Pti4 Is Induced by Ethylene and Salicylic Acid, and Its Product Is Phosphorylated by the Pto Kinase

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The tomato *Pti4* gene encodes a transcription factor that was identified on the basis of its specific interaction with the product of the *Pto* disease resistance gene in a yeast two-hybrid system. We show here that the Pti4 protein specifically binds the GCC-box *cis* element, which is present in the promoter region of many pathogenesis-related (*PR*) genes. Expression of the *Pti4* gene in tomato leaves was rapidly induced by ethylene and by infection with *Pseudomonas syringae* pv *tomato,* and this induction preceded expression of GCC-box-containing *PR* genes. Although salicylic acid also induced *Pti4* gene expression, it did not induce GCC-box *PR* genes. Rather, salicylic acid antagonized ethylene-mediated expression of GCC-box *PR* genes. We demonstrate that the Pti4 protein is specifically phosphorylated by the Pto kinase and that this phosphorylation enhances binding of Pti4 to the GCC box. In addition, induced overexpression of Pto and Pti4 in tomato leaves resulted in a concomitant increase in GCC-box *PR* genes. Our results support a model in which phosphorylation of the Pti4 protein by the Pto kinase enhances the ability of Pti4 to activate expression of GCC-box *PR* genes in tomato.

INTRODUCTION

A well-characterized plant defense response associated with pathogen attack is the expression of pathogenesis-related (*PR*) genes. *PR* genes are activated in both resistant and susceptible plants in response to pathogen attack. However, they often are expressed more rapidly and to a greater extent in incompatible interactions in which a resistant plant is challenged with an avirulent pathogen (Voisey and Slusarenko, 1989; van Kan et al., 1992; Jia and Martin, 1999). This implies that enhanced *PR* gene expression is mediated by a recognition event involving a disease resistance (*R*) gene and its cognate avirulence *(avr)* gene. The roles of *PR* genes in plant defense responses against pathogens have been widely investigated (Cutt and Klessig, 1992). The best-characterized plant defense genes include those that encode glucanases and chitinases. These enzymes appear to be antimicrobial, based on their ability to degrade cell wall components of pathogens (Mauch et al., 1988; Sela-Buurlage et al., 1993) and have been shown to enhance disease resistance when overexpressed in plants (Broglie et al., 1991; Zhu et al., 1994). Despite the established biological function in plant defense responses for some *PR* genes, the molecular mechanisms responsible for their expression in *R–avr* recognition remain largely unclear.

The expression of many defense-related genes is regulated by signaling molecules such as salicylic acid (SA) and ethylene. Increases in SA and its conjugates have been associated with the activation of defense genes in many plant species (reviewed in Dempsey et al., 1999). Compelling evidence for a key role of SA in defense gene regulation comes from analysis of tobacco and Arabidopsis NahG transformants in which SA is converted to an inactive form. The NahG plants fail to induce several *PR* genes such as *PR-1, PR-2,* and *PR-5* and show increased susceptibility to pathogens (Delaney et al., 1994). Additional evidence for a role of SA in defense comes from certain Arabidopsis mutants in which the SA signaling pathway is blocked (Cao et al., 1994; Delaney et al., 1995; Shah et al., 1997). These plants are unable to accumulate several *PR* gene transcripts when infected by pathogens and show increased susceptibility to disease. Most of the *PR* genes induced by SA encode acidic, extracellular forms of PR proteins (Friedrich et al., 1995). Another large class of *PR* genes encodes basic proteins, and many of these genes do not appear to be regulated by SA (Vidal et al., 1997).

Many PR genes that are induced by plant–pathogen interactions also are upregulated by exposure to ethylene (Deikman, 1997). Ethylene-regulated *PR* genes often encode basic proteins that are localized primarily in vacuoles (Eyal et al., 1993). Recent evidence indicates that expression of some of these *PR* genes is independent of SA but requires an ethylene-dependent signaling pathway. For example, the *PDF1.2* gene, which encodes an antifungal peptide, is

activated when Arabidopsis is infected by *Alternaria brassicicola* or exposed to ethylene; however, SA does not activate *PDF1.2* (Penninckx et al., 1996). Activation of *PDF1.2* was abolished in ethylene-insensitive mutants of Arabidopsis, whereas its induction by pathogens or ethylene was not affected in either NahG plants or a nonexpresser of *PR* genes (*npr1*) mutant (Penninckx et al., 1998).

An important approach to understanding *PR* gene regulation is to identify defense-responsive promoter regions and isolate their cognate DNA binding proteins. Several defense-related transcription factors have been identified by using this method, including the WRKY transcription factors (Rushton et al., 1996), a basic leucine zipper transcription factor G/HBF-1 (Dröge-Laser et al., 1998), and the ethylene-responsive element binding proteins (EREBPs; Ohme-Takagi and Shinshi, 1995). Isolation of these factors has provided new insights into *PR* gene regulation but also has raised the additional question of how these transcription factors themselves are regulated. In particular, because many *PR* genes are expressed rapidly during gene-for-gene incompatible interactions, it is important to understand the role of *R* genes in the regulation of defense-related transcription factors.

In tomato, the *R* gene *Pto* confers resistance to *Pseudomonas syringae* pv *tomato* strains expressing the *avrPto* gene. The molecular cloning of the *Pto* gene and the corresponding avirulence gene, *avrPto*, permits extensive investigation of the mechanism of *R* gene–mediated defense responses. *Pto* encodes an active serine/threonine protein kinase (Martin et al., 1993; Loh and Martin, 1995), and *avrPto* encodes a small hydrophilic protein that probably is delivered into the plant cell by way of a bacterial type III secretion system (Ronald et al., 1992; Alfano and Collmer, 1996). We and others have demonstrated that the Pto kinase interacts with AvrPto protein, and mutations in Pto or AvrPto that disrupt this interaction also abolish disease resistance (Scofield et al., 1996; Tang et al., 1996; Frederick et al., 1998). Therefore, a direct interaction between Pto and AvrPto determines gene-for-gene specificity in this plant–pathogen interaction. This recognition event appears to initiate multiple signal transduction pathways that lead to the hypersensitive response, oxidative burst, and enhanced expression of *PR* genes (Gu and Martin, 1998).

Several proteins with putative roles in *Pto*-mediated signaling pathways have been identified from a yeast two-hybrid screen by using the Pto kinase as bait (Zhou et al., 1995, 1997). Among them, *Pti1* (for Pto-interacting 1), which encodes a serine/threonine kinase, appears to be a direct downstream component because Pto phosphorylates Pti1 but not vice versa. Overexpression of Pti1 in tobacco indicates that the protein is involved in the development of the hypersensitive response (Zhou et al., 1995). Three other genes encoding Pto-interacting proteins, *Pti4*, *Pti5*, and *Pti6*, encode related putative transcription factors; each has a DNA binding domain, a nuclear localization sequence, and a possible transactivation domain rich in acidic amino acids

(Zhou et al., 1997). The proteins encoded by *Pti4*, *Pti5*, and *Pti6* share extensive similarity with the amino acid sequences of EREBPs (Ohme-Takagi and Shinshi, 1995). Along with the EREBPs, Pti5 and Pti6 have been shown to bind the GCC-box *cis* element present in the promoter region of many ethylene-regulated *PR* genes (Ohme-Takagi and Shinshi, 1995; Zhou et al., 1995). Thus, the discovery of *Pti4*, *Pti5*, and *Pti6* represents a definitive link between an *R* gene and the specific induction of genes involved in plant defense. In this article, we have further characterized the regulation of *Pti4* gene expression and the relationship between the Pto kinase and the Pti4 protein.

RESULTS

Pti4 Protein Specifically Binds the GCC-Box *cis* Element

To examine whether Pti4 binds the same *cis* element as Pti5 and Pti6 (Zhou et al., 1997), we overexpressed Pti4 as a tagged protein with a region of six histidine residues in *Escherichia coli* and purified to near homogeneity by using a nickel–agarose affinity column (see Methods). The purified His-Pti4 protein was used in a mobility shift gel assay. As shown in Figure 1, in which glutathione *S*-transferase (GST)–Pti5 was used as a positive control, the His-Pti4 fusion protein was able to bind the GCC box. To test the specificity of this binding, we performed a competition experiment by adding the unlabeled GCC-box DNA to the mobility shift assay. The addition of unlabeled GCC-box to the binding assay decreased the binding of His-Pti4 to the labeled GCC-box, and 100-fold excess of unlabeled GCC-box DNA resulted in the complete loss of binding of the labeled GCC-box to the His-Pti4 protein. Furthermore, as was seen with GST-Pti5, the His-Pti4 protein did not bind a mutated GCC (mGCC) box containing two G-to-T substitutions in the GCC-GCC core sequence (Ohme-Takagi and Shinshi, 1995; Zhou et al., 1997). From these results, we conclude that Pti4 specifically binds the GCC-box *cis* element.

Ethylene Rapidly Increases Abundance of *Pti4* but Not of *Pti5* or *Pti6* Transcripts

Pti4, Pti5, and Pti6 are similar to the tobacco EREBPs in both amino acid sequence and DNA binding properties (Ohme-Takagi and Shinshi, 1995; Zhou et al., 1997). We were interested, therefore, in whether ethylene is involved in the transcriptional regulation of the *Pti4*, *Pti5*, and *Pti6* genes the way it is for some of the genes encoding EREBPs (Ohme-Takagi and Shinshi, 1995). For these experiments, we used two lines of the tomato cultivar Rio Grande that are isogenic except for the *Pto* locus: Rio Grande–PtoR (RG-PtoR) and Rio Grande–PtoS (RG-PtoS). RNA gel blot experiments (Figure 2A) revealed that in plants from both lines,
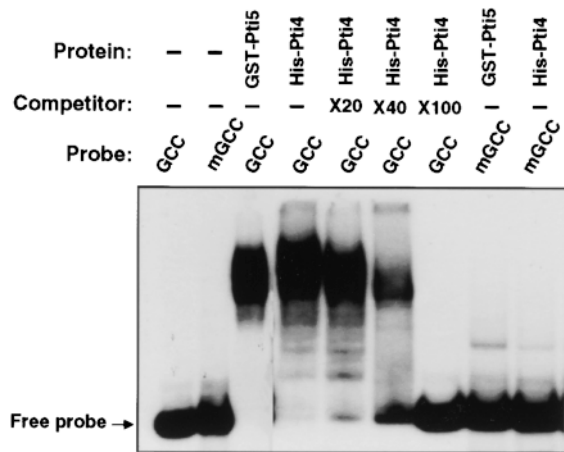
Figure 1. Pti4 Specifically Binds the GCC-Box Sequence.

