

Tomato Protein Kinase 1b Mediates Signaling of Plant Responses to Necrotrophic Fungi and Insect Herbivory^W

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The tomato protein kinase 1 (TPK1b) gene encodes a receptor-like cytoplasmic kinase localized to the plasma membrane. Pathogen infection, mechanical wounding, and oxidative stress induce expression of TPK1b, and reducing TPK1b gene expression through RNA interference (RNAi) increases tomato susceptibility to the necrotrophic fungus *Botrytis cinerea* and to feeding by larvae of tobacco hornworm (*Manduca sexta*) but not to the bacterial pathogen *Pseudomonas syringae*. TPK1b RNAi seedlings are also impaired in ethylene (ET) responses. Notably, susceptibility to *Botrytis* and insect feeding is correlated with reduced expression of the proteinase inhibitor II gene in response to *Botrytis* and 1-aminocyclopropane-1-carboxylic acid, the natural precursor of ET, but wild-type expression in response to mechanical wounding and methyl-jasmonate. TPK1b functions independent of JA biosynthesis and response genes required for resistance to *Botrytis*. TPK1b is a functional kinase with autophosphorylation and Myelin Basic Protein phosphorylation activities. Three residues in the activation segment play a critical role in the kinase activity and in vivo signaling function of TPK1b. In sum, our findings establish a signaling role for TPK1b in an ET-mediated shared defense mechanism for resistance to necrotrophic fungi and herbivorous insects.

INTRODUCTION

Plants recognize attempted pathogen infection and activate defense responses to limit damage caused by disease with the mechanisms of recognition and response depending on the nature of the pathogen encountered. For necrotrophic pathogens, mechanisms of recognition and response are poorly understood. Current molecular models of pathogen response signaling have been derived largely from studies that center on plant interactions with biotrophic pathogens. In general, plants recognize pathogens through race-specific effectors or general elicitors referred to as pathogen-associated molecular patterns (PAMPs) (Jones and Dangl, 2006). Direct or indirect recognition of effectors by plant R proteins triggers a cascade of defense responses culminating in the hypersensitive response (HR) and resistance to specific races of biotrophic pathogens. However, there are no known race-specific effectors or corresponding R proteins described for necrotroph–plant interactions, and the HR may in fact enhance infection by necrotrophic pathogens (Govrin and Levine, 2000). Recently, an R protein was implicated in susceptibility to a necrotrophic pathogen (Lorang et al., 2007). PAMP-triggered basal responses are triggered by diverse pathogens, including necrotrophs, but neither the pattern recognition receptors nor the elicitors are characterized in many plant–

necrotroph interactions. In the case of necrotrophic pathogens, toxins, damage to the infected plant cell walls by hydrolytic enzymes, and the oligosaccharides released are all likely to play important roles in perception and response signaling. *Botrytis cinerea* and other necrotrophs produce toxins and cell wall degrading enzymes, including pathogen polygalacturonases (Prins et al., 2000). Oligogalacturonide (OG) fragments released from cell walls during pathogen attack or insect herbivory induce plant defense responses (Bishop et al., 1981; Doares et al., 1995; Ferrari et al., 2007). Consistent with this, plants may sense damage to the cell wall or recognize OGs through pattern recognition receptors. Additionally, PAMPs and race-specific elicitors induce systemic resistance against biotrophic pathogens (Dempsey et al., 1999; Mishina and Zeier, 2007), although the extent of resistance against necrotrophic pathogens is unclear. Thus, plant responses to necrotrophs and biotrophs are regulated through distinct and overlapping recognition and response mechanisms.

