The Tomato Kinase Pti1 Contributes to Production of Reactive Oxygen Species in Response to Two Flagellin-Derived Peptides and Promotes Resistance to Pseudomonas syringae Infection

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The Pti1 kinase was identified from a reverse genetic screen as contributing to pattern-triggered immunity (PTI) against Pseudomonas syringae pv. tomato (Pst). The tomato genome has two Pti1 genes, referred to as Pti1a and Pti1b. A hairpin-Pti1 (hpPti1) construct was developed and was used to generate two independent stable transgenic tomato lines that had reduced transcript abundance of both genes. In response to P. syringae pv. tomato inoculation, these hpPti1 plants developed more severe disease symptoms, supported higher bacterial populations, and had reduced transcript accumulation of PTI-associated genes, as compared with wild-type plants. In response to two flagellin-derived peptides, the hpPti1 plants produced lesser amounts of reactive oxygen species (ROS) but showed no difference in mitogen-activated protein kinase (MAPK). Synthetic Pti1a and Pti1b genes designed to avoid silencing were transiently expressed in the hpPti1 plants and restored the ability of the plants to produce wild-type levels of ROS. Our results identify a new component of PTI in tomato that, because it affects ROS production but not MAPK signaling, appears to act early in the immune response.

The interaction of tomato (Solanum lycopersicum) with P. syringae pv. tomato both causes a persistent disease of tomatoes and also provides a powerful model system for understanding bacterial pathogenesis and the plant immune system (Jones 1991; Pedley and Martin 2003; Young et al. 1986). In common with other plants, tomato resists attack by microbial pathogens by employing a sophisticated, two-layered immune system (Cook et al. 2015; Dodds and Rathjen 2010; Jones and Dangl 2006). The first layer of defense involves pattern recognition receptors (PRRs) located at the plasma membrane that are able to perceive extracellular microbe-associated molecular patterns (MAMPs) (Boller and Felix 2009; Couto and Zipfel 2016; Hofmann et al. 2017; Zipfel 2014). These MAMPs are typically conserved components present in essential pathogen structures, such as the flagellin protein in the bacterial flagellum, which contains two MAMPs, flg22 and flgI1-28 (Bent and Mackey 2007; Cai et al. 2011; Clarke et al. 2013; Felix et al. 1999).

One of the best-characterized PRRs is FLS2, which detects flg22 and occurs in Arabidopsis, tomato, and other plants (Boller and Felix 2009; Chinchilla et al. 2006; Gómez-Gómez and Boller 2000; Robatzek et al. 2007). FLS2, upon binding flg22, initiates a signaling cascade, leading to a variety of responses, including production of reactive oxygen species (ROS), activation of mitogen-activated protein kinase (MAPK) cascades, and induction of defense-related genes (Zipfel 2014). These responses along with production of antimicrobial compounds and cell-wall reinforcement halt pathogen invasion and are collectively referred to as pattern-triggered immunity (PTI) (Boller and Felix 2009). The flgI1-28 peptide is recognized by tomato, potato, and pepper, and the gene encoding the cognate PRR, referred to as FLS3, was recently identified using a mapping-by-sequencing approach (Clarke et al. 2013; Hind et al. 2016).

To overcome these defense responses and cause disease, pathogenic microbes have evolved virulence proteins (effectors) that are typically translocated into the plant cell to interfere with pathogen detection or interrupt PTI signaling (Dou and Zhou 2012; Macho and Zipfel 2015). P. syringae pv. tomato, like many other bacterial pathogens, employs a type III secretion system, which acts as a molecular syringe to enable injection of approximately 30 effector proteins into the plant cell (Block and Alfano 2011; Buell et al. 2003; Cunnac et al. 2011; Lindeberg et al. 2012). Two of these, AvrPto and AvrPtoB, act early during the PTI response, to interfere with the FLS2 and FLS3 receptor complexes to prevent an effective induction of PTI (Cheng et al. 2011; He et al. 2012).

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flagellin perception, influencing the expression of defense-
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plays a role in PTI, then immunity is not fully induced by the
sequences from 129 receptor-like cytoplasmic kinases (RLCKs)
RESULTS

Pti1a

The Pti1 serine-threonine kinase was originally identified
from a yeast two-hybrid screen as a Pto interactor and was
implicated in ETI (Zhou et al. 1995). Overexpression of tomato
Pti1 in a stable transgenic tobacco line resulted in enhanced cell
death in response to P. syringae pv. tabaci carrying avrPto, and
it was concluded that Pti1 amplifies the Pto signaling response
(Zhou et al. 1995). A possible role for Pti1 in ETI was sup-
ported by the observation that Pto specifically phosphorylates
Pti1 but Pti1 does not phosphorylate Pto, suggesting that Pti1
functions directly downstream of Pto (Sessa et al. 1998, 2000;
Zhou et al. 1995). However, subsequent mutational analysis of
Pto revealed several substitution mutants that are unable to
phosphorylate Pti1 but still trigger Prf-mediated cell death when
transiently overexpressed in Nicotiana benthamiana (Wu et al.
2004). Importantly, a stable transgenic tomato line overexpressing
PtoG50S, a variant that lacks kinase activity and does not interact
with Pti1 in yeast, confers resistance to P. syringae pv. tomato strains
expressing avrPto (Mathieu et al. 2014; Xiao et al. 2003). Later
work found that the response to AvrPto in tobacco differs from
tomato and depends on an unidentified resistance protein (Nguyen
et al. 2010a; Shan et al. 2000; Yeam et al. 2010). Collectively, these
observations suggest that Pti1 does not play a role in ETI.

Here, we describe the discovery that Pti1 appears to act early
in the PTI response by inducing ROS production in response
to flagellin perception, influencing the expression of defense-
related genes and enhancing resistance to P. syringae pv. tomato.

RESULTS

Cell death suppression assay identifies
Pti1 as contributing to PTI.