A mobility shift assay was performed as described previously (Zhou et al., 1995), using 50 ng of purified His-Pti4 or GST-Pti5 fusion protein mixed with 5 fmol of ^{32}P-labeled wild-type GCC-box or mGCC-box oligonucleotides (see Methods). The competitor consisted of the unlabeled wild-type GCC oligonucleotide. The notations $\times20$, $\times40$, and $\times100$ indicate that the amount of unlabeled wild-type GCC-box oligonucleotide added to these reactions was 20-, 40-, or 100-fold that of the labeled GCC-box oligonucleotide. The ($-$) signs indicate that no competitor, or no protein, was added to these reactions. The position of the unbound (Free) probe is shown.

Pti4 transcript abundance increased as early as 15 min after exposure to 10 ppm ethylene gas and continued to increase over the next 2 to 4 hr before gradually decreasing. Thus, *Pto* had no effect on the expression of the *Pti4* gene in response to ethylene. In contrast to the rapid induction of *Pti4* by ethylene, *Pti5* and *Pti6* were not responsive to ethylene treatment. The transcript levels of *Pti5* and *Pti6* remained low throughout the 8-hr ethylene treatment.

A key role for the GCC box in mediating ethylene-responsive gene expression has been demonstrated in tobacco (Eyal et al., 1993; Ohme-Takagi and Shinshi, 1995). Recently, we identified a GCC box in the promoter regions of several tomato *PR* genes, including glucanase B *(GluB)*, chitinase B *(ChiB)*, and osmotin *(Osm)*, and showed that expression of these genes was enhanced in an incompatible interaction involving *Pto* and *avrPto* (Jia and Martin, 1999). To determine whether these GCC-box-containing *PR* genes also were induced by ethylene, we hybridized the RNA gel blots with *GluB* and *Osm* probes (Figure 2A). The accumulation of *GluB* and *Osm* transcripts first was detected 2 hr after ethylene treatment and increased to a maximum by 4 to 6 hr. In a control experiment in which plants were similarly manipulated but not exposed to ethylene, neither *Pti4* nor *GluB* transcripts were induced (data not shown). The first appearance of *GluB* and *Osm* transcripts thus was ~1.5 hr after that of *Pti4* transcripts. This result is consistent with

Pti4 playing a role in the expression of these tomato GCC-box-containing *PR* genes. No difference in ethylene induction of *GluB* and *Osm* was observed between the RG-PtoR and RG-PtoS plants; therefore, the *Pto* gene does not appear to be involved in ethylene-regulated expression of these *PR* genes.

Effect of Cycloheximide on Ethylene Regulation of the *Pti4* and *GluB* Genes

The rapid induction of *Pti4* transcripts by ethylene suggests that de novo protein synthesis is not required for this
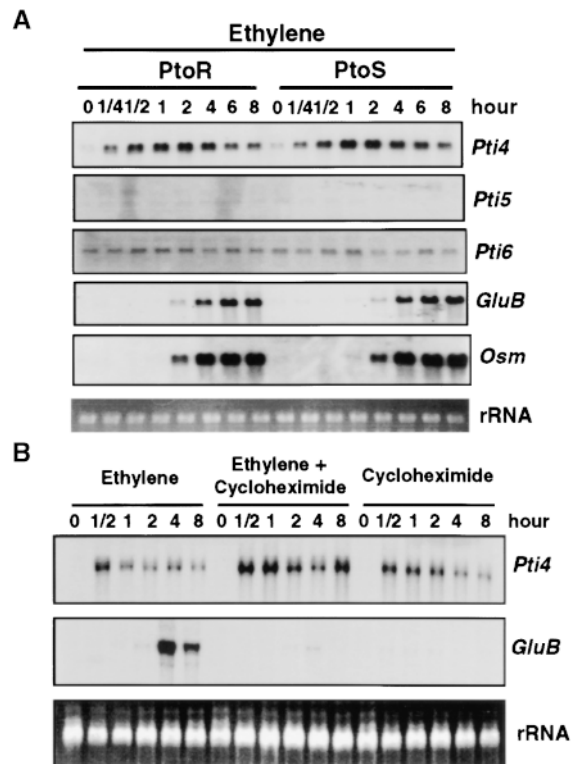

Figure 2. Effect of Ethylene and Cycloheximide on Expression of *Pti4, Pti5, Pti6,* and GCC-Box-Containing *PR* Genes.

Fully expanded leaves from 4-week-old tomato plants were used for analysis of gene expression. Equal loading of lanes in all gels was verified by visualizing the rRNA in the RNA gels stained with ethidium bromide.

(A) RG-PtoR or RG-PtoS tomato plants were treated with 10 μL/L ethylene gas for the times indicated. Total RNAs were isolated from leaves and analyzed as duplicate RNA gel blots by hybridization with radiolabeled *Pti4, Pti5, Pti6, GluB,* or *Osm* probes.

(B) RG-PtoR plants were treated with ethylene (10 μL/L), cycloheximide (5 μg/mL), or a combination of both for the times indicated. Total RNAs were isolated from leaves and analyzed as duplicate RNA gel blots by hybridization with radiolabeled *Pti4* or *GluB* probes.

process. This is typical for the induction of many immediate-early transcription factors, including those that appear to be involved in regulation of plant defense genes (Yang and Klessig, 1996). To examine whether de novo protein synthesis is involved in ethylene-induced Pti4 function, we blocked protein synthesis by pretreatment of tomato plants with 5 μg/mL cycloheximide for 30 min before applying ethylene. Cycloheximide commonly is observed to induce transcription factor genes (Suzuki et al., 1998; Yang and Klessig, 1996; Horvath et al., 1998), and as shown in Figure 2C, it increased *Pti4* expression with timing and transcript levels similar to those induced by ethylene. Treatment with a combination of ethylene and cycloheximide further increased the abundance of *Pti4* transcripts, possibly as a result of the additive effect of ethylene and cycloheximide. These findings suggest that transcriptional activation of *Pti4* by ethylene occurs by way of preformed transactivator molecules in the plant cell. Alternatively, cycloheximide may inhibit the de novo protein synthesis of an unstable protein that negatively regulates the *Pti4* transcripts, or cycloheximide might simply stabilize *Pti4* transcripts (Li et al., 1994). Despite the increase in *Pti4* transcripts in these experiments, *GluB* expression was not induced. In fact, cycloheximide treatment blocked the induction of *GluB* by ethylene, as observed after the combination treatment of cycloheximide and ethylene (Figure 2C). Therefore, induction of *GluB* by ethylene apparently requires de novo synthesis of the protein or proteins that activate its expression.

Effect of SA on Expression of *Pti4*, *Pti5*, *Pti6*, and GCC-Box *PR* Genes

SA induces the expression of a wide array of plant genes that can be classified on the basis of how quickly they are induced (Dempsey et al., 1999). Several transcription factor genes, including tobacco *EREBP-1*, are immediate-early SA-responsive genes, being activated within 10 min of SA treatment (Horvath et al., 1998). We examined possible effects of SA on *Pti4*, *Pti5*, and *Pti6* transcript abundance and found, as shown in Figure 3A, that *Pti4* transcripts accumulated as early as 30 min after treatment, whereas *Pti5* and *Pti6* transcript levels were not responsive to SA. In a control experiment in which plants were treated with either water or 3-hydroxybenzoic acid (an inactive SA analog), induction of *Pti4* transcripts was not detected (data not shown). To test whether SA-induced accumulation of *Pti4* correlated with GCC-box *PR* gene accumulation, we hybridized an RNA gel blot with the *GluB* and *Osm* probes (Figure 3B). An increase in transcript abundance of these genes was not detected until 8 hr after treatment, and even then, the increase was negligible. To ensure that the SA-signaling pathway was activated by our SA treatment, we used a known SA-inducible gene, *Le-eli3* (R.L. Thilmony and G.B. Martin, unpublished data), as a marker. Transcripts of *Le-eli3* were detected 2 hr after treatment.

Several recent reports indicate that both SA-dependent and SA-independent pathways are responsible for activation of distinct sets of defense-related genes (Penninckx et al., 1996; Vidal et al., 1997; Thomma et al., 1998). In addition, certain components might be shared by both pathways. SA also has been reported to sensitize ethylene-inducible *PR* gene expression, and ethylene may enhance SA-induced expression of some *PR* genes (Lawton et al., 1994; Xu et al., 1994). To examine a possible interaction between SA and ethylene in the activation of *Pti4*, *Pti5*, and *Pti6* and in the expression of *PR* genes containing GCC boxes, we treated tomato plants simultaneously with SA and ethylene. As shown in Figure 3B, this simultaneous treatment increased *Pti4* transcripts by approximately twice that seen with either treatment alone and might reflect an additive effect. Ethylene did not activate *Le-eli3* and did not affect *Le-eli3* expression induced by SA in comparison with that induced by SA treatment alone. Interestingly, however, we found that SA substantially suppressed ethylene induction of *GluB* and *Osm* expression (Figure 3B). Together with our results shown in Figure 3A, we conclude that SA does not cause early induction of these *PR* genes and, in fact, has a negative effect on their activation by ethylene.

Induction of *Pti4*, *Pti5*, and *Pti6* by the Wound Response Pathway

In tomato, ethylene is required for activation of proteinase inhibitor genes by the wound response pathway, although ethylene itself is not sufficient to induce wound-responsive gene expression (O'Donnell et al., 1996). Wounding of tomato leaves results in increased ethylene production (O'Donnell et al., 1996), and some *PR* genes are induced by mechanical wounding (Pastuglia et al., 1997). Furthermore, a GCC-box *cis* element is present in the promoter of at least some members of the 1-aminocyclopropane-1-carboxylate oxidase gene family, which is involved in ethylene biosynthesis (Jia and Martin, 1999). These observations suggest that *Pti4*, *Pti5*, and *Pti6*, singly or in combination, might be involved in the regulation of ethylene production in the plant and in turn play a role in wound-responsive gene expression. As shown in Figure 4A, wounding tomato leaves rapidly induced *Pti4* transcripts but not *Pti5* or *Pti6* transcripts. *Pti4* transcript abundance was very low, however, and quickly decreased to near background values just 6 hr after wounding. This corresponded to very low induction of *GluB* transcripts beginning 6 hr after wounding. By contrast, transcripts of proteinase inhibitor 2 gene (*Pin2*), a molecular marker for the wound response, increased markedly 4 hr after wounding and continued to accumulate to high amounts 8 hr after wounding. To further examine the possible involvement of Pti4 in the wound response pathway, we treated tomato plants with systemin, a well-characterized, potent signaling molecule involved in the wound response through the octodecanoid pathway (Schaller and Ryan,
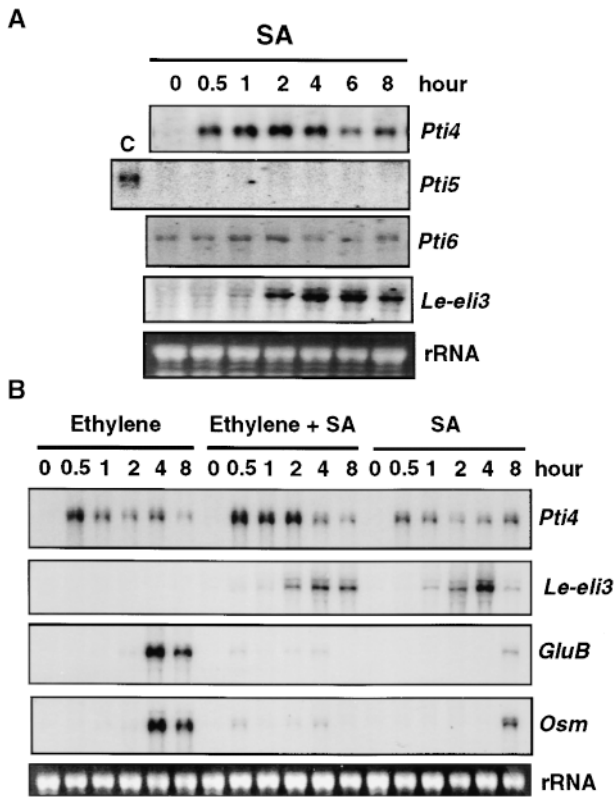
A