Networks of regulatory factors and defense molecules mediate responses to pathogens. Receptor-like protein kinases (RLKs) regulate recognition and early responses to diverse internal and external signals and frequently play critical roles in defense and symbiosis. RLKs are structurally diverse and constitute a large gene family in many plants with >600 members in *Arabidopsis thaliana* and ~1130 in rice (*Oryza sativa*) (Shiu et al., 2004). The majority of RLKs contain transmembrane and extracellular domains for sensing extracellular signals such as pathogen-derived molecules (Torii, 2000). *Xa21* encodes a rice leucine-rich repeat receptor-like kinase (LRR-RLK) that confers resistance to the bacterial blight pathogen *Xanthomonas oryzae* pv *oryzae* (Song et al., 1995). The *Arabidopsis* FLS2 and BAK1 proteins are both LRR-RLKs involved in recognition and

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signaling of flagellin (Chinchilla et al., 2007). Interestingly, BAK1 is also implicated in cell death control and resistance to necrotrophic pathogens (He et al., 2007; Kemmerling et al., 2007). LysM receptor-like kinase1 (LysM RLK1) from *Arabidopsis* is required for responses to chitin (Miya et al., 2007; Wan et al., 2008), a component of fungal cell walls recognized as a PAMP by plant cells. Disruption of LysM RLK1 impairs resistance to *Alternaria brassicicola* and *Erysiphe cichoracearum*, indicating its role in defense signaling. The tomato (*Solanum lycopersicum*) RLK SR160 is a receptor for systemin, whereas the SYMRK and HAR1 RLKs are involved in fungal and/or bacterial symbiosis (Krusell et al., 2002; Stracke et al., 2002). Receptor-like cytoplasmic kinases (RLCKs) are a subgroup of RLKs that lack transmembrane and extracellular domains but share a common monophyletic origin with RLKs (Shiu and Bleeker, 2001) and have also been implicated in defense responses. Pto and PBS1 are well-studied RLCKs involved in race-specific resistance to bacterial pathogens in tomato and *Arabidopsis*, respectively (Martin et al., 1993; Swiderski and Innes, 2001), whereas *Arabidopsis* BIK1 is a RLCK that mediates crosstalk between defense pathways in *Arabidopsis* (Veronese et al., 2006). All of these RLCKs act early in recognition or signaling of pathogen defense pathways.

Perception of pathogen-derived molecules initiates a variety of downstream events, including activation of mitogen-activated protein kinase (MAPK) cascades, phosphorylation of downstream targets, and activation of general and specific defense responses (Asai et al., 2002). FLS2-mediated MAPK activation regulates defense gene transcription and resistance to *Pseudomonas syringae* and *Botrytis* (Asai et al., 2002), suggesting that defense initiated by a bacterial PAMP can confer resistance to necrotrophic fungi. Perception of systemin by the membrane-bound receptor SR160 activates MPK1 and MPK2 and initiates the synthesis of jasmonate (JA) leading to the induction of defense gene expression, suggesting a role for MAPKs downstream of recognition in insect defense (Kandath et al., 2007). MAPKs, wound-induced protein kinase, and salicylic acid-induced protein kinase are activated during Cf-9/Avr9 interaction in tobacco (*Nicotiana tabacum*; Ludwig et al., 2005), and their *Arabidopsis* orthologs MPK3/6 function in activation of PAMP (Asai et al., 2002) and effector-triggered responses (Menke et al., 2004) in addition to signaling of reactive oxygen intermediates (Rentel et al., 2004). Other studies have demonstrated the activation of MAPKs by wounding, low temperature, drought, salinity, and reactive oxygen intermediates, suggesting extensive crosstalk in plant responses to external cues (Zhang et al., 1998; Romeis, 2001; Zhang and Klessig, 2001). Thus, signal transduction components may be shared among defense responses induced by effectors, PAMPs, insect herbivory, and even abiotic stress factors, and signals initiated by diverse pathogens may converge into conserved MAPK cascades. Downstream from MAPKs are transcription factors and defense molecules. In *Arabidopsis*, ERF, WRKY, and MYB transcription factor genes have been implicated in plant defense to necrotrophic pathogens (Berrocal-Lobo et al., 2002; Mengiste et al., 2003; Coego et al., 2005; Zheng et al., 2006). The functions of some, but not all, of these transcription factor genes are associated with the expression of *PDF1.2*, a plant defensin molecule with antifungal activities implicated in resistance to necrotrophic pathogens.