Protein kinases are known to play various roles in PTI (Couto
Therefore, to identify new components of PTI, we cloned DNA
sequences from 129 receptor-like cytoplasmic kinases (RLCKs)
and other protein kinase–encoding genes from tomato and used
these for virus-induced gene silencing (VIGS) in N. benthamiana.
Each gene-silenced plant was then examined for altered defense
responses, using an assay in which PTI is induced by a non-
pathogenic bacterial strain, followed by overlap-infiltration of a
pathogenic strain (Chakravarthy et al. 2010). If the silenced gene
plays a role in PTI, then immunity is not fully induced by the
nonpathogenic strain and disease-associated cell death occurs
more rapidly in the overlapping area. The Pti1 gene was identified
in this screen, which was unexpected, because it had previously
been identified as playing a role in ETI (Zhou et al. 1995).

Inspection of the tomato genome sequence revealed that there
are two Pti1 genes, which we refer to as SlPti1a (Solyc12g098980) and
SlPti1b (Solyc05g053230) (Supplementary Table S1). The genes are 88% identical at the nucleotide level and their predicted
proteins are 93% identical (Supplementary Fig. S1). Pti1a encodes the protein originally identified as interacting with Pto (Zhou et al.
1995). From RNA sequencing (RNA-Seq) data generated pre-
viously (Rosli et al. 2013), we determined that Pti1a is expressed
in tomato leaves, though its expression is not specifically affected
by inducers of PTI (it is induced in mock-inoculations after 6 h, as
compared with the initial 30 min time point, suggesting possible
stress responsiveness [Supplementary Fig. S2]). Pti1b is expressed
at a basal level in leaves and its transcript abundance increased
significantly in response to the MAMPs eps22 (a 22-amino acid
peptide from a bacterial cold-shock protein) and flgII-28 as well as
P. fluorescens (having both flg22 and flgII-28) but not in response
to Agrobacterium tumefaciens, which has a flagellin that does not trigger transcriptional change in tomato (Rosli et al. 2013). Pti1b transcript abundance also increased in response to inoculation with P. syringae pv. tomato and was reduced in the presence of the effectors AvrPto and AvrPtoB.
N. benthamiana is an allotetraploid and, as expected, we
identified four Pti1 genes in the genome sequence of this species, which we refer to as NbPti1a (Niben101Scf01236g02003), NbPti1b
(Niben101Scf01334g04008), NbPti1c (Niben101Scf01671g04002),
and NbPti1d (Niben101Scf01820g00026) (Bombarely et al. 2012)
(Fig. 1A). To further characterize Pti1 in N. benthamiana, we used these gene sequences and the Sol Genomics Network (SGN) VIGS
tool (Fernandez-Pozo et al. 2015a) to design two constructs,
designated NbP1 and NbP2, each of which was expected to silence all four Pti1 genes in N. benthamiana (Supplementary Table S2).
These two constructs were used for VIGS, along with an NbFLS2
construct (as a positive control) and an Escherichia coli–derived DNA fragment (EC1, as a negative control) (Rosli et al. 2013).

The cell death suppression assay was performed by inducing PTI
with P. fluorescens 55, followed 7 h later by overlap-infiltration
of DC3000ShopQ1-1, which causes disease in N. benthamiana
(Chakravarthy et al. 2010; Rosli et al. 2013). As expected, sili-
cencing of FLS2, which is known to diminish the response to
bacterial flagellin, prevented full induction of PTI, thus resulting
in increased disease-associated cell death in the overlapping area
compared with EC1 control plants, whose PTI response was not
impaired (Fig. 1B and C). Importantly, silencing with either NbP1 or
NbP2 caused increased disease in the overlapping area, as compared
with the negative control, indicating a compromised PTI response,
although the disease was less than that observed in FLS2-silenced
plants (Fig. 1B and C). To evaluate the silencing efficiency of the two
VIGS constructs, we performed quantitative real-time polymerase
chain reaction (qPCR) on NbP1- and NbP2-silenced plants and found
that Pti1 transcript abundance was reduced to less than 20% of the
level in the EC1 control plants (Fig. 1D). Although our Pti1
primers were expected to amplify transcripts of the N. benthamiana
Pti1a, Pti1b, and Pti1c genes, sequencing of the qPCR products
from the EC1 control plants revealed only transcripts derived from
NbPti1b and NbPti1c. Using previously generated RNA-Seq data
(Pombo et al. 2014), we examined reads per kilobase of transcript
per million mapped reads (RPKM) and discovered that both NbPti1b
and NbPti1c are highly expressed in N. benthamiana leaves, whereas
NbPti1a and NbPti1d have a much lower transcript abundance
(Fig. 1A). Based on the VIGS experiments and these expression
levels, we conclude that, among the Pti1 genes in N. benthamiana,
one or both NbPti1b or NbPti1c are the main contributors to PTI,
and the robust silencing of these genes likely explains the observed
impact on cell death suppression in the PTI assay.

Transgenic tomato plants silenced for Pti1a and Pti1b are more susceptible to P. syringae pv. tomato infection.

To test whether the Pti1 genes contribute to PTI in tomato,
we developed a hairpin RNA interference (RNAi) construct
designed to silence the two Pti1 genes in this species (Fig. 2A). The construct was used to develop two independent stable transgenic lines in the Rio Grande-prf3 background (RG-prf3, lacking a functional Prf) (Salmeron et al. 1996). One line (F27-36) has a single-copy, homozygous, hpPti1 integration and the other line (F10-10) carries multiple copies of the hpPti1 construct. For each of these two lines, we identified an ‘azygous’ control line that was derived from the original transformation event but which had lost the one or more of the transgenes in subsequent generations, due to segregation. To determine the degree of Pti1 silencing and to characterize the transcriptome of these plants, we performed RNA-Seq on F27-36 and F10-10 hpPti1 plants (and on one of the azygous controls) that had been inoculated 3 or 6 h earlier with DC3000 ΔavrPtoΔavrPtoB, a P. syringae pv. tomato strain that has reduced virulence and is used to detect subtle changes in host response (Lin and Martin 2005; Kvitko et al. 2009; Rosli et al. 2013). In the hpPti1 lines, the transcript levels of Pti1a and Pti1b were reduced to about 35 and 25%, respectively, of the level in the azygous control line (Fig. 2B; Supplementary Fig. S3A). We also inoculated plants of each hpPti1 line with DC3000 ΔavrPtoΔavrPtoB to compare their disease symptoms and bacterial populations with azygous and RG-prf3 control plants. Severe disease symptoms were observed on hpPti1 plants compared with azygous and RG-prf3 plants, which developed only moderate disease (Fig. 2C). The hpPti1 plants supported about threefold greater bacterial populations compared with the azygous and RG-prf3 controls (Fig. 2D). Together, these observations indicate that, in tomato, one or both of the Pti1 kinases contribute to PTI in response to DC3000 ΔavrPtoΔavrPtoB.