B

Figure 3. Effect of SA and Ethylene on Expression of *Pti4, Pti5, Pti6, Le-eli3*, and the GCC-Box-Containing *PR* Genes *GluB* and *Osm*.

Fully expanded leaves from 4-week-old tomato plants were used for analysis of gene expression. Equal loading was verified by visualizing the rRNA in the RNA gels stained with ethidium bromide.

(A) RG-PtoR tomato plants were treated with 1 mM SA for the times indicated. Total RNAs were isolated from leaves and analyzed as duplicate RNA gel blots by hybridization with radiolabeled *Pti4, Pti5, Pti6*, or *Le-eli3* probes. Note that lane C for *Pti5* contains total RNA isolated from leaves of 8-week-old tomato plants in which the *Pti5* transcript is detectable.

(B) RG-PtoR tomato plants were treated with ethylene (10 µL/L), SA (1 mM), or a combination of both for the times indicated. Total RNAs were isolated from leaves and analyzed as duplicate RNA gel blots by hybridization with radiolabeled *Pti4, Le-eli3, GluB*, or *Osm* probes.

Developmental and Organ-Specific Expression of *Pti4, Pti5,* and *Pti6*

Expression of *PR* genes varies in different plant tissues and during different developmental stages (Cutt and Klessig, 1992). To investigate the possible roles of Pti4, Pti5, and Pti6 in regulation of *PR* gene expression in different tissues and organs at different developmental stages, we isolated tomato RNAs and analyzed them by RNA gel blot hybridization. As shown in Figure 5, *Pti4* transcript abundance was less in young leaves than in old leaves. Low amounts of *Pti4*
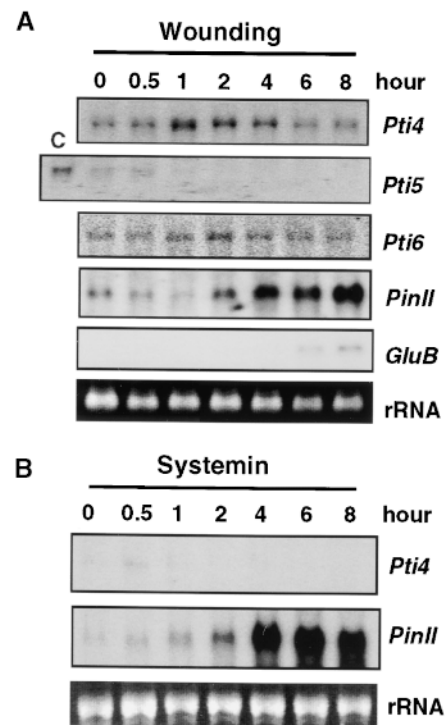
A

B

Figure 4. Effect of Wounding or Systemin on Expression of *Pti4, Pti5, Pti6, Pin2,* and *GluB*.

Fully expanded leaves from 4-week-old tomato plants were used for analysis of gene expression. Equal loading was verified by visualizing the rRNA in the RNA gels stained with ethidium bromide. Pin II, Pin2.

(A) Tomato RG-PtoR plants were wounded by rubbing leaves with carborundum, after which total RNAs were isolated from leaves at the times indicated. Duplicate RNA gel blots were hybridized with radiolabeled *Pti4, Pti5, Pti6, Pin2*, or *GluB* probes. Note that lane C for *Pti5* contains total RNA isolated from leaves of 8-week-old tomato plants in which *Pti5* transcript is detectable.

(B) Tomato RG-PtoR plants were treated with 1 nM systemin, after which total RNAs were isolated from leaves at the times indicated. Duplicated RNA gel blots were hybridized with radiolabeled *Pti4* or *Pin2* probes.

1995). As shown in Figure 4B, systemin strongly increased *Pin2* transcripts but it did not increase *Pti4* transcripts. In related experiments, jasmonic acid also was shown not to induce *Pti4* accumulation (Thara et al., 1999). Thus, the induction of *Pti4* by wounding might not result from activation of the octadecanoid pathway. Rather, similar to tobacco *EREBP* genes, induction of *Pti4* by wounding might result from a separate mechanism of wound-responsive transcriptional activation (Suzuki et al., 1998).

transcripts also were found in stems and green fruits, whereas high amounts were detected in reproductive organs, roots, red fruits, and undifferentiated callus cells. In contrast, *Pti5* transcripts were present only in low or undetectable amounts in all tissues, except for older leaves. The amounts of *Pti6* transcripts were similar to those of *Pti4* in all cases except red fruits, in which they were less abundant. Because *Pti4* is induced by ethylene, the high amount of *Pti4* transcripts in red fruits may have resulted from the high amounts of this hormone known to occur in ripening fruits. Consistent with this observation, *Pti4* transcripts also were increased in senescent leaves and reproductive organs in which ethylene biosynthesis is increased (Abeles et al., 1992). However, because *Pti5* and *Pti6* are not induced by ethylene (Figure 2), the expression of these genes in some of the same tissues as *Pti4* suggests that other developmental cues are involved in regulation of *Pti5* and *Pti6* expression.

To examine the correlation of *Pti4, Pti5,* and *Pti6* transcript levels with *PR* gene expression during development, we probed the same RNA gel blots with *GluB* and *GluA*. Expression of the GCC-box-containing gene *GluB* correlated well with *Pti4* expression, whereas the *GluA* transcripts did not. The promoter region of *GluA* has not been characterized, but on the basis of these results, we speculate that it does not contain a GCC box.
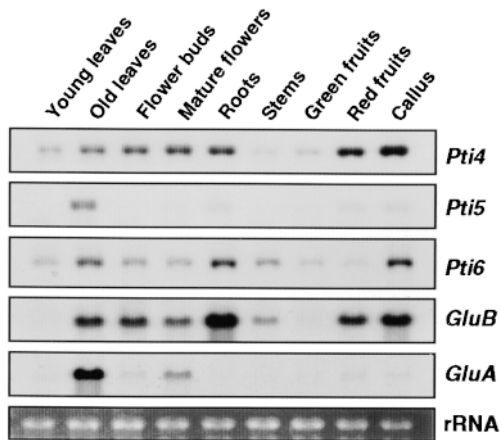

Figure 5. Expression of *Pti4, Pti5, Pti6, GluB,* and *GluA* in Various Tissues at Different Developmental Stages.

The third and fourth leaves of 3- to 4-week-old or 11- to 12-week-old tomato plants were harvested as young and old leaves, respectively. Unopened and fully opened flowers are referred to as flower buds and mature flowers, respectively. Root and stem tissues were harvested from 3- to 4-week-old plants. Green fruits were immature fruits, and red fruits were red ripe fruits. Total RNAs were isolated from these tissues, and duplicate RNA gel blots were hybridized with the *Pti4, Pti5, Pti6, GluB,* or *GluA* probes. Equal loading was verified by visualizing the rRNA in the RNA gel stained with ethidium bromide.

Expression of *Pti4* and *Pti6* in Compatible or *Pto*-Mediated Incompatible Interactions

We observed previously that expression of GCC-box-containing *PR* genes is enhanced in an incompatible interaction involving *Pto*-containing tomato and the bacterial pathogen *P. s. tomato* expressing *avrPto* (Jia and Martin, 1999). Because the Pti4, Pti5, and Pti6 proteins all interact with Pto kinase in a yeast two-hybrid system and specifically bind the GCC box, we wanted to examine the possible mechanisms by which interaction of Pto kinase with these transcription factors regulates *PR* gene expression. We began by determining whether induction of *Pti4, Pti5,* or *Pti6* transcripts correlated with an increase in *PR* gene expression during an incompatible interaction. Leaves of tomato cultivars RG-PtoR and RG-PtoS were vacuum-infiltrated with *P. s. tomato* DC3000 expressing the *avrPto* gene, and RNA was isolated over the time course shown in Figure 6A. *Pti4* transcripts accumulated 30 min after inoculation with *P. s. tomato*, regardless of the expression of the *Pto* gene in the tomato leaves.

Experiments with virulent *P. s. tomato* T1 produced similar observations and further showed that this induction of *Pti4* does not require SA or ethylene (Thara et al., 1999). In addition, *Pti5* transcripts also are induced in both compatible and incompatible interactions with only slightly enhanced expression in the latter interaction (Thara et al., 1999). *Pti6* transcripts were not induced by pathogen inoculation in either RG-PtoR or RG-PtoS plants (Figure 6A). As observed previously (Jia and Martin, 1999), expression of the *GluB* and *Osm* genes was enhanced substantially in the incompatible interaction (RG-PtoR) compared with their expression in the compatible interaction (RG-PtoS) (Figure 6A). These results indicate that enhanced expression of these GCC-box-containing *PR* genes during interactions with *P. s. tomato* is not directly correlated with the transcript amounts of *Pti4, Pti5,* and *Pti6*.

Because *Pti4* transcripts were moderately induced by wounding (Figure 4A), a mock inoculation was included as a control for comparing the difference of *Pti4* transcripts induced by wounding and by vacuum infiltration of *P. s. tomato.* As shown in Figure 6B, *Pti4* transcripts did increase during the early sampling points, but the transcript amounts declined markedly 4 hr after mock infiltration. This corresponded to a slight increase of *GluB* transcripts at the 4-, 6-, and 8-hr times. Plants inoculated with bacteria, in contrast, showed substantially greater and more prolonged expression of *Pti4* transcripts (Figure 6A).