We studied the function of *tomato protein kinase 1b* (*TPK1b*) in basal resistance of tomato. We show that *TPK1b* encodes a membrane associated functional kinase required for resistance against *Botrytis* and insect herbivory in tomato. *TPK1b* is a homolog of the *Arabidopsis protein kinase 1b* (*APK1b*) (Hirayama and Oka, 1992) that has no known biological function and the *Brassica M* locus protein kinase (*MLPK*) implicated in self-incompatibility (Murase et al., 2004). Interestingly, *TPK1b* cDNA rescues the disease susceptibility of the *Arabidopsis bik1* mutant, suggesting that TPK1b and *Arabidopsis* BIK1 perform overlapping or redundant functions. Furthermore, *TPK1b* is required for ethylene (ET) responses of tomato seedlings, suggesting that the impaired defense against insects and *Botrytis* is due to attenuated activation of ET-dependent defense. These altered pathogen and ET responses in *TPK1b* RNA interference (RNAi) plants correlated with reduced expression of the tomato *proteinase inhibitor II* (*PI-II*) gene in response to *Botrytis* and 1-aminocyclopropane-1-carboxylic acid (ACC), but expression of *PI-II* in response to mechanical wounding and methyl-jasmonate (MeJA) was unaffected. Our results demonstrate that *TPK1b* is involved in ET-dependent but JA-independent defense, thus establishing TPK1b and ET as key regulators of insect and pathogen defense responses.

RESULTS

Cloning and Characterization of *TPK1b*

The *TPK1b* gene was identified during a screen for the tomato homolog of the *Arabidopsis Botrytis Induced Kinase1* (*BIK1*) gene (Veronese et al., 2006). We cloned several cDNAs that are predicted to encode receptor-like cytoplasmic protein kinases (RLCKs) sharing various levels of sequence similarity to *Arabidopsis BIK1*. Among these, *TPK1b* (SGN-U319927, SGN-E344457) is induced early during *Botrytis* infection, consistent with possible function early in a defense response pathway. *TPK1b* is induced as early as 12 h after *Botrytis* inoculation in tomato (Figure 1A). In addition, *P. syringae*, mechanical wounding, and oxidative stress caused by the herbicide paraquat strongly induce *TPK1b* gene expression (Figures 1B to 1D). *TPK1b* transcripts are abundant in leaves and are moderately expressed in stems (Figure 1E). Basal expression is low in green fruits but increases as the fruit ripens. Thus, *TPK1b* is transcriptionally regulated by biotic and abiotic agents, which suggests its role in plant responses to diverse signals.

The full-length *TPK1b* cDNA was cloned by 5' and 3' random amplification of cDNA ends (RACE). The gene-specific region of *TPK1b* was hybridized, under high-stringency conditions, to a DNA blot of tomato genomic DNA digested with different restriction enzymes (Figure 2A). A single hybridizing band was observed for *TPK1b* consistent with a single-copy gene in the genome. The *TPK1b* cDNA contains an open reading frame of 404 amino acids encoding an RLCK (Shiu and Bleeker, 2001) with an estimated molecular mass of 44.6 kD. TPK1b contains a consensus plant *N*-myristoylation motif [MGXXS/T(K)] (Boisson et al., 2003). Protein *N*-myristoylation is the covalent attachment of myristic acid to the N-terminal Gly residue of a nascent

