated to 10% transmittance. Detectors were set at 610 nm for chlorophyll and 530 nm for GFP fluorescence. Serial confocal sections (2 μm thick) were collected. Images were exported as TIFF files and processed for printing using Adobe Photoshop (Mountain View, CA).

Arabidopsis Protoplast Transient Expression and Reporter Gene Activity Assay

Isolation and transfection of Arabidopsis protoplasts were performed according to a modified polyethylene glycol method as described by Abel and Theologis (1994). Typically, in a cotransfection assay, 5×10^5 protoplasts in 200 μL were transfected with 16 μg of effector plasmids, 8 μg of reporter plasmids, and 2 μg of internal control plasmids. The transfected protoplasts were incubated at 22°C for 16 to 20 hr, harvested by centrifugation at 80*g* for 3 min, and then quickly frozen and stored at −80°C. For reporter gene activity assays, either the protoplasts were lysed in passive lysis buffer (Promega) and luciferase activity was measured using a dual-luciferase assay kit according to the manufacturer's instructions (Promega), or proteins

were extracted in extraction buffer (100 mM potassium phosphate and 1 mM DTT, pH 7.5) and GUS and LUC activity were determined as described by Sprenger-Haussels and Weisshaar (2000).

Arabidopsis Transformation

The plasmids pBTEX35S::Pti4, pBTEX35S::Pti5, and pBTEX35S:: Pti6 were introduced into *Agrobacterium tumefaciens* strain GV3101 and used to transform Arabidopsis (ecotype Columbia) using an in planta transformation method (Bechtold et al., 1993). Putative transformants (T1 plants) were selected by plating seed on Murashige and Skoog (1962) medium containing 50 mg/L kanamycin. After selection for 2 weeks, kanamycin-resistant seedlings were transferred to soil. Homozygous lines for the transgenes were identified in the T3 generation by segregation for kanamycin resistance and confirmed by DNA gel blot analysis.

SDS-PAGE and Immunoblotting Assay

Transfected protoplasts were harvested as described above, and total proteins were extracted by adding 200 μL of 1 × SDS sample buffer. Protein electrophoresis and transfer to polyvinylidene difluoride membranes were described previously (Frederick et al., 1998). For the immunoblotting assay, the blots were incubated overnight with anti-HA antibody at a concentration of 0.1 μg/mL, and proteins were visualized using an enhanced chemiluminescence kit (Amersham).

RNA Extraction and RNA Gel Blot Analysis

Total RNA was isolated according to the method described previously (Gu et al., 2000), separated by electrophoresis on formaldehyde-agarose gels, and transferred onto nylon membranes (Hybond N^+; Amersham). ^{32}P labeling of cDNA probes was performed using a random hexamer labeling kit (Ambion, Austin, TX). The procedure for RNA gel blot hybridization has been described previously (Gu et al., 2000). Radioactivity was detected by either autoradiography or phosphorimaging (for the qualitative assays).

Bacterial and Fungal Infection

For the infection of plants with the fungal pathogen *Erysiphe orontii* (recently renamed *Golovinomyces orontii*; Braun, 1999), Arabidopsis plants were grown in Metro-Mix 200 (Scotts-Sierra Horticultural Products, Marysville, OH) under a 12-hr light/dark cycle in a greenhouse with supplemental fluorescent lighting (19 ± 2°C). Four- to 4.5-week-old plants were infected with a moderately heavy inoculum (conidia from two infected leaves) of Erysiphe using a settling tower and scored for disease symptoms at 14 days after infection as described by Reuber et al. (1998). Transgenic plants were compared with wild-type Columbia plants in the same box. Three boxes, each containing nine transgenic plants and three wild-type plants, were assessed per transgenic line. The experiment was repeated with similar results.

For bacterial inoculation, plants were grown in a light room under a 16-hr photoperiod at 22°C. Four-week-old plants were dipped for 30 sec in a suspension of virulent *Pseudomonas syringae* pv *tomato* strain DC3000 (10^6 colony-forming units/mL). Leaves were photographed 4 days after inoculation.

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