RNA-Seq analysis reveals overlap between genes induced by the Pti1 proteins and PTI.

To gain insight into the possible roles of Pti1a and Pti1b, we further analyzed our RNA-Seq data from the two hpPti1 lines treated with DC3000 ΔavrPtoΔavrPtoB, to identify genes whose transcript abundance is either reduced or increased in the absence of these kinases. We observed that more genes were affected in the multicopy hpPti1 line (F10-10) than in the single-copy line (F27-36) (Fig. 3A; Supplementary Dataset S1). Because both hpPti1 lines showed the same degree of compromised resistance to P. syringae pv. tomato, we focused on the subset of genes that were affected in both lines. There were only 26 genes whose transcript abundance was less in both hpPti1 lines (i.e., their expression was induced in the presence of Pti1a and Pti1b), and only 11 genes whose transcript abundance was greater in both

Fig. 1. Silencing Pti1 compromises pattern-triggered immunity (PTI) in Nicotiana benthamiana. A, Phylogenetic tree based on nucleotide sequences showing the relationship of the four Pti1 genes in N. benthamiana, i.e., NbPti1a, NbPti1b, NbPti1c, and NbPti1d. B, Transcript abundance was less in both hpPti1 lines (i.e., their expression was induced in the presence of Pti1a and Pti1b), and only 11 genes whose transcript abundance was greater in both

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hpPti1 lines (i.e., their expression was suppressed in the presence of Pti1a and Pti1b [Fig. 3A]). Some of the genes with reduced transcript abundance in the hpPti1 lines have been implicated in defense responses, including the pathogenesis-related (PR)-1b gene (Chen et al. 2014) (Solyc02g076980) and the cathespin L-like cysteine protease genes Rcr3 (Solyc02g077040) and Pip1 (Solyc02g077040) that were recently reported to play an important role in P. syringae pv. tomato resistance in tomato (Ilyas et al. 2015; Richau et al. 2012; Shindo et al. 2016) (Fig. 3B). To more generally investigate whether these differentially expressed genes are associated with PTI, we compared them with our previously published set of genes that are induced or suppressed after treatment with the MAMP flgII-28 (Cai et al. 2011; Rosli et al. 2013). Of the 26 genes with increased transcript abundance when the Pti1 kinases were present, 19 were also induced by flgII-28 (73%). Of the 11 genes with reduced transcript abundance, none were suppressed by flgII-28; there are also five Pti1-suppressed genes induced by flgII-28. Gene Ontology (GO) term analysis of the 26 Pti1-induced and 11 Pti1-suppressed genes showed the former to be predominantly associated with plant defense, whereas the latter are associated with biosynthetic and metabolic processes (Fig. 3C; Supplementary Dataset S2). These analyses are consistent with our disease and bacterial population assays in supporting a role for one or both of the Pti1 kinases in the PTI response to P. syringae pv. tomato.

Silencing of Pti1a and Pti1b negatively impacts ROS production associated with PTI.

Flagellin-derived peptides (i.e., flg22 and flgII-28) are important MAMPs associated with P. syringae pv. tomato–mediated PTI in both tomato and N. benthamiana (Chakravarthy et al. 2010; Rosli et al. 2013). To further investigate a role of the Pti1 kinases in PTI, we used two standard assays to test for the host response to these peptides, i.e., activation of MAPK cascades and generation of ROS (Nguyen et al. 2010b). Because we observed no difference in silencing efficiency or disease susceptibility between the single- and the multicopy hpPti1 lines, we performed these assays with only the single-copy line (F27-36). To detect MAPK activation, leaf discs were incubated with either flg22, flgII-28, or water, as a control, and phosphorylated MAPKs were detected with an antibody. Although both flg22 and flgII-28 induced MAPK phosphorylation, there was no difference in this response between the hpPti1 and azygous control plants at the lowest concentration of each peptide that reliably activated MAPKs (10 nM for flg22 and 25 nM for flgII-28 [Fig. 4A]). To measure ROS production, we used leaf discs in a 1. 

**Fig. 2.** Transgenic tomato plants silenced for Pti1a and Pti1b are more susceptible to infection by DC3000ΔavrPtoΔavrPtoB. A, Schematic representation of the tomato Pti1a and Pti1b genes, with the kinase domain highlighted in black. The numbers indicate nucleotide positions of the 5′ and 3′ ends and the location of the kinase domain. The origin of the hairpin (hp) fragment is shown below the gene. B, Relative transcript abundance of Pti1a and Pti1b in homozygous single-copy hairpin-Pti1 (hpPti1) plants (F27-36) compared with azygous control plants. ATPase is shown as a control. Transcript levels are based on RNA-Seq reads from the 6-h dataset of three plants per genotype normalized to CBL1 (Pombo et al. 2014). Bars show mean ± 99% confidence interval. Asterisks indicate significant differences between hpPti1 and azygous plants. P values are <0.0001 for both Pti1a and Pti1b (based on a false discovery rate correction). C, Four-week-old transgenic hpPti1 plants (F27-36) along with azygous control plants and progenitor RG-prf3 plants were vacuum-infiltrated with 5 × 10⁵ CFU of DC3000ΔavrPtoΔavrPtoB per milliliter and disease symptoms were monitored. Photographs of the same representative plants are shown before infiltration (Day 0) and 5 days after infiltration (Day 5). Seven plants for each genotype were tested. D, Bacterial populations were determined in the plants shown in C. Samples were taken after infiltration (Day 0) and 2 days later (Day 2). Bars show the mean ± 99% confidence interval. Different letters indicate significant differences based on a one-way analysis of variance followed by Tukey’s honest significant difference post hoc test. P values are 0.0002 for hpPti1 versus azygous and 0.0019 for hpPti1 versus RG-prf3. This experiment was performed four times with similar results.