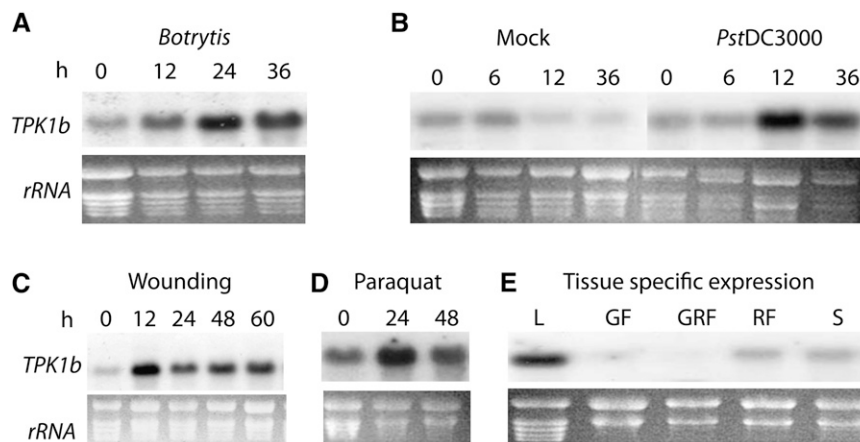


Figure 1. Basal and Induced Expression of the *TPK1b* Gene.

RNA gel blot analysis showing upregulation of *TPK1b* gene expression by

(A) *B. cinerea*.

(B) *P. syringae*.

(C) Mechanical wounding.

(D) Oxidative stress (paraquat, methyl viologen).

(E) Basal expression in fruit and leaf tissues.

Total RNA (15 μ g) was loaded per lane. rRNA staining is shown as a loading control. The experiments were repeated at least three times with similar results. Wild-type plants of the tomato cultivar CastlemartII were inoculated with pathogens or were treated with chemicals as described in Methods. h, h after inoculation or chemical treatment; L, leaf; GF, green fruit; GRF, green-red fruit; RF, red fruit; S, stem.

polypeptide that suggests potential membrane localization (Johnson et al., 1994). In addition, TPK1b has a protein kinase catalytic domain (KD, residues 68 to 353) that includes the protein kinase ATP binding region I and a Ser/Thr kinase active-site signature (VIYRDFKTSNILL, residues 197 to 209) (Figure 2B). The KD occupies the central part of TPK1b with all 11 conserved subdomains of protein kinases (Hardie, 1999). Similar to other Ser/Thr kinases, TPK1b subdomain VI contains a Lys residue in the DFKTSN motif, and subdomain VIII contains a G-T/S-XX-Y/F-X-APE motif (Hardie, 1999), suggesting that TPK1b is a Ser/Thr kinase (Stone and Walker, 1995). TPK1b KD contains a structural element known as the activation segment that lies between the conserved sequence motifs DFG (domain VII) and APE (domain VIII) (Johnson et al., 1996). Also, TPK1b is an RD-type protein kinase because it has the Asn residue in subdomain VIb (D-203) and an Arg immediately preceding this Asn residue (Dardick and Ronald, 2006).

Sequence and phylogenetic analyses were performed to decipher the exact relationship between TPK1b and related RLCKs. TPK1b is closely related to RLCKs from different plant species with overall sequence identities ranging from 60 to 80%. A predicted RLCK (CAO21648) from grapevine (*Vitis vinifera*) (Jaillon et al., 2007) shows the highest sequence identity (80%) to TPK1b. Two related *Arabidopsis* kinases, APK1b (At2g28930) and APK1a (At5g02290), are also closely related to TPK1b, sharing 75 and 68% sequence identities, respectively, to TPK1b. TPK1b is also highly similar to the *Brassica* MLPK (68% identity), the *Arabidopsis* proteins NAK (70%, At5g02290) and BIK1 (66%, At2g39660), and the BIK1-like protein (61%, At3g55450) (Figure 2B).