Pto Kinase Phosphorylates Pti4 Protein

In mammalian systems, the activity of transcription factors often is regulated by phosphorylation or dephosphorylation events (Hunter and Karin, 1992). Because the product of the *Pto* resistance gene is an active serine/threonine kinase (Loh
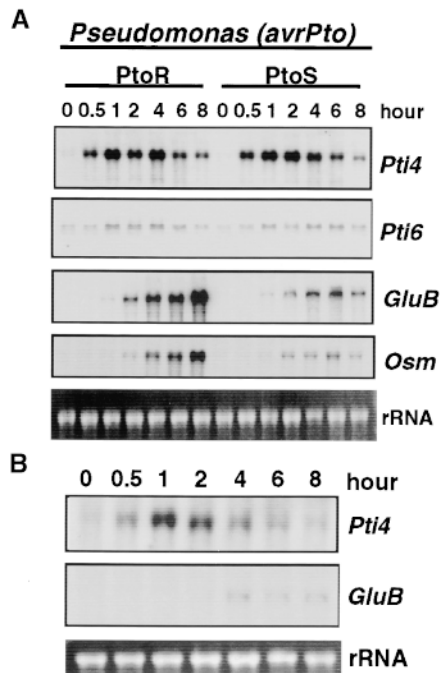
A

Pseudomonas (avrPto)

B

Figure 6. Expression of *Pti4, Pti6, GluB*, and *Osm* in an Incompatible or Compatible Interaction Involving *P. s. tomato*.

(A) RG-PtoR or RG-PtoS tomato plants were vacuum-infiltrated with 2.2×10^7 colony-forming units per milliliter of *P. s. tomato* DC3000 expressing the *avrPto* gene in 10 mM MgCl$_2$ and 0.005% Silwet.
(B) RG-PtoR tomato plants were vacuum-infiltrated with 10 mM MgCl$_2$ and 0.005% Silwet as a control.
Leaf tissues were harvested at the indicated times after vacuum infiltration. Total RNAs were isolated, and duplicate RNA gel blots were hybridized with radiolabeled *Pti4, Pti6, GluB*, or *Osm* probes. Equal loading was verified by visualizing the rRNA in the RNA gels stained with ethidium bromide.

and Martin 1995), it is possible that this kinase phosphorylates Pti4, Pti5, or Pti6 and thereby increases their activities. To examine this possibility, we conducted in vitro phosphorylation assays by using Pto kinase with Pti4, Pti5, and Pti6 as substrates. However, although expression and purification of GST-Pti5 and GST-Pti6 as described previously yielded proteins that were suitable for DNA binding assays (Zhou et al., 1997), they were neither sufficiently abundant nor pure enough for kinase assays (data not shown). Expression of Pti5 or Pti6 as a His-tagged fusion at the N terminus in *E. coli* also gave low yields. We finally succeeded in expressing and purifying Pti5 (but not Pti6) as a fusion protein with a His tag at its C terminus (see Methods). To test whether the Pti5-His fusion protein was functional, we performed a mobility shift assay. As shown in Figure 7B, the Pti5-His fusion protein specifically binds to a DNA fragment containing a wild-type GCC box but not to a mGCC box (Figure 7B).

For kinase assays, Pto was overexpressed as a myelin basic protein (MBP)–Pto fusion in *E. coli* and purified as described previously (Loh and Martin, 1995). Figure 7A shows that MBP-Pto strongly phosphorylated the His-Pti4 fusion protein but did not phosphorylate the Pti5-His protein. To determine the specificity of Pto phosphorylation of Pti4, we included the serine/threonine kinases Fen and Pti1 as controls. Fen has 80% amino acid identity to Pto kinase; Pti1 is phosphorylated by Pto and plays a role in the hypersensitive response (Martin et al., 1994; Zhou et al., 1995). Substrates for Pti1 and Fen have not been identified. In our experiments, neither Fen nor Pti1 phosphorylated Pti4, indicating that phosphorylation of Pti4 by Pto is specific (Figure 7A). Moreover, the Pti5-His protein was not phosphorylated by Fen or Pti1. Coomassie blue staining indicated that similar amounts of each kinase and His-Pti4 or Pti5-His protein were used in each reaction (Figure 7A, bottom gel).

Pto Phosphorylates Pti4 on at Least Four Threonine Residues

Pto autophosphorylates serine/threonine residues by way of an intramolecular mechanism (Loh and Martin, 1995; Sessa et al., 1998). To determine which amino acids of Pti4 are phosphorylated by Pto, we subjected the Pto-phosphorylated Pti4 protein to phosphoamino acid analysis (Figure 7C). Pti4 was exclusively phosphorylated on threonine residues. To determine how many threonine residues on Pti4 were phosphorylated by Pto, we digested the phosphorylated Pti4 with trypsin and separated the resulting peptides by two-dimensional electrophoresis/chromatography. Figure 7D shows that four radioactive peptides were detected, indicating that at least four threonine residues in Pti4 are phosphorylated by Pto.

Phosphorylation of Pti4 by Pto Influences Its DNA Binding Activity in Vitro

As shown in Figure 1, His-Pti4 specifically binds to the GCC box. To investigate whether phosphorylation of Pti4 affects its DNA binding activity in this assay, we first phosphorylated Pti4 by using the Pto kinase and then examined its binding to the GCC box by using a mobility shift assay. In preliminary experiments, we found that the presence of kinase buffer in the mobility shift assay reduced the binding of Pti4 to the GCC box; therefore, we increased the amount of this protein (to 0.25 μg) used in our experiments. As shown in Figure 8, phosphorylation of Pti4 with either of two different Pto fusion proteins, MBP-Pto or GST-Pto (Sessa et al., 1998), increased the binding of Pti4 to the GCC box. Control experiments conducted without the Pto kinase (lane1), with an inactive Pto protein (MBP-Pto [K69Q]; lane 4), or with the active kinase MBP-Fen, which does not phosphorylate Pti4 (lane 7), showed no effect on Pti4 binding of the GCC box. A
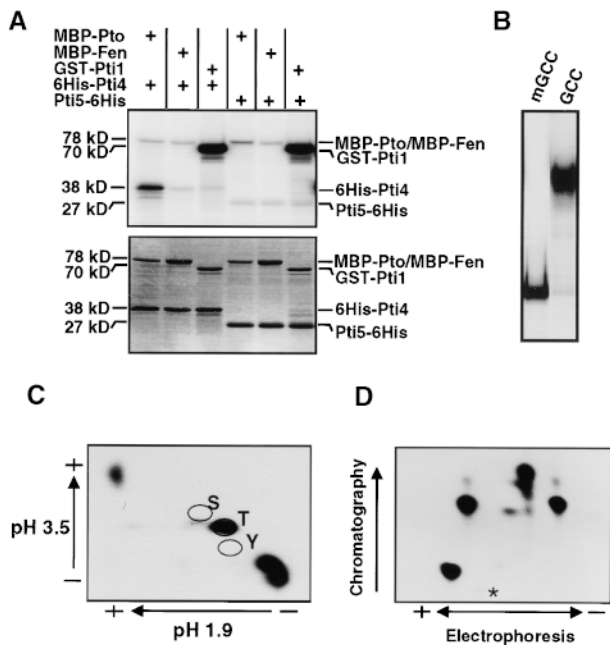
Figure 7. Phosphorylation of the Pti4 Protein by Pto Kinase.

(A) His-Pti4, Pti5-His, MBP-Pto, MBP-Fen, and GST-Pti1 proteins were purified as described in Methods. Two micrograms of His-Pti4 or Pti5-His protein was incubated with 2 μg of MBP-Pto, MBP-Fen, or GST-Pti1 protein in an in vitro kinase assay. Total proteins from each reaction were separated by SDS-PAGE and analyzed by autoradiography. The autoradiograph (top) shows the phosphorylated proteins, and the Coomassie blue–stained gel (bottom) shows the protein profile. The (+) signs denote that the indicated protein was present in the reaction. Protein masses are shown in kilodaltons.

(B) A mobility shift assay was performed as described previously (Zhou et al., 1995), using 50 ng of purified Pti5-His fusion protein mixed with 5 fmol of ^{32}P-labeled wild-type GCC-box or mGCC-box oligonucleotides (see Methods).

(C) Radiolabeled His-Pti4 protein that had been phosphorylated by Pto was transferred to a polyvinyl difluorine membrane, eluted, and hydrolyzed to its compositional amino acids as described in the Methods. The amino acids were separated by two-dimensional thin-layer chromatography. The (+) and (−) signs indicate the positions of the positive and negative electrodes. The locations of standards of serine (S), threonine (T), and tyrosine (Y) residues as determined by addition of ninhydrin are indicated. Radiolabeled, phosphorylated residues were detected by autoradiography.

(D) Radiolabeled His-Pti4 protein that had been phosphorylated by Pto was digested with trypsin, as described by Sessa et al. (1998). Peptides were separated horizontally by thin-layer electrophoresis at pH 4.7 and then vertically by ascending chromatography. The (+) and (−) signs indicate the positions of the positive and negative electrodes. Peptides were detected by autoradiography. The origin is indicated by an asterisk.

kinase reaction of Pti4 with MBP-Pto in the absence of ATP also had no effect on Pti4 binding of the GCC box (lane 6). When the kinase buffer was used in the mobility shift assay, we observed unbound (free) probe only in the lane containing Pto (lane 2). We believe that the lack of free probe in the lanes containing Pti4 results from the excess amount of this protein used in the reactions; the protein may have bound loosely to the GCC box and dissociated during electrophoresis. This explanation is consistent with the slightly higher background observed in the lanes with Pti4 than in the lane with Pto alone (lane 2).

Overexpression of Pto Overcomes SA Inhibition of Expression of *PR* Genes Having a GCC Box

The phosphorylation of Pti4 by Pto raises the possibility that this post-translational modification affects the activity of Pti4 in the induction of *PR* genes with GCC boxes. One way to test this hypothesis is to increase the expression of Pto and Pti4 concomitantly and to determine whether there is a direct correlation between this increase and the expression of *PR* genes having a GCC box. To perform these experiments, we used a pair of tomato lines, R11-12 and R11-13, derived from a tomato line carrying a 35S::*Pto* transgene. R11-12 is homozygous for the transgene, and R11-13 is a sibling line that by segregation has lost the transgene (Tang et al., 1999). We took advantage of three observations in these experiments: (1) *Pti4* is inducible by SA (Figure 3A); (2) SA does not induce expression of *PR* genes having the GCC box and, in fact, suppresses ethylene induction of *GluB* and *Osm* expression (Figure 3B); and finally, (3) SA is known to increase expression from the cauliflower mosaic virus 35S promoter that controls expression of *Pto* in R11-12 (Qin et al., 1994).