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chemiluminescence-based assay (Chakravarthy et al. 2010; Clarke et al. 2013). We observed a reduction in ROS production to approximately 50% in hpPti1 plants, as compared with azygous control plants, despite using relatively high concentrations of flg22 and flgII-28 (100 nM each [Fig. 4B]). These observations suggest that one or both of the tomato Pti1 kinases function in a signaling pathway activated by flagellin perception upstream of ROS production but independent of the MAPKs monitored by this assay.

Transient complementation demonstrates that either Pti1a or Pti1b can restore ROS production to the hpPti1 plants.

In addition to the one or more intended genes, RNAi can silence other nontarget genes, possibly leading to misinterpretations (Senthil-Kumar and Mysore 2011). To determine if the reduced ROS production in response to flg22 and flgII-28 is indeed due to silencing of the Pti1 genes, we developed synthetic Pti1a and Pti1b genes that would not be silenced by the hpPti1 construct (Kumar et al. 2006; Wu et al. 2016). Specifically, the 5′ portion of each gene was altered so that it did not have a contiguous region of more than 18 nucleotides identical to the hpPti1 fragment that could trigger RNA silencing. The synthetic regions encoded identical amino acid sequences as the wild-type proteins and were fused to the 3′ wild-type sequence of the respective gene (Fig. 5A; Supplementary Fig. S4). To verify that these synthetic Pti1 variants, referred to as synPti1a and synPti1b, are expressed and evade silencing, we transiently expressed synPti1a and synPti1b or wild-type Pti1a and Pti1b in N. benthamiana leaves in the presence of the hpPti1 construct (Fig. 5B). Accumulation of the synthetic proteins was not significantly different from that of the wild-type proteins when coexpressed with the unrelated hpBti9 construct (Zeng et al. 2011). However, when coexpressed with the hpPti1 construct, accumulation of the wild-type Pti1 proteins was strongly diminished, whereas the synthetic Pti1 variants accumulated to similar levels as with the hpBti9 construct, indicating the synthetic Pti1 genes are not targeted by the hpPti1 fragment (Fig. 5B). We then transiently expressed synPti1a and synPti1b in leaves of single-copy hpPti1 tomato plants; the gene encoding yellow fluorescent protein (YFP) was expressed in hpPti1 and azygous plants as negative and positive control, respectively (Fig. 5C).

![Fig. 3](image-url) Genes whose expression is induced by Pti1 after *Pseudomonas syringae* pv. *tomato* treatment are associated with pattern-triggered immunity. A, Number of genes whose expression is significantly induced or suppressed by Pti1 (P ≤ 0.05, based on a false discovery rate correction) after treatment with DC3000avrPtoavrPtoB in either the single-copy hairpin-Pti1 (hpPti1) line (F27-36) or the multicopy hpPti1 line (F10-10). Differentially expressed genes from the 3-h and 6-h datasets were combined for each line and duplicate genes were removed. The number of genes in common between the two lines is shown as patterned boxes (i.e., 26 induced and 11 suppressed). B, Transcript abundance of three genes with reduced expression in both hpPti1 lines (i.e., genes that are normally induced by Pti1 after *P. syringae* pv. *tomato* treatment), i.e., *PR-1b* (Solyc00g174340) (Chen et al. 2014) and cysteine proteinase genes Pip1 (Solyc02g077040) and Rcr3 (Solyc02g076980) (Ilyas et al. 2015; Richau et al. 2012; Shindo et al. 2016). Expression levels were normalized to *CBL1* (Pombo et al. 2014) and *ATPase* is shown as an internal control for each time point (3 or 6 h). Bars show mean ± 95% confidence interval calculated from the RNA-Seq reads from the 3-h or 6-h dataset from the single-copy hpPti1 line (F27-36) compared with the azygous control line. Asterisks indicate significant differences in transcript abundance between hpPti1 and azygous control plants. P values are 0.0027 for *PR-1b*, 0.0291 for *Pip1*, and 0.0018 for *Rcr3* (based on a false discovery rate correction). C, The genes shown in A that are in common between the two hpPti1 lines were subjected to a Gene Ontology (GO) term analysis. Shown are the top five GO terms for both the Pti1-induced genes and the Pti1-suppressed genes. The group percentage shows the frequency of a given GO term in the analyzed set of genes. The genome percentage shows the overall frequency of that GO term in the tomato genome. P values are based on a false discovery rate correction.
ROS production in response to flg22 increased in the presence of either synPti1a or synPti1b, comparable to the level in the positive control (azygous plants transiently expressing YFP). Thus, the silencing of Pita and Pitiib in the hpPti1 lines and not silencing of nontarget genes leads to decreased ROS production.

Pti1 proteins localize to the cell periphery and S-acylation on cysteine residues 6 and 7 is implicated in this localization.

Localization experiments of Pti1-like proteins in maize determined that ZmPti1a localizes to the plasma membrane (PM) upon treatment with flg22 or flgII-28. This localization was dependent on the predicted acylation sites C6 and C7 (Matsui et al. 2014). The C6 and C7 residues are conserved in the tomato Pti1 proteins and we introduced serine substitutions to test their importance in subcellular localization. We transiently expressed wild-type Pita and Pitiib along with variants encoding C6S/C7S substitutions fused to YFP in leaves of N. benthamiana (Supplementary Fig. S5). The wild-type proteins localized to the cell periphery, whereas the C6S/C7S mutants showed a more diffuse localization with obvious accumulation in the nuclei. YFP, included as a control, also accumulated in the nucleus in addition to the cytoplasm. Because the C6 and C7 residues are predicted S-acylation sites, it is possible that the peripheral localization of wild-type Pti1 is due to S-acylation. Tomato Pti1a was previously reported to have a diffuse localization when transiently expressed in onion epidermal cells and this observed discrepancy might be due to differences in the experimental system (Herrmann et al. 2006).

Neither Pti1a nor Pti1b play a demonstrable role in Pto-mediated resistance to P. syringae pv. tomato in tomato.