The highest conservation between TPK1b and related RLCK sequences is in the KD with 80 to 87% identities to the related RLCKs. TPK1b KD is flanked by two short nonkinase domains that have no obvious similarities to proteins of known function. The N-terminal region shows a sequence identity of 40 to 54% to related RLCKs. By contrast, the TPK1b C-terminal region consisting of 50 amino acids appears to be specific and shows no homology to any protein in the database, including *Arabidopsis* APK1b, MPLK, and CAO21648.

Next, we examined the phylogenetic relationship between TPK1b and other RLCKs (Figure 2C). A rooted phylogenetic tree was constructed using the neighbor-joining phylogeny (Saitou and Nei, 1987) based on the full KD of TPK1b and related kinases aligned with ClustalW (Thompson et al., 1994). The tomato PTO kinase or the PBS1 kinases were used as an outgroup. PTO and PBS1 are RLCKs and yet are not closely related to the TPK1b based on sequence analysis. The phylogenetic analysis clusters TPK1b, APK1b, APK1a, grapevine CAO2648, and MLPK kinases in the same clade, consistent with the sequence analysis above (Figure 2B). The next related clade contains *Arabidopsis* NAK1, BIK1, and the BIK1-like proteins (Figure 2C). The functions of most of these related RLCKs have not been determined. APK1a and APK1b show kinase activities in vitro, but their biological function is not determined (Hirayama and Oka, 1992), whereas MLPK is involved in self-incompatibility in *Brassica rapa* (Murase et al., 2004).

TPK1b Is Required for Resistance to *Botrytis*

To study the biological function of *TPK1b*, we generated tomato *TPK1b* RNAi plants that are reduced in *TPK1b* gene expression.

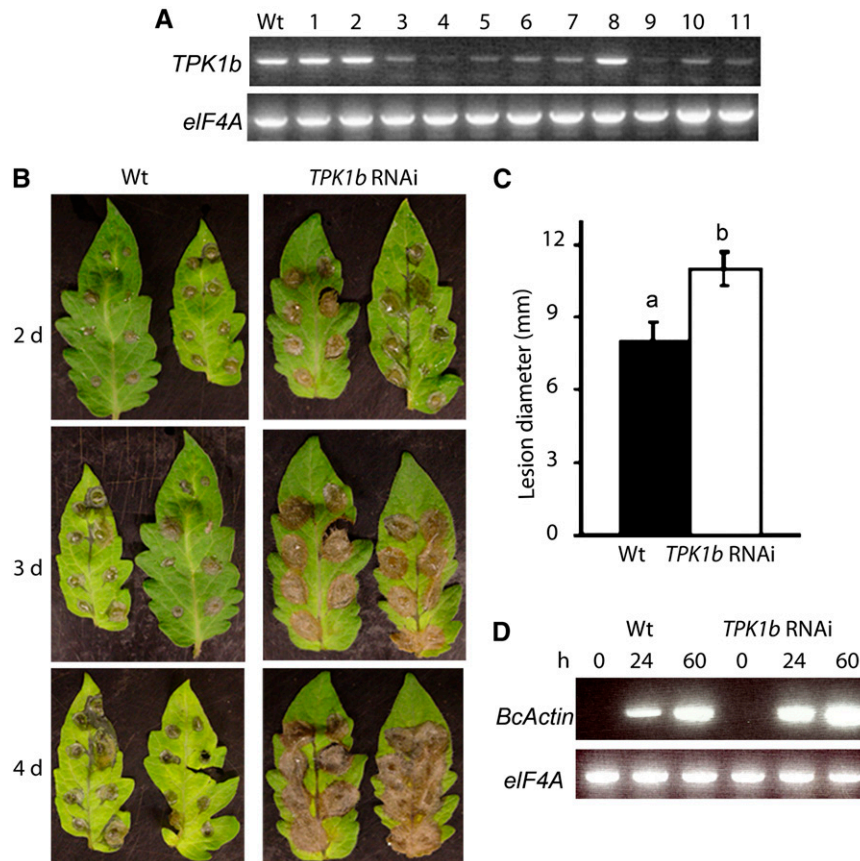


Figure 3. *TPK1b* Is Required for Resistance to *Botrytis*.