Tomato lines R11-12 and R11-13 plants were treated with SA, and their RNA was isolated over a 4-hr period. Figure 9 shows that transcript abundance of *Pti4* increased within 30 min and reached similar amounts in both lines. As expected, SA also induced expression of the 35S::*Pto* transgene in line R11-12, and *Pto* transcripts reached their maximum at 2 hr after SA treatment. Importantly, increases in the transcripts for *GluB* and *Osm* occurred only in the *Pto*-overexpressing line, and the timing of this increase correlated with the appearance of the *Pti4* and *Pto* transcripts. As described previously (Tang et al., 1999), some *PR* genes are constitutively expressed in R11-12 and other *Pto*-overexpressing lines when the plants are older (>6 weeks) or grown under high light conditions. We purposely used younger plants (4 weeks old) grown under lower light conditions to avoid this constitutive expression (see Methods). Thus, although *Pto* and *PR* gene transcripts were low at time 0, treatment with SA greatly increased the amounts of their transcripts (Figure 9). In summary, the ability of *Pto* overexpression to overcome the SA inhibition of *PR* gene expression (Figure 3) and the phosphorylation of Pti4 by Pto support a role for Pti4 in the activation of *PR* genes having a GCC box.
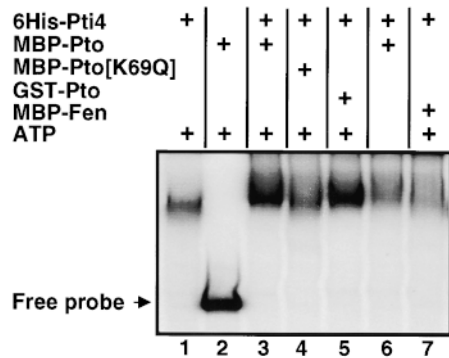
Figure 8. Phosphorylation of Pti4 by Pto Affects Its DNA Binding Activity.

Components as indicated in each lane were preincubated at 25°C for 30 min in 10 μL of kinase buffer. For each kinase reaction, 0.25 μg of His-Pti4 and kinase was used. The final concentration of unlabeled ATP was 1 mM. After incubation, the volume in each tube was adjusted to 30 μL, with the mobility shift assay buffer containing the radiolabeled probe, and assayed as described in Methods. The (+) signs indicate that the corresponding protein, or ATP, was present in the reaction. The position of the unbound (Free) probe is shown.

DISCUSSION

To identify components involved in *Pto*-mediated signal transduction pathways leading to disease resistance, we previously had used the product of the *Pto* disease resistance as a bait in the yeast two-hybrid system to search for Pti proteins. Among them, we found that *Pti4, Pti5,* and *Pti6* encode putative transcription factors strikingly similar to those of the EREBPs both in structure and GCC-box binding properties (Figure 1; Zhou et al., 1997). These observations prompted us to investigate the responsiveness of one of these genes, *Pti4*, to ethylene and other possible inducers and to investigate the relationship between the Pto kinase and the Pti4 protein. As depicted in Figure 10, our results indicate that Pti4 is a component of an ethylene signaling pathway leading to expression of several ethylene-regulated genes. Although SA rapidly induced transcription of *Pti4*, it antagonized ethylene-regulated expression of GCC-box-containing *PR* genes. Overexpression of *Pto* and concomitant induction of *Pti4* after SA application correlated with increased expression of GCC-box-containing *PR* genes. Moreover, the Pto kinase specifically phosphorylated Pti4 on a few of the threonine residues, phosphorylation that enhanced Pti4 binding of the GCC box. Given these observations, we postulate that phosphorylation of Pti4 by Pto modulates Pti4 activity, which then increases expression of *PR* genes during resistance to bacterial speck disease (i.e., more than that observed in a compatible interaction; Figure 10).

Pti4 Is Associated with Ethylene Regulation of GCC-Box-Containing *PR* Genes

Pti4 transcripts rapidly accumulated in response to ethylene, an increase that preceded expression of the GCC-box-containing *GluB* and *Osm* genes. Furthermore, transcriptional activation of *GluB* by ethylene required de novo synthesis of protein factors, which might be required for interacting with GCC-box *cis* elements. However, no synthesis of new proteins was required for the rapid accumulation of *Pti4* transcripts induced by ethylene. This suggests that *Pti4* is an immediate-early response gene, the product of which is required for GCC-box *cis* element binding to activate *PR* genes in the ethylene signaling pathway (Figure 10).

The correlation of the expression of *Pti4* and *PR* genes having a GCC box in plant tissues known to produce increased amounts of ethylene provides further support for a role of Pti4 in ethylene-regulated *PR* gene expression. In addition, in preliminary experiments with transgenic plants overexpressing the *Pti4* transgene, we have found that several ethylene-regulated *PR* genes are constitutively expressed (Y.-Q. Gu and G.B. Martin, unpublished data). In tobacco, induction of *EREBP* genes by wounding is insufficient for activation of GCC-box-containing *PR* genes (Suzuki et al., 1998), and an Arabidopsis EREBP, AtEBP, has been shown to cross-couple with a basic leucine zipper transcription factor (Büttner and Singh, 1997). It is possible therefore that the activity of Pti4 is modulated by interaction
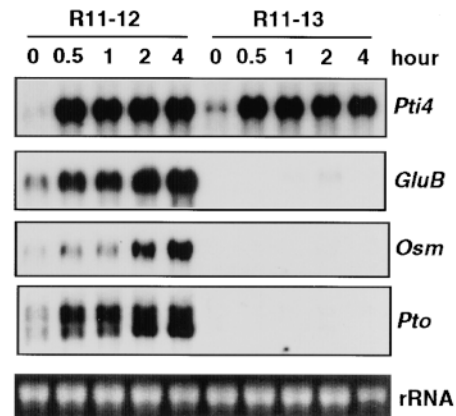

Figure 9. Expression of *Pti4,* the GCC-Box-Containing *PR* Genes, and *Pto* Induced by SA in Tomato Plants with or without a 35::*Pto* Transgene.

A tomato line that is homozygous for a 35S::*Pto* transgene (R11-12) and a sibling line lacking the transgene (R11-13) were treated with 1 mM SA, and leaves were harvested at the times indicated. Total RNAs were isolated, and duplicate RNA gel blots were hybridized with radiolabeled *Pti4, GluB, Osm,* or *Pto* probes. Equal loading was verified by visualizing the rRNA in the RNA gel stained with ethidium bromide.
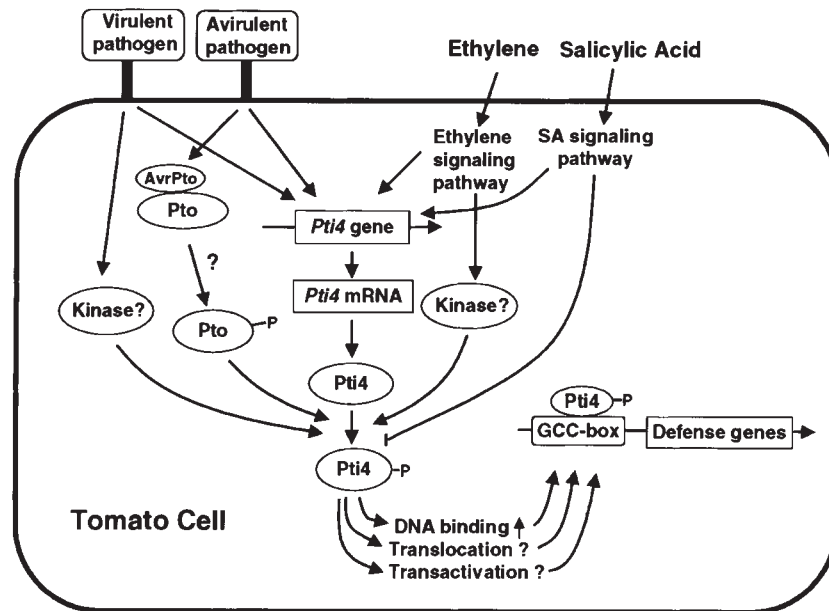
Figure 10. Proposed Model for Activation of GCC-Box-Containing *PR* Genes by Transcription Factor Pti4 in *Pto*-Mediated and Other Defense Response Signaling Pathways.

?, mechanistic steps that are still conjectural; P, phosphorylation.

with other transcription factors in the activation of GCC-box promoters.

Pti4, Pti5, and Pti6 proteins belong to a large family of APETALA2/EREBP–type transcription factors in plants. This family of transcription factors has been proposed to play various roles in plant growth, development, and responses to different environmental stimuli (Okamuro et al., 1997). The differential expression of *Pti4, Pti5,* and *Pti6* in various plant tissues implies that they respond to different endogenous signals and have distinct roles in plants. Thus, it is not surprising that *Pti4*, but not *Pti5* or *Pti6*, responds to ethylene treatment. Recently, several genes were isolated from an Arabidopsis cDNA library by using tobacco *EREBP-1* as a probe; only one, ethylene-responsive factor 1 (*ERF1),* was induced rapidly by ethylene, and its role in regulating ethylene-inducible genes was demonstrated (Solano et al., 1998). Perhaps Pti4 is a tomato functional homolog of ERF1 in mediating ethylene-regulated expression of *PR* genes containing a GCC box.

SA Induces Pti4 but Antagonizes Ethylene-Induced *PR* Gene Expression

SA plays an important signaling role in activation of *PR* genes in plant defense responses during pathogen attack. Genes activated by SA can be categorized into immediate-early and late genes, based on their response time to SA treatment (Dempsey et al., 1999). Some immediate-early SA-responsive genes encode transcription factors that when activated, may be responsible for the expression of late SA-responsive genes. For example, transcripts of the tobacco *myb1* transcription factor gene rapidly accumulate when plants are treated with SA; then Myb1 proteins specifically bind the H-box *cis* element in the promoter of *PR-1a*, a late SA-responsive gene (Yang and Klessig, 1996). SA also might regulate *PR* gene expression by modulating the activity of transcription factors. For example, SA treatment has been shown to increase the ASF-1 element binding activity present in tobacco nuclear extracts (Stange et al., 1997). This enhanced binding activity is likely mediated by protein phosphorylation events because treatment of SA-induced tobacco nuclear extracts with alkaline phosphatase decreases ASF-1 binding activity. Currently, the mechanism by which SA modulates the activity of these transcription factors remains unclear.