Pti1a was originally discovered in a yeast two-hybrid screen as an interactor of Pto and was thought to function in Pto-mediated resistance (Zhou et al. 1995). In light of our finding that one or both of the Pti1 kinases play a role in PTI, we used our hpPti1 line to examine a possible role of these kinases in the Pto resistance pathway in tomato. Recognition of AvrPto in tomato requires both Pto and the NB-LRR protein Prf (Martin et al. 1993; Salmeron et al. 1994, 1996; Scofield et al. 1996; Tang et al. 1996). The hpPti1 line was made in the susceptible RG-prf3 background, which is homozygous for Pto but has a deletion in Prf, rendering the gene nonfunctional (Salmeron et al. 1996). Therefore, we crossed RG-PtOR (homozygous for both Pto and Prf) with hpPti1 plants to obtain F1 plants that contain a single functional copy of Prf, one copy of the hpPti1-silencing fragment, and two copies of Pto. As a control, we crossed RG-PtOR with RG-prf3 plants, which resulted in F1 plants identical to the RG-PtOR × hpPti1 cross, except that no hpPti1 construct is present.

The F1 plants were inoculated with DC3000 (expressing avrPto and avrPtoB) at 2 titers (10^5 and 10^6 CFU/ml), and disease symptoms were documented and bacterial populations were measured on days 3 and 2, respectively. No difference in Pto-mediated resistance was observed between the F1 plants either carrying or lacking the hpPti1 construct (Fig. 6A and B). At the lower inoculum concentration, all of the plants exhibited strong Pto-Prf-mediated resistance to P. syringae pv. tomato whereas, as expected, at the higher inoculum concentration, the plants carrying only a single copy of Prf developed mild disease symptoms and showed increased bacterial growth since Pto-Prf-mediated resistance is known to be semidominant (Carland and Staskawicz 1993) (Fig. 6A and B).

It is possible that residual Pti1 protein is present due to incomplete silencing and is sufficient to fully activate the Pto-Prf pathway. Therefore, we performed a second set of inoculations, using the same bacterial concentrations with resistant RG-PtOR plants and a transgenic line containing an RNAi construct that silences Pto in the RG-PtOR background (hpPto) (Pascuzzi 2006). RG-PtOR has two copies of Pto and Prf and showed no signs
of disease at either inoculum level (Fig. 6A). The hpPto plants were highly susceptible, showing that silencing a gene directly involved in the Pto pathway leads to a complete loss of Pto-Prf-mediated resistance even when two copies of Pto and Prf are present (Fig. 6A).

The questions remained whether a single copy of the hpPti1 fragment is sufficient to effectively silence the two Pti1 genes and whether the level of silencing is comparable to silencing of Pto by the hpPto construct. To address these issues, we first compared Pti1 transcript abundance in RG-PtoR × hpPti1 F1 plants and the transgenic hpPti1 line and found that there is no significant difference in silencing efficacy, as both lines have reduced Pti1a and Pti1b mRNA levels of about 30% compared with the RG-PtoR × RG-prf3 control F1 plants (Fig. 6C). Second, we compared the hpPto plants to RG-PtoR plants and found that transcript abundance of Pto was reduced to about 10% in the presence of the silencing fragment (Supplementary Fig. S6). In conclusion, although Pti1 transcripts are reduced to 30% of wild-type levels in the RG-PtoR × hpPti1 F1 plants, we see no effect on Pto-Prf-mediated resistance, indicating that neither Pti1 nor Pti1b demonstrably contribute to this ETI response.

DISCUSSION

We have found that transgenic tomato plants silenced for Pti1 are more susceptible to infection by P. syringae pv. tomato, fail to induce the expression of several defense-related genes associated with the PTI response, and have reduced ROS production in response to flagellin-derived peptides. Collectively, these observations support a role for Pti1 in PTI. Here, we place our findings in the context of previous work on Pti1 and Pti1-related genes and discuss possible mechanisms by which the tomato Pti1 kinase might contribute to PTI. We also speculate on why Pti1 was originally identified as an interactor of the Pto kinase.

Our data support a role for Pti1 in the PTI signaling pathway in response to flagellin perception and indicate it acts either as part of the FLS2 and FLS3 receptor complexes or downstream of these complexes. Silencing of Pti1a and Pti1b affected ROS production in response to flg22 and flgII-28 and had a limited effect on the transcriptome but did not impact MAPK activation. The mechanism by which Pti1 contributes to ROS production is unknown, but it is possible that it regulates the NADPH oxidase RBOHD (RBOHb in tobacco), which is localized to the plasma membrane and has a well-studied role in immunity-associated ROS generation (Adachi et al. 2015; Li et al. 2014). The Pti1 proteins are localized to the cell periphery, which places them in a position to be physically associated with the FLS2 and FLS3 receptor complexes or with an RBOH protein. In Arabidopsis, RBOHD is activated by direct Ca2+ binding and by phosphorylation by calcium-dependent protein kinases and BIK1 on partially overlapping residues (Boudsocq et al. 2010; Dubiella et al. 2013; Kadota et al. 2015). In N. benthamiana, RBOHB is required for flagellin-induced ROS production (Segonzac et al. 2011). In the future, we will test the hypothesis that the Pti1 kinase might phosphorylate RBOHB and thereby contribute to its activation and ROS production.

A large number of genes are differentially expressed during the host response to individual MAMPs or to P. syringae pv. tomato (Pombo et al. 2014; Rosli et al. 2013). It is striking, then, that, despite the role of Pti1 in enhancing PTI against P. syringae pv. tomato, our RNA-Seq analysis indicated the kinase plays a minor role in inducing gene expression in response to inoculation with P. syringae pv. tomato. Immunity-associated gene expression