(A) RT-PCR from wild-type and *TPK1b* RNAi lines showing *TPK1b* transcript levels.

(B) *TPK1b* RNAi plants show increased susceptibility to *Botrytis*.

(C) Disease lesion size in *Botrytis*-inoculated leaves at 2 DAI.

(D) Accumulation of *Botrytis ActinA* mRNA as a measure of fungal growth in drop-inoculated leaves.

In **(A)**, numbers above the lanes indicate independent RNAi transgenic lines, and the RNA used for the RT-PCR was extracted from *Botrytis*-inoculated leaves. In **(C)**, the values represent the mean \pm SE from a minimum of 60 lesions. Analysis of variance and Duncan's multiple range test were performed to determine the statistical significance of the differences in the mean disease lesion sizes using SAS software (SAS Institute, 1999). Bars with different letters are significantly different from each other at $P = 0.05$. In **(D)**, RT-PCR was performed using the *Botrytis ActinA* gene-specific primers. Experiments were repeated at least three times with similar results. h, h after inoculation; d, days after inoculation; *BcActin*, *B. cinerea ActinA* gene.

TPK1b RNAi plants showed increased susceptibility to *Botrytis* with larger disease lesions than wild-type plants at 2 to 4 d after inoculation (DAI) (Figures 3B and 3C). At 3 to 4 DAI, the lesions expanded rapidly and coalesced in *TPK1b* RNAi leaves, whereas lesions expanded more slowly and remained discrete at 4 DAI in wild-type leaves. The *TPK1b* RNAi plants also supported more *Botrytis* growth as measured by the amount of the fungal *ActinA* mRNA in the inoculated plants (Figure 3D; Benito et al., 1998). Similarly, when we suppressed *TPK1b* gene expression in the Micro-Tom cultivar using virus-induced gene silencing (VIGS) (Liu et al., 2002), we observed increased susceptibility to *Botrytis* (see Supplemental Figure 1A online). In the silenced plants, disease developed faster and resulted in tissue collapse in leaves that emerged after the VIGS treatment, consistent with the pattern of silencing in control plants silenced for the tomato phytotene desaturase gene (see Supplemental Figure 1B online,

right). *TPK1b* RNAi plants inoculated with the virulent strain of the bacterial pathogen *P. syringae* showed no altered responses (see Supplemental Figure 2 online). Thus, our data suggest that *TPK1b* plays an important role in limiting both disease symptoms and fungal growth in tomato plants inoculated with *Botrytis* but plays no role in resistance to *P. syringae*.

TPK1b Is Required for Resistance to Insect Feeding

Induction of *TPK1b* gene expression by wounding and previous reports on tomato defense response to *Botrytis* (Diaz et al., 2002) suggest a mechanistic link between insect and necrotrophic pathogen defense. Therefore, we tested tomato *TPK1b* RNAi lines for susceptibility to feeding by tobacco hornworm (*Manduca sexta*) larvae. In a detached leaf assay, newly hatched tobacco hornworm larvae were placed on leaves of 8-week-old

tomato plants to initiate a feeding trial. Larvae and leaf weights were determined when the foliage of *TPK1b* RNAi plants was ~90% consumed. In parallel, control detached leaves were kept without larvae to determine leaf weight due to desiccation. The larvae consumed significantly more foliage of *TPK1b* RNAi than from the wild type (see Supplemental Figure 3A online). After 5 d of larval feeding, 72% of *TPK1b* RNAi leaves were consumed relative to only 35% of the wild-type plants (Figure 4A). Losses of leaf weight due to desiccation were relatively minor and comparable in both genotypes. The average weights of larvae feeding on *TPK1b* RNAi plants were 2.3-fold more than larvae recovered

from wild-type leaves (Figure 4B; see Supplemental Figure 3B online). In an independent assay, larvae were weighed and placed on whole plants growing in the greenhouse (Figure 4C, left). The *TPK1b* RNAi plants were defoliated due to the larval feeding (Figure 4C, right). The average weight gain of larva feeding on *TPK1b* RNAi plants was ~2.4-fold more than those feeding on wild-type plants regardless of the duration of feeding (Figure 4D), suggesting a clear loss of resistance to larval feeding in the *TPK1b* RNAi plants. Thus, *TPK1b* functions in tomato resistance to insects and suggests a shared mechanism against pathogens and insect pests.