In this study, we found that although SA rapidly increased *Pti4* transcripts, it did not induce expression of GCC-box-containing *PR* genes. This suggests that SA might have a negative effect on the expression of GCC-box-containing *PR* genes. In support of this hypothesis, we found that SA treatment suppressed the ethylene induction of these *PR* genes. One possible mechanism for this suppression is a blockage of ethylene biosynthesis, given that SA antago-

nizes the activity of 1-aminocyclopropane-1-carboxylate oxidase, an enzyme that catalyzes the production of ethylene (Fan et al., 1995). That mechanism, however, is unlikely to be operative here because ethylene was provided constantly during the simultaneous treatment with SA. Alternatively, SA might negatively modulate components involved in the ethylene signaling pathway, thereby suppressing the expression of GCC-box-containing *PR* genes. Given the observation that simultaneous treatment with ethylene and SA further increased the amount of *Pti4* RNA, perhaps SA exerts a negative effect on the activity of the Pti4 protein (Figure 10). The mechanism responsible for such negative regulation, if one exists, remains to be determined.

Investigators using kinase and phosphatase inhibitors have demonstrated that protein phosphorylation/dephosphorylation plays a vital role in the SA-signaling pathways that lead to the activation of *PR* genes (Conrath et al., 1997). Recently, a tobacco 48-kD mitogen-activated protein kinase, the activity of which is induced by SA, was found to be involved in plant defense response pathways (Zhang et al., 1998). These results suggest that a phosphorylation/dephosphorylation cascade involved in SA signaling might change the activity of transcription factors in mediating *PR* gene expression. In addition, the negative regulatory role of SA on DNA binding proteins has been reported (Buchel et al., 1996). The binding activity of GT-1–like protein, which binds several fragments of tobacco *PR-1a* promoter, was severely decreased in nuclear extracts from SA-treated leaves (Buchel et al., 1996). Therefore, Pti4 could be a target for SA-activated protein kinases/phosphatases, and modulation of Pti4 by those enzymes may downregulate its activity.

Possible Role of Pti4 Phosphorylation by Pto in Regulation of *PR* Genes

R gene–dependent transcript accumulation of defense-related genes has been observed in several plant–pathogen interaction systems (e.g., Kiedrowski et al., 1992; Reuber and Ausubel, 1996). We found previously that expression of GCC-box-containing *PR* genes was enhanced by *Pto–avrPto* recognition (Figure 6A; Jia and Martin, 1999). Because Pti4, Pti5, and Pti6 bind specifically to the GCC-box element, *Pto–avrPto* recognition possibly could cause rapid transcriptional activation of these genes, the products of which then might mediate expression of GCC-box-containing *PR* genes. However, our results indicate that although *Pti4* transcripts rapidly accumulate after pathogen attack, this induction of *Pti4* is not specific for *Pto–avrPto* recognition but occurs in both compatible and incompatible interactions. In addition, although the abundance of *Pti5* transcripts is slightly enhanced in an incompatible interaction (Thara et al., 1999), constitutive expression of *Pti5* in transgenic tomato plants does not result in increased expression of GCC-box *PR* genes (Y.-Q. Gu and G.B. Martin, unpublished data). Moreover, *Pti6* was not transcriptionally

activated by pathogen attack. Thus, the enhanced expression of GCC-box-containing *PR* genes in the *avrPto–Pto* incompatible interaction is not correlated simply with increased *Pti4*, *Pti5*, and *Pti6* transcripts. An alternative possibility is that recognition of *avrPto* by *Pto* plays a role in modulating the activity of Pti4, Pti5, and Pti6 proteins. This finding is consistent with the observation that the Pto kinase physically interacts with Pti4, Pti5, and Pti6 proteins (Zhou et al., 1997) and with our present results demonstrating that Pto phosphorylates Pti4.

Phosphorylation of transcription factors is a common mechanism for regulation of gene expression in eukaryotes (Hunter and Karin, 1992). However, regulation of plant transcription factors by phosphorylation has been reported in only a few cases (Yu et al., 1993; Després et al., 1995; Subramaniam et al., 1997; Dröge-Laser et al., 1998). In all of these cases, the identity of the kinases involved is not known. Several lines of evidence suggest that phosphorylation of Pti4 by the Pto kinase plays an important role in *Pto*-mediated regulation of expression of GCC-box-containing *PR* genes during plant resistance to the bacterial speck pathogen. First, a kinase-deficient Pto that does not confer disease resistance fails to interact with Pti4 in a yeast two-hybrid system (Jia et al., 1997; Zhou et al., 1997). Second, the phosphorylation of Pti4 by Pto is highly specific; two other kinases, although closely related, do not phosphorylate the Pti4 protein. Third, *Pti4* transcripts accumulate in both compatible and incompatible *Pseudomonas* spp–tomato interactions, but enhanced expression of GCC-box-containing *PR* genes is seen only in the incompatible interaction involving Pto and AvrPto. Fourth, phosphorylation of Pti4 by Pto enhances its binding to the GCC box. Finally, the negative effect of SA on the expression of GCC-box-containing *PR* genes is overcome by overexpression of Pto and concomitant expression of *Pti4*. Our experiments did not exclude a role for Pti5 and Pti6 (or other transcription factors) in Pto-mediated regulation of *PR* genes. However, because Pti5 is not phosphorylated by Pto in vitro, and because we were not successful in isolating sufficient Pti6 for phosphorylation analysis, we were unable to investigate further any possible mechanisms whereby Pto might regulate the activity of these proteins.

The increased binding of phosphorylated Pti4 to the GCC box provides one explanation for the enhanced expression of *PR* genes during disease resistance. How AvrPto might be involved in this event, however, remains unclear. *Pto–avrPto* recognition occurs by a physical interaction of the two proteins (Scofield et al., 1996; Tang et al., 1996), an interaction that might stimulate Pto kinase activity and in turn allow phosphorylation of Pti4. Alternatively, a small amount of the Pto kinase might exist in a complex with a negative regulator such that interaction with AvrPto would release active Pto for Pti4 phosphorylation. Our results with Pto overexpression (in the absence of AvrPto) favor the latter explanation (Figure 9). Further support for this model can be derived from the observation that Pto kinase autophosphorylates by

means of an intramolecular mechanism and phosphorylates the Pti1 and Pti4 substrates in the absence of AvrPto (Figure 7; Zhou et al., 1995; Sessa et al., 1998).

Pto phosphorylates at least four threonine residues in Pti4 (and no serine residues). The Pti4 protein contains 10 threonine residues (Zhou et al., 1997); two of these are located in the putative DNA binding domain, two in a possible transactivation domain, and one near a nuclear localization sequence. Phosphorylation is known to affect cellular localization, binding activity to cognate *cis* elements, transactivation potential, and turnover of transcription factors (Hunter and Karin, 1992; Papavassiliou et al., 1992). An alteration in any of these processes potentially could affect Pti4 regulation of *PR* gene expression. We have shown that Pto phosphorylation of Pti4 enhances the binding of the transcription factor to the GCC box. However, we cannot exclude the possibility that other mechanisms (e.g., localization or transactivation) also play an important role in modulating Pti4 activity. By mapping the Pti4 phosphorylation sites and examining their functional relevance, we should gain further insights into the role of Pto kinase in activating defense genes during the incompatible interaction.

METHODS

Plant Materials, Growth Conditions, and Treatment

Tomato (*Lycopersicon esculentum*) cultivars Rio Grande–PtoR (RG-PtoR) and Rio Grande–PtoS (RG-PtoS), which are near-isogenic tomato lines for the *Pto* locus, were grown in the greenhouse with 16 hr of light and temperatures of 26 to 28°C. Three- to 4-week-old tomato plants of similar sizes were used for the experiments. For the experiments with *Pto*-transgenic line R11-12 and its sibling line R11-13, plants were grown in the greenhouse with low light intensity, and seedlings of 3-week-old plants were used for treatment. Under this growth condition, the R11-12 plants did not show the spontaneous cell death caused by a combination of overexpression of *Pto* and high light intensity (Tang et al., 1999).

The bacterial pathogen expressing the *avrPto* gene, *Pseudomonas syringae* pv *tomato* DC3000, was grown at 30°C with shaking in King's medium B (Martin et al., 1993) containing 100 μg/mL rifampicin. Bacteria were pelleted by centrifugation, washed once with 10 mM $MgCl_2$, and resuspended at a concentration of 2.2×10^7 colonyforming units per milliliter in 10 mM $MgCl_2$ and 0.005% Silwet (Union Carbide, Southbury, CT). Leaves of RG-PtoR plants that were infiltrated at this inoculum level displayed a hypersensitive response ~12 hr after inoculation. RG-PtoS leaves infiltrated with this same inoculum showed disease symptoms beginning at 12 hr after inoculation. The infiltration of plants was performed according to Jia et al. (1997). Plants were submerged in the bacterial suspension in a bell jar, and a vacuum was applied for 2 min and then released rapidly to ensure uniform infiltration of leaves.

Ethylene treatment of plants was performed in a plexiglass chamber. A volume of ethylene was injected into the chamber to give a final concentration of 10 μL/L. For chemical compound treatments, solutions were prepared as follows: salicylic acid (SA) and cycloheximide were directly dissolved in water at concentrations of 1 mM and

5 μg/mL, respectively; a systemin stock solution was prepared by dissolving synthetic mature systemin peptide (a gift from C.A. Ryan, Washington State University, Pullman) in water at 1 mg/mL; a working solution was made by diluting the stock solution 5000-fold to give a final concentration of 200 ng/mL. The compounds were applied to tomato leaves by stem feeding with the method of O'Donnell et al. (1996). Wounding was performed by rubbing tomato leaves with carborundum according to the method described by Zhang and Klessig (1998). For all the experiments, leaf tissues were harvested at the specified times, frozen in the liquid nitrogen, and stored at −80°C before isolation of RNA.

Overexpression and Purification of Recombinant His-Pti4 and Pti5-His Proteins

For expression and purification of Pti4 protein, the coding sequence was amplified by polymerase chain reaction (PCR), introducing a BamHI site in front of the ATG codon and immediately after the stop codon. The PCR product was gel purified, digested with BamHI, and ligated in frame into the His-tagged pEQ9 expression vector (Qiagen, Chatsworth, CA). The resulting plasmid, pEQ9Pti4, was sequenced to ensure that no PCR-induced errors were introduced into the Pti4 sequence. DH12S *Escherichia coli* cells were transformed with pEQ9Pti4 and grown to an OD_{600} of 0.6. Bacteria were induced with 1 mM isopropyl β-D-thiogalactopyranoside for 5 hr at 37°C. Most of the His-Pti4 fusion protein was insoluble, being localized in inclusion bodies (data not shown). Therefore, the bacterial pellet was resuspended in buffer A (50 mM Tris-HCl, pH 7.5, and 200 mM NaCl) containing 8 M urea. The supernatant was loaded onto a nickel–agarose column and washed with 20 column volumes of 8 M urea in buffer A. Protein (His-Pti4) was renatured on the column by further washing with a urea gradient from 8 to 0 M in buffer A. Protein (His-Pti4) was eluted from the column with 300 mM imidazole in buffer A and then dialyzed against buffer A overnight at 4°C.