![Fig. 5. Synthetic Pti1 genes restore reactive oxygen species (ROS) production in hairpin-Pti1 (hpPti1) plants in response to flg22. A, A schematic illustration showing where the hairpin fragment anneals to the wild-type Pti1 transcript. Synthetic versions of the tomato Pti1 genes, referred to as synPti1a and synPti1b, were made by synthesizing the 5' portion of each gene with alternate codons (black) so that the hairpin fragment is no longer able to anneal. This synthetic portion was then fused to the 3' wild-type sequence (white) by polymerase chain reaction. The synPti1 genes encode the same amino acid sequences as the wild-type Pti1 genes and all constructs included a FLAG tag at the C-terminus. B, Wild-type and synthetic versions of Pti1 and Pti1b were transiently expressed in Nicotiana benthamiana leaves, using agroinfiltration together with either the hpPti1 fragment or the unrelated hpBti9 fragment (Zeng et al. 2011). Tissue samples were collected 2 days after infiltration, total protein was extracted, and Pti1 proteins were detected by anti-FLAG immunoblotting. Coomassie brilliant blue staining (CBB) shows equal loading of protein. C, Agroinfiltration was used to transiently express synPti1a and synPti1b in leaves of hpPti1 tomato plants along with the yellow fluorescent protein gene (YFP) (negative control). Azygous plants transiently expressing YFP served as a positive control. Leaf discs were treated with 100 nM flg22 and production of ROS was measured. The graph shows the cumulative relative luminescence (area under the curve). Bars show mean ± 99% confidence interval of eight plants per group. Different letters indicate significant differences based on a one-way analysis of variance, followed by Tukey’s honest significant difference post hoc test. P values are 0.0030 for hpPti1+synPti1a versus hpPti1+YFP, 0.0375 for hpPti1+synPti1b versus hpPti1+YFP, and 0.0002 for hpPti1+YFP versus azygous+YFP. This experiment was performed twice with similar results.](image-url)
changes are typically regulated by MAPK cascades (Meng and Zhang 2013). We found no evidence for an effect of Pti1 on MAPK activation, although it is possibly small changes do occur and are below the sensitivity of our pMAPK assay. It is, perhaps, more likely that another signaling pathway is affected in the hpPti1 plants, possibly one normally triggered by ROS production (Apel and Hirt 2004). It is interesting that one of the genes most highly induced by Pti1 is PR-1b, which was recently shown to encode a propeptide, with its cleaved peptide, CAPE1, activating defense responses enhancing resistance to \textit{P. syringae pv. tomato} (Chen et al. 2014). It is possible that perception of flagellin results in Pti1 activation, which, in turn, leads to \textit{PR-1b} expression through an unknown mechanism, and the resulting CAPE1 peptide further stimulates plant defense responses. Also of interest, our RNA-Seq data shows that the expression of two genes encoding cathapsin L-like cysteine proteinases, Rcr3 and Pip1, is induced by Pti1. It has been reported recently that the secreted \textit{P. syringae pv. tomato} protein Cip1 (C14-inhibiting protein-1)

![Fig. 6](image)

**Fig. 6.** Pto-mediated resistance is not detectably impaired in hairpin-Pti1 (hpPti1) plants. A, Four-week-old RG-PtoR \times RG-prf3 and RG-PtoR \times RG-prf3, F1 plants were vacuum-infiltrated with DC3000 at 10^5 or 10^6 CFU/ml and disease symptoms were monitored. RG-PtoR and hpPto plants were included as resistant and susceptible controls, respectively. Photographs were taken 3 days after infiltration. Experiments involving RG-PtoR F1 plants used four plants per genotype, and the RG-PtoR and hpPto experiments used three plants each. The dashed line indicates experiments performed on different days. B, Bacterial populations were determined in the plants used in A. Tissue samples were taken 2 days after infiltration. Bars show mean \pm 99% confidence interval. No significant difference was found in either treatment. This experiment was performed twice with similar results. C, Quantitative polymerase chain reaction to monitor transcript levels of Pti1a and Pti1b in the F1 plants. Expression data were normalized to CBL1 (Pombo et al. 2014) and are shown in relation to the RG-PtoR \times RG-prf3 control group. ATPase is shown as an internal control. Bars show mean \pm 95% confidence interval of four plants per group. Homozygous hpPti1 plants were included for comparison. Different letters indicate significant differences based on a Brown-Forsythe test, followed by a Games-Howell post hoc test. \(P\) values for Pti1a are 0.024 for RG-PtoR \times RG-prf3 versus RG-PtoR \times hpPti1 and 0.014 for RG-PtoR \times RG-prf3 versus hpPti1, and \(P\) values for Pti1b are 0.054 for RG-PtoR \times RG-prf3 versus RG-PtoR \times hpPti1 and 0.040 for RG-PtoR \times RG-prf3 versus hpPti1.
inhibits Rcr3 and Pip1 and a DC3000 

Several studies have examined a role for the rice kinase OsPt1a in response to the blast fungus Magnaporthe grisea as well as to Xanthomonas oryzae pv. oryzae, the causal agent of bacterial blight (Matsui et al. 2010a and b; Takahashi et al. 2007). Rice plants with transposon-induced mutations in OsPt1a have a dwarf phenotype, form spontaneous lesions, show enhanced resistance to a compatible race of M. grisea and have increased PR gene expression. These observations suggested that OsPt1a negatively regulates PTI responses in rice. Overexpression of OsPt1a led to lesion development (disease) by an incompatible race of M. grisea and enhanced lesion formation after inoculation with a compatible strain of X. oryzae pv. oryzae, indicating that OsPt1a also represses ETI responses and further supported its role as a negative regulator of PTI (Takahashi et al. 2007). Silencing RAR1, whose protein is a component of several R protein complexes (Shirasu 2009), negated the dwarf phenotype of rice Pt1a plants and abolished both constitutive PR gene expression and the enhanced lesion formation by the compatible race of M. grisea, thus showing that repression of immune signaling by OsPt1a is dependent on RAR1 (Takahashi et al. 2007).

The results regarding OsPt1a are in seeming conflict with our data showing a positive regulatory function for Pt1 in the tomato PTI response. Despite the apparent opposite function of Pt1 in rice and tomato, the two proteins function interchangeably in complementing the pt1a rice plants (Takahashi et al. 2007). One proposed explanation for this discrepancy is that the signaling pathways downstream of Pt1 have diverged in rice and tomato (Takahashi et al. 2007). Given the large evolutionary distance between the two species, this is a plausible hypothesis. However, an alternative explanation is that the lesion-mimic and dwarf phenotypes in pt1a rice plants are due to the effects of an R protein that normally guards OsPt1a. Absence of OsPt1a in null mutants might trigger an ETI response that results in the observed autoimmune phenotypes. Such a scenario is reminiscent of RIN4 in Arabidopsis, which is guarded by two R proteins, RPM1 and RPS2 (Axtell and Staskawicz 2003). One possible explanation for why Pt1 was initially found to interact with Pto is that the latter kinase might have evolved as a ‘decoy’ of a host protein that plays a role in PTI by interacting with Pt1. A search of the predicted tomato proteome identified two malectin-like receptor kinases, Mal1 and Mal2 (Solyce11g072910 and Solyce06g005230), whose kinase domains have the highest sequence similarity to Pto. Malectin-like receptor kinases (also called Catharanthus roseus receptor-like kinases1-like kinases [CRLK1Ls]) have an extracellular region containing two subdomains with similarity to the carbohydrate-binding domain of the animal malectin protein, a transmembrane domain, and an intracellular kinase domain (Lindner et al. 2012). One well-studied CRLK1L in Arabidopsis is FERONIA (FER) (At3g51550), which plays a role in cell-to-cell communication during pollination but which also impacts host responses to certain bacterial and fungal pathogens (Keinath et al. 2010; Kessler et al. 2010; Masachis et al. 2016). Interestingly, FER becomes rapidly phosphorylated upon treatment of tissue with flg22 and it accumulates along with FLS2 in PM ‘rafts’ during PTI (Benschop et al. 2007; Keinath et al. 2010). At early time points, Arabidopsis fer mutants support slightly increased bacterial growth; however, at later time points, fer plants allowed less bacterial proliferation than wild-type plants (Keinath et al. 2010). Based on these results, it was hypothesized that FER may act with FLS2 (Keinath et al. 2010). Indeed, it was reported recently that, in Arabidopsis, FER promotes ROS production in response to flg22, associates with both FLS2 and BAK1, and plays a positive role in regulating PTI (Stegmann et al. 2017). We are currently investigating whether Mal1 and Mal2 play a role in PTI in tomato and, if they do, whether Pt1 proteins might facilitate their function.

MATERIALS AND METHODS

Plant material.

Nicotiana benthamiana accession Nm-1 (Bombarely et al. 2012) was grown for 4 to 6 weeks in a controlled environment chamber with 16 h of light and 65% relative humidity, with
temperatures of 24°C during light and 22°C during dark periods. The hpPti1 lines were generated by cloning a segment of the tomato Pti1 gene sequence (Fig. 2A) into pHELLSGATE8 (Helliwell et al. 2002) to obtain the hpPti1 silencing construct. Tomato RG-prf3 (Salmeron et al. 1996) plants were transformed by the Center for Plant Biotechnology Research at the Boyce Thompson Institute (Ithaca, NY, U.S.A.). We obtained one single-copy line (F27-36) and one multicopy line (F10-10) as well as two corresponding ‘azygous’ control lines that had lost one or more transgenes due to segregation. The hpPto line was made in the RG-PtoR background and was described previously (Pascuzzi 2006). All tomato plants were grown in a greenhouse without supplemental light for 4 to 5 weeks before use in pathogen assays or RNA-Seq experiments.

DNA cloning.

The constructs for VIGS were developed as previously described (Rosli et al. 2013). Suitable sequences were selected using the Sol Genomics Network (SGN) VIGS tool (Fernandez-Pozo et al. 2015a) and were cloned into pCR8/GW/TOPO (Invitrogen), and transformed using the LR Clonase II enzyme mix (Invitrogen), and transformed into Agrobacterium tumefaciens GV2260 (Hellens et al. 2000). Full-length tomato Pti1 genes were PCR-amplified and ligated into the pJLSmart Gateway entry vector (Invitrogen), and transformation into Agrobacterium tumefaciens was performed (Mathieu et al. 2014). Synthetic Pti1 versions were obtained by changing the codons of the 5’ gene portions to prevent binding of the hpPti1 fragment without altering the amino acid sequence. These synthetic portions were ordered from Integrated DNA Technologies and were fused to the wild-type 3’ sequences by PCR and were cloned into pJLSmart. Cysteine-to-serine substitutions were introduced, by PCR using complementary custom DNA oligonucleotides (Integrated DNA Technologies), following standard protocols. All Pti1 constructs were recombined into the binary plant expression vectors pGWB411 or pGWB541 (Nakagawa et al. 2007), using the LR Clonase II enzyme mix to obtain C-terminal FLAG and enhanced YFP fusions, respectively. These expression constructs were transformed into A. tumefaciens strains 1D1249 (for tomato) and GV3101 (for N. benthamiana) (Hellens et al. 2000; Wrobleswki et al. 2005). Vectors and constructs are shown in Supplementary Table S3.

Cell death suppression assay.

The cell death suppression assay in silenced N. benthamiana plants was performed as described previously (Chakravarthy et al. 2010; Clarke et al. 2013), with modifications. Leaf discs of tomato hpPti1 and azygous control plants were floated overnight in water in white, flat-bottom, 96-well plates (Greiner Bio-One). The water was removed 12 h later and a solution containing 100 nM flg22, 34 µg/ml luminol (Sigma-Aldrich), and 20 µg of horseradish peroxidase per milliliter (type VI-A; Sigma-Aldrich) was added. ROS production was quantified by means of luminescence output from each well over time. Luminescence was measured using a Synergy 2 microplate reader (BioTek).

Agrobacterium-mediated transient expression.

Preparation of Agrobacterium strains and plant infiltrations were performed as described previously (Mathieu et al. 2014; Kraus et al. 2016). Briefly, confirmed Agrobacterium strains were grown on lysogeny broth plates with the appropriate antibiotics for 36 to 48 h at 30°C. Cells were collected and suspended in infiltration buffer containing 10 mM MgCl₂, 10 mM MES (pH 5.7), and 200 µM acetoxyxynone (Sigma-Aldrich), the OD₆₀₀ for each strain was adjusted to 0.3, and cells were incubated for 1 h at room temperature. Leaves of N. benthamiana and tomato plants were infiltrated with needle-less syringes and the plants were placed in a controlled environment chamber (described above).

Protein detection.

Discs of Agrobacterium-transformed N. benthamiana leaf tissue were collected 2 days after agroinfiltration, were frozen, and were ground in liquid nitrogen. Proteins were extracted as described above, except that no phosphatase inhibitor was used for the protein extraction. FLAG-tagged proteins were detected using anti-FLAG-HRP (Sigma-Aldrich).