For overexpression and purification of Pti5 protein, the coding sequence was amplified by PCR, introducing an NcoI site in front of the ATG and immediately before the stop codon. This PCR product was cloned in-frame into the NcoI site of pET-15b expression vector (Novagen, Madison, WI). The resulting plasmid, pETPti5, from which Pti5 was expressed with 6 histidines at its C terminus, was transformed into *E. coli* BL21. The procedure for induction and purification of Pti5-His fusion protein was the same as that for His-Pti4. Purification of GST-Pti5 and the gel mobility shift assays were performed as described previously (Zhou et al., 1997). The sequence of the GCC oligonucleotide was 5'-CATAAGA**G**CC**G**CCACTAAAATAAGACC-GATCAAATAAGA**G**CC**G**CCAT-3' and that of the mutated mGCC oligonucleotide was 5'-CATAAGA**T**CC**T**CCACTAAAATAAGACCGAT-CAAATAAGA**T**CC**T**CCAT-3' (the boldface type indicates the nucleotides that differ between the two oligonucleotides).

RNA Isolation and RNA Gel Blot Hybridization

RNA isolation was performed according to Perry and Francki (1992) with some modifications. Approximately 1 g of frozen tissue was ground in liquid nitrogen with a mortar and pestle to a fine powder. The leaf powder was extracted by vigorous vortex mixing for 30 sec in a mixture of 2 mL of water-saturated phenol and an equal volume of extraction buffer (100 mM Tris-HCl, pH 8.0, 100 mM LiCl, 10 mM EDTA, and 1% SDS) preheated at 75°C. Two milliliters of 24:1 (v/v)

chloroform–isoamyl alcohol was added to the sample and vortex mixed again for 30 sec. The mixture was centrifuged at 12,000g for 10 min at 4°C, and 2 mL of supernatant was transferred to a new 15-mL tube. RNA was precipitated by adding an equal volume of 4 M LiCl and incubating in a −70°C freezer for 30 min. RNA was pelleted by centrifugation at 12,000g for 20 min at 4°C, washed once with 70% ethanol, and resuspended in nuclease-free water. Ten micrograms of total RNA from each sample was separated by electrophoresis on a 1.2% formaldehyde–agarose gel, transferred to Hybond C membrane, and immobilized by UV light cross-linking.

RNA gel blot hybridizations were performed according to the manufacturer's instructions (Amersham). Briefly, the Hybond C membrane was prehybridized for at least 4 hr in hybridization solution containing 50% formamide, 2 × SSPE (1 × SSPE is 0.15 M NaCl, 10 mM sodium phosphate, and 1 mM EDTA, pH 7.4), and 5 × Denhardt's solution (1 × Denhardt's solution is 0.02% Ficoll, 0.02% PVP, and 0.02% BSA) (Ausubel et al., 1987). Hybridization was initiated by adding denatured DNA probes labeled with ^{32}P-dATP by using a random hexamer labeling kit (Ambion, Austin, TX). After a minimum 16-hr incubation, the membrane was washed three times (20 min each time) at 55°C in 2 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate) (Ausubel et al., 1987) plus 0.1% SDS. Radioactivity was detected by autoradiography.

In Vitro Kinase Assay, Phosphoamino Acid Analysis, and Peptide Mapping

For the phosphorylation assays, His-Pti4 and Pti5-His were purified as described above, and the myelin basic protein (MBP)–Pto, glutathione *S*-transferase (GST)–Pti1, MBP-Fen, and GST-Pto kinases were expressed and purified from *E. coli* as previously described (Loh and Martin, 1995; Zhou et al., 1995; Sessa et al., 1998). The phosphorylation assay was performed in solution at room temperature for 20 min. Each reaction contained 2 μg of kinase and 4 μg of His-Pti4 in a 50-μL volume with final concentrations of 50 mM Tris-HCl, pH 7.0, 1 mM DTT, 10 mM MnCl$_2$, 20 μM unlabeled ATP, and 4 μCi of γ-^{32}P-ATP (6000 Ci/mmol; Amersham Pharmacia Biotech). The reaction was stopped by adding 15 μL of 4 × SDS sample buffer (1 × SDS sample buffer is 62.5 mM Tris-HCl, 5% glycerol, and 2.5% β-mercaptoethanol) and boiling for 10 min before loading onto an SDS–polyacrylamide gel. After electrophoresis, the gel was stained with Coomassie Brilliant Blue G 250, dried, and subjected to autoradiography. Phosphoamino acid analysis and peptide mapping of phosphorylated Pti4 by Pto were performed by the procedure described by Sessa et al. (1998, 2000).

ACKNOWLEDGMENTS

REFERENCES

Abeles, F.B., Morgan, P.W., and Saltveit, M.E., Jr. (1992). Ethylene in Plant Biology. (San Diego, CA: Academic Press).

Alfano, J.R., and Collmer, A. (1996). Bacterial pathogens in plants: Life up against the wall. Plant Cell **8,** 1683–1698.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Smith, J.A., Seidman, J.G., and Struhl, K., eds (1987). Protocols in Molecular Biology. (New York: John Wiley).

Broglie, K.E., Chet, I., Holliday, M., Cressman, R., Biddle, P., Knowlton, S., Mauvais, C.J., and Broglie, R. (1991). Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani.* Science **254,** 1194–1197.

Buchel, A.S., Molemkamp, R., Bol, J.F., and Linthorst, H.J.M. (1996). The *PR-1a* promoter contains a number of elements that bind GT-1–like nuclear factors with different affinity. Plant Mol. Biol. **30,** 493–504.

Büttner, M., and Singh, K. (1997). *Arabidopsis thaliana* ethylene-responsive element binding protein (AtEBP), an ethylene-inducible, GCC-box DNA-binding protein, interacts with an Ocs element binding protein. Proc. Natl. Acad. Sci. USA **94,** 5961–5966.

Cao, H., Bowling, S.A., Gordon, A.S., and Dong, X. (1994). Characterization of an Arabidopsis mutant that is nonresponsive to inducers of systemic acquired resistance. Plant Cell **6,** 1583–1592.

Conrath, U., Silva, H., and Klessig, D.F. (1997). Protein dephosphorylation mediates salicylic acid–induced expression of *PR-1* genes in tobacco. Plant J. **11,** 747–757.

Cutt, J.R., and Klessig, D.F. (1992). Pathogenesis-related proteins. In Genes Involved in Plant Defense, T. Boller and F. Meins, eds (New York: Springer-Verlag), pp. 209–243.

Deikman, J. (1997). Molecular mechanism of ethylene regulation of gene transcription. Physiol. Plant. **100,** 561–566.

Delaney, T.P., Uknes, S., Vernooij, B., Friendrich, L., Weymann, K., Negrotto, D., Gaffney, T., Gut-Rella, M., Kessmann, H., Ward, E., and Ryals, J. (1994). A central role of salicylic acid in plant disease resistance. Science **266,** 1247–1250.

Delaney, T.P., Friedrich, L., and Ryals, J. (1995). Arabidopsis signal transduction mutant defective in chemically and biologically induced disease resistance. Proc. Natl. Acad. Sci. USA **92,** 6602–6606.

Dempsey, D.A., Shah, J., and Klessig, D.F. (1999). Salicylic acid and disease resistance in plants. Crit. Rev. Plant Sci. **18,** 547–575.

Després, C., Subramaniam, R., Matton, D.P., and Brisson, N. (1995). The activation of the potato *PR-10a* gene requires the phosphorylation of the nuclear factor PBF-1. Plant Cell **7,** 589–598.

Dröge-Laser, W., Kaiser, A., Lindsay, W.L., Kalkier, B.A., Loake, G.J., Doerner, P., Dixon, R.A., and Lamb, C. (1998). Rapid stimulation of a soybean protein-serine kinase that phosphorylates a novel bZIP DNA-binding protein, G/HBF-1, during the induction of early transcription-dependent defenses. EMBO J. **16,** 726–738.

Eyal, Y., Meller, Y., Lev-Yadum, S., and Fluhr, R. (1993). A basic-type *PR-1* promoter directs ethylene responsiveness, vascular and abscission zone-specific expression. Plant J. **4,** 225–234.

Fan, X., Mattheis, J.P., and Fellman, J.K. (1995). Inhibition of apple ACC oxidase activity and respiration by acetylsalicylic acid. Plant Physiol. **108** (suppl.), 69.

Frederick, R.D., Thilmony, R.L., Sessa, G., and Martin, G.B. (1998). Recognition specificity for the bacterial avirulence protein AvrPto is determined by Thr-204 in the activation loop of the tomato Pto kinase. Mol. Cell **2**, 241–245.

Friedrich, L., Vernooij, B., Gaffney, T., Morse, A., and Ryals, J. (1995). Characterization of tobacco plants expressing a bacterial salicylate hydroxylase gene. Plant Mol. Biol. **19**, 959–968.

Horvath, D.M., Huang, D.J., and Chua, N.-H. (1998). Four classes of salicylate-induced tobacco genes. Mol. Plant-Microbe Interact. **11**, 895–905.

Hunter, T., and Karin, M. (1992). The regulation of transcription by phosphorylation. Cell **70**, 375–387.

Jia, Y., and Martin, G.B. (1999). Rapid transcript accumulation of pathogenesis-related genes during an incompatible interaction in bacterial speck disease-resistant tomato plants. Plant Mol. Biol. **40**, 455–465.

Jia, Y., Loh, Y.-T., Zhou, J., and Martin, G.B. (1997). Allele of Pto and Fen occur in bacterial speck-susceptible and fenthion-insensitive tomato and encode active protein kinases. Plant Cell **9**, 61–73.

Kiedrowski, S., Kawalleck, P., Hahlbrock, K., Somssich, I.E., and Dangl, J. (1992). Rapid activation of a novel plant defense gene is strictly dependent on the Arabidopsis *RPM1* disease resistance. EMBO J. **11**, 4677–4684.

Lawton, K.A., Potter, S.L., Uknes, S., and Ryals J. (1994). Acquired resistance signal transduction in Arabidopsis is ethylene independent. Plant Cell **6**, 581–588.

Li, Y., Strabala, T.J., Hagen, G., and Guilfoyle, T. (1994). The soybean SAUR open reading frame contains a *cis* element responsible for cycloheximide-induced mRNA accumulation. Plant Mol. Biol. **24**, 715–723.

Loh, Y.-T., and Martin, G.B. (1995). The *Pto* bacterial resistance gene and the *Fen* insecticide sensitivity gene encode functional protein kinases with serine/threonine specificity. Plant Physiol. **108**, 1735–1739.

Martin, G.B., Brommonschenkel, S., Chunwongse, J., Frary, A., Ganal, M.W., Spivey, R., Wu, T., Earle, E.D., and Tanksley, S.D. (1993). Map-based cloning of a protein kinase gene conferring disease resistance in tomato. Science **262**, 1432–1436.

Martin, G.B., Frary, A., Wu, T., Brommonschenkel, S., Chunwongse, J., Earle, E.D., and Tanksley, S.D. (1994). A member of the *Pto* gene family confers sensitivity to fenthion resulting in rapid cell death. Plant Cell **6**, 1543–1552.

Mauch, F., Mauch-Mani, B., and Boller, T. (1988). Antifungal hydrolase in pea tissue. II. Inhibition of fungal growth by combinations of chitinase and β-1,3-glucanase. Plant Physiol. **88**, 936–942.

O'Donnell, P.J., Calvert, C., Atzorn, R., Wasternack, C., Leyser, H.M.O., and Bowles, D.J. (1996). Ethylene as a signal mediating the wound response of tomato plants. Science **274**, 1914–1917.

Ohme-Takagi, M., and Shinshi, H. (1995). Ethylene-inducible DNA binding proteins that interact with an ethylene-responsive element. Plant Cell **7**, 173–182.

Okamuro, J.K., Caster, B., Villarroel, R., Van Montagu, M., and Jofuku, K.D. (1997). The AP2 domain of APETALA2 defines a large new family of DNA binding proteins in Arabidopsis. Proc. Natl. Acad. Sci. USA **94**, 7076–7081.

Papavassiliou, A.G., Treier, M., Chavrier, C., and Bohmann, D. (1992). Targeted degradation of c-Fos, but not v-Fos by a phosphorylation-dependent signal on c-Jun. Science **258**, 1941–1944.

Pastuglia, I., Roby, D., Dumas, C., and Cock, J.M. (1997). Rapid induction by wounding and bacterial infection of an *S* gene family receptor-like kinase gene in *Brassica oleracea*. Plant Cell **9**, 49–60.

Pennickx, I.A.M.A., Eggermont, K., Terras, F.R., Thomma, B.P.H.J., De Samblanx, G.W., Buchala, A., Métraux, L.-P., Manners, J.M., and Broekaert, W.F. (1996). Pathogen-induced systemic activation of a plant defensin gene in Arabidopsis follows a salicylic acid-independent pathway. Plant Cell **8**, 2309–2323.

Penninckx, I.A.M.A., Thomma, B.P.H.J., Buchala, A., Metraux, J.-P., and Broekaert, W.F. (1998). Concomitant activation of jasmonate and ethylene response pathways is required for induction of a plant defensin gene in Arabidopsis. Plant Cell **10**, 2103–2113.

Perry, L.K., and Francki, R.I.B. (1992). Insect-mediated transmission of mixed and reassorted cucumovirus genomic RNAs. J. Gen. Virol. **73**, 2105–2114.

Qin, X.-F., Holuigue, L., Horvath, D.M., and Chu, N.-H. (1994). Immediate-early transcription activation by salicylic acid via the cauliflower mosaic virus *as-1* element. Plant Cell **6**, 863–874.

Reuber, T.L., and Ausubel, F.M. (1996). Isolation of Arabidopsis genes that differentiate between resistance response mediated by the *RPS2* and *RPM1* disease resistance genes. Plant Cell **8**, 241–249.

Ronald, P.C., Salmeron, J.M., Oldroyd, G.E.D., and Staskawicz, B.J. (1992). The cloned avirulence gene *avrPto* induces disease resistance in tomato cultivars containing the *Pto* resistance gene. J. Bacteriol. **174**, 1604–1611.

Rushton, P.J., Torres, J.T., Parniske, M., Wernert, P., Hahlbrock, K., and Somssich, I.E. (1996). Interaction of elicitor-induced DNA-binding proteins with elicitor response elements in the promoters of parsley *PR-1* genes. EMBO J. **15**, 5690–5700.

Schaller, A., and Ryan, C.A. (1995). Systemin—A polypeptide defense signal in plants. Bioessays **18**, 27–33.

Scofield, S.R., Tobias, C.M., Rathjen, J.P., Chang, J.H., Lavelle, D.T., Michelmore, R.W., and Staskawicz, B.J. (1996). Molecular basis of gene-for-gene specificity in bacterial speck disease of tomato. Science **274**, 2063–2065.

Sela-Buurlage, M., Ponstein, A.S., Bres-Vloemans, S.A., Melchers, L.S., van den Elzen, P.J.M., and Cornelissen, B.J.C. (1993). Only specific tobacco (*Nicotiana tabacum*) chitinase and β-1,3-glucanases exhibit antifungal activity. Plant Physiol. **101**, 857–863.

Sessa, G., D'Ascenzo, M., Loh, Y.T., and Martin, G.B. (1998). Biochemical properties of two protein kinases involved in disease resistance signaling in tomato. J. Biol. Chem. **273**, 15860–15865.

Sessa, G., D'Ascenzo, M., and Martin, G.B. (2000). Thr38 and Ser198 are Pto autophosphorylation sites required for the AvrPto-Pto–mediated hypersensitive response. EMBO J. **19**, in press.

Shah, J., Tsui, F., and Klessig, D.F. (1997). Characterization of a salicylic acid–insensitive mutant (*sai1*) of *Arabidopsis thaliana*, identified in a selective screen utilizing the SA-inducible expression of the *tms2* gene. Mol. Plant-Microbe Interact. **10**, 69–78.

Solano, R., Stepanova, A., Chao, Q., and Ecker, J.R. (1998). Nuclear events in ethylene signaling: A transcriptional cascade mediated by ETHYLENE-INSENSITIVE3 and ETHYLENE-RESPONSIVE-FACTOR1. Genes Dev. **12,** 3703–3714.

Stange, C., Ramirez, I., Gomez, I., Jordana, X., and Holuigue, L. (1997). Phosphorylation of nuclear proteins directs binding to salicylic acid–responsive elements. Plant J. **11,** 1315–1324.

Subramaniam, R., Despres, C., and Brisson, N. (1997). A functional homolog of mammalian protein kinase C participates in the elicitor-induced defense response in potato. Plant Cell **9,** 653–664.

Suzuki, K., Suzuki, N., Ohme-Takagi, M., and Shinshi, H. (1998). Immediate early induction of mRNAs for ethylene-responsive transcription factors in tobacco leaf strips after cutting. Plant J. **15,** 657–665.

Tang, X., Frederick, R.D., Zhou, J., Halterman, D.A., Jia, Y., and Martin, G.B. (1996). Initiation of plant disease resistance by physical interaction of AvrPto and the Pto kinase. Science **274,** 2060–2063.

Tang, X., Xie, M., Kim, Y.J., Zhou, J., Klessig, D.F., and Martin, G.B. (1999). Overexpression of *Pto* activates defense responses and confers broad resistance. Plant Cell **11,** 15–29.

Thara, V.K., Tang, X., Gu, Y., Martin, G., and Zhou, J.-M. (1999). *Pseudomonas syringae* pv. *tomato* induces the expression of tomato *EREBP*-like genes *Pti4* and *Pti5* independent of ethylene, salicylate and jasmonate. Plant J. **20,** 475–484.

Thomma, B.P.H.J., Eggermont, K., Penninckx, I.A.M.A., Mauch-Mani, B., Vogelsang, R., Cammue, B.P.A., and Broekaert, W. (1998). Separate jasmonate-dependent and salicylate-dependent defense-response pathways in Arabidopsis are essential for resistance to distinct microbial pathogens. Proc. Natl. Acad. Sci. USA **95,** 15107–15111.

van Kan, J.A.L., Joosten, M.H.A.J., Wagemakers, C.A.M., van den Berg-Velthuis, G.C.M., and de Wit, P.J.G.M. (1992). Differential accumulation of mRNAs encoding extracellular and intracellular PR proteins in tomato induced by virulent and avirulent races of *Cladosporium fulvum.* Plant Mol. Biol. **20,** 513–527.

Vidal, S., Ponce de Leon, I., Denecke, J., and Palva, E.T. (1997). Salicylic acid and the plant pathogen *Erwinia carotovora* induce defense genes via antagonistic pathways. Plant J. **11,** 115–123.

Voisey, C.R., and Slusarenko, A.J. (1989). Chitinase mRNA and enzyme activity in *Phaseolus vulgaris* (L.) increase more rapidly in response to avirulent than to virulent cells of *Pseudomonas syringae* pv. *phaseolicola.* Physiol. Mol. Plant Pathol. **35,** 403–412.

Xu, Y., Chang, P.-F.L., Liu, D., Narasimhan, M., Raghothama, K.G., Hasegawa, P.M., and Bressan, R.A. (1994). Plant defense genes are synergistically induced by ethylene and methyl jasmonate. Plant Cell **6,** 1077–1085.

Yang, Y., and Klessig, D.F. (1996). Isolation and characterization of a tobacco mosaic virus–inducible *myb* oncogene homolog from tobacco. Proc. Natl. Acad. Sci. USA **93,** 14972–14977.

Yu, L.M., Lamb, C.L., and Dixon, R.A. (1993). Purification and biochemical characterization of proteins which bind to the H-box *cis* element implicated in transcriptional activation of plant defense genes. Plant J. **3,** 805–816.

Zhang, S., and Klessig, D.F. (1998). The tobacco wound–activated mitogen-activated protein kinase is encoded by *SIPK.* Proc. Natl. Acad. Sci. USA **95,** 7225–7230.

Zhang, S., Du, H., and Klessig, D.F. (1998). Activation of the tobacco kinase by both a cell wall–derived carbohydrate elicitor and purified proteinaceous elicitins from *Phytophthora* spp. Plant Cell **10,** 435–449.

Zhou, J., Loh, Y.-T., Bressan, R.A., and Martin, G.B. (1995). The tomato gene *Pti1* encodes a serine/threonine kinase that is phosphorylated by Pto and is involved in the hypersensitive response. Cell **83,** 925–935.

Zhou, J., Tang, X., and Martin, G.B. (1997). The Pto kinase conferring resistance to tomato bacterial speck disease interacts with proteins that bind a *cis* element of pathogenesis-related genes. EMBO J. **16,** 3207–3218.

Zhu, Q., Maher, E.A., Masoud, S., Dixon, R.A., and Lamb, C.J. (1994). Enhanced protection against fungal attack by constitutive co-expression of chitinase and glucanase in transgenic tobacco. BioTechnology **12,** 807–812.