Fluorescence imaging.

Sections of N. benthamiana leaves expressing Pti1-YFP fusions were mounted on microscopy slides 2 days after agroinfiltration and were analyzed on a Leica DM5500 epifluorescence microscope. Images were acquired with a Retiga 2000R CCD camera (QImaging) using QCapture Pro software (QImaging).

qPCR.

Total RNA was extracted from N. benthamiana and tomato leaf tissue, using Plant RNA purification reagent (Invitrogen) according to the manufacturer’s instructions. RNA samples were additionally purified using RNeasy Mini columns (Qiagen).
and the isolated RNA was treated with RNase-free TURBO DNase (Ambion), following the respective manufacturers’ protocols. First-strand cDNA synthesis and qPCR were performed exactly as described previously (Breuillin-Sessoms et al. 2015). Cycle numbers of each plant were normalized (ΔCT) to PPA2 (Liu et al. 2012) for N. benthamiana and CBL1 (Pombo et al. 2014) for tomato. Means and confidence intervals of the transformed cycle numbers (2^(-ΔΔCT)) to form exactly as described previously (Breuillin-Sessoms et al. 2015). First-strand cDNA synthesis and qPCR were performed using tomato genome sequence version 2.4.2 as described previously (Rosli et al. 2013). The chosen cutoffs for differentially regulated genes were ≥3 RPKM in at least one of the treatments, ≥1.5-fold expression change, and P ≤ 0.05. To capture the number of differentially expressed genes in the two hpPt1 lines, the 3- and 6-h datasets were combined for each hpPt1 line and duplicate genes were removed. The RNA-Seq reads for visualizing Pt1a and Pt1b silencing were taken from the 6-h dataset, were normalized to CBL1 (Pombo et al. 2014), and were expressed in relation to the azygous control line. The PTI ‘marker genes’ were visualized the same way, based on the 3-h dataset. The Pt1a and Pt1b expression data in response to different PTI inducers (Rosli et al. 2013) were simply visualized in graph form.

Phylogenetic analysis.
Coding sequences for the Pt1 genes from tomato and N. benthamiana were obtained from SGN (Fernandez-Pozo et al. 2015b). Alignment and tree construction were performed with MEGA7, with the guidance of a step-by-step protocol (Hall 2013; Kumar et al. 2016). Specifically, DNA sequences were aligned using the MUSCLE method (align codons, default settings). Maximum likelihood (ML) substitution models were predicted using the default settings. The phylogenetic tree was estimated using the ML method (Tamura 3-parameter model, gamma distributed rates among sites, partial deletion of gaps/missing data, default settings otherwise). Reliability of the tree was estimated using the bootstrap method (1,000 replicates). The alignment file is provided as Supplementary Dataset S3.

Gene sequences.
Gene sequences are available from the SGN database under the following accession numbers: SfPt1a (Soly:0501223), NbPt1a (Niben101Scf01236g02003), NbPt1b (Niben101Scf01334g04008), NbPt1c (Niben101Scf01671g04002), and NbPt1d (Niben101Scf01820g00026). RNA-Seq reads have been deposited in the National Biotechnology Information Sequence Read Archive under accession number SRP076863, and analyzed data are available from the Tomato Functional Genomics database under accession number D014.

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Benschop, J. J., Mohammed, S., O’Flaherty, M., Heck, A. J., Slijper, M., and Menke, F. L. 2007. The isolation, RNA library preparation, and RNA-Seq analysis were performed using tomato genome sequence version 2.4.2 as described previously (Rosli et al. 2013).

Supplementary Table S4.

RNA sequencing analysis.
Tomato hpPt1 lines F27-36 (single hpPt1 copy) and F10-10 (multiple hpPt1 copies) along with an azygous control line were vacuum-infiltrated with DICh3000AvrPtoΔAvrPtoB at 5 × 10^5 CFU/ml. Tissue samples were taken 3 and 6 h after infiltration. The treatments were repeated in three successive weeks (three biological replicates). RNA isolation, library preparation, and RNA-Seq analysis were performed using tomato genome sequence version 2.4.2 as described previously (Rosli et al. 2013). The chosen cutoffs for differentially regulated genes were ≥3 RPKM in at least one of the treatments, ≥1.5-fold expression change, and P ≤ 0.05. To capture the number of differentially expressed genes in the two hpPt1 lines, the 3- and 6-h datasets were combined for each hpPt1 line and duplicate genes were removed. The RNA-Seq reads for visualizing Pt1a and Pt1b silencing were taken from the 6-h dataset, were normalized to CBL1 (Pombo et al. 2014), and were expressed in relation to the azygous control line. The PTI ‘marker genes’ were visualized the same way, based on the 3-h dataset. The Pt1a and Pt1b expression data in response to different PTI inducers (Rosli et al. 2013) were simply visualized in graph form.

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Gene sequences.
Gene sequences are available from the SGN database under the following accession numbers: SfPt1a (Soly:1212g099890), SfPt1b (Soly1205g053230), NbPt1a (Niben101Scf01236g02003), NbPt1b (Niben101Scf01334g04008), NbPt1c (Niben101Scf01671g04002), and NbPt1d (Niben101Scf01820g00026). RNA-Seq reads have been deposited in the National Biotechnology Information Sequence Read Archive under accession number SRP076863, and analyzed data are available from the Tomato Functional Genomics database under accession number D014.

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effector is directly phosphorylated by the chitin receptor and mediates rice immunity. Cell Host Microbe 13:347-357.


Author-Recommended Internet Resources

National Center for Biotechnology Information Sequence Read Archive: https://www.ncbi.nlm.nih.gov/sra
Nicotiana benthamiana Resources: http://btiscience.org/our-research/enabling-technologies/nicotiana-benthamiana
Pseudomonas-Plant Interaction database: http://www.pseudomonas-syringae.org
Sol Genomics database: https://solgenomics.net
Tomato Functional Genomics database: http://ted.bti.cornell.edu
Tomato Functional Genomics Digital Expression (RNA-seq) Experiment List database: http://ted.bti.cornell.edu/cgi-bin/TFGD/digital/home.cgi