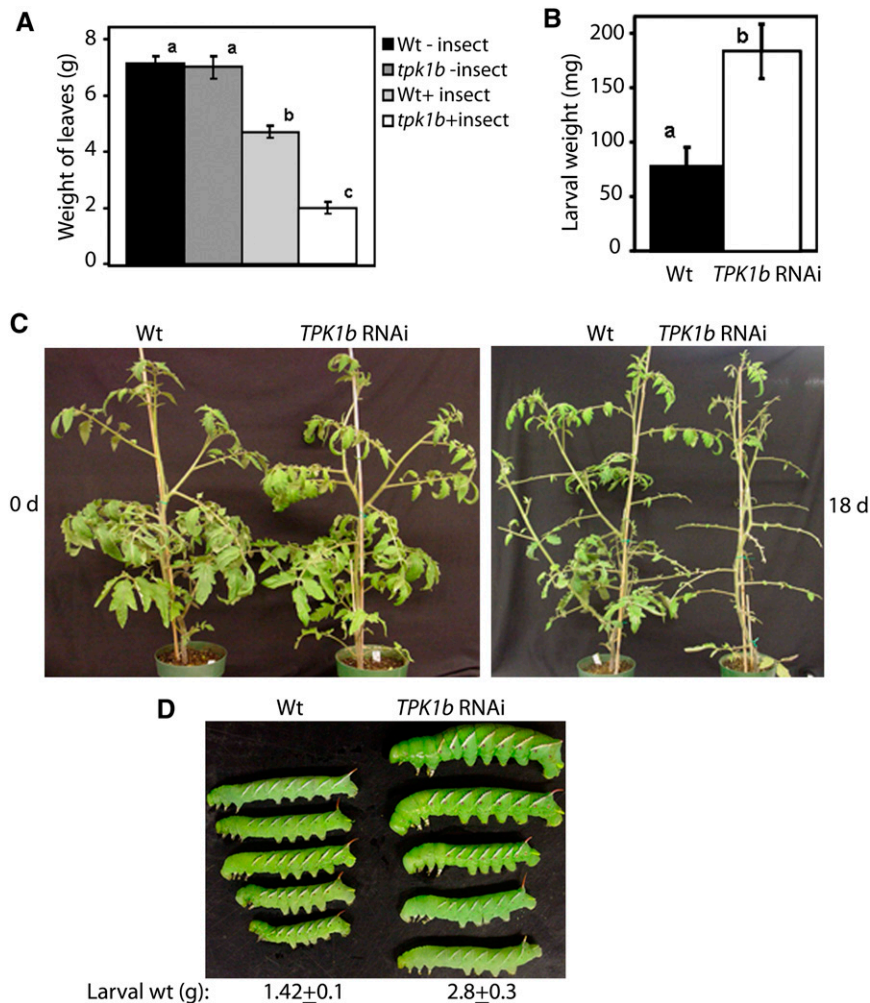


Figure 4. *TPK1b* RNAi Plants Show Reduced Resistance to Tobacco Hornworm.

- (A) Leaf weight recovered at the end of 5 d of feeding trial on detached leaves ($n = 25$).
- (B) Larval weight recovered at the end of 5 d of feeding trial on detached leaves ($n = 10$).
- (C) Wild-type and *TPK1b* RNAi plants at the beginning (left) and end (right) of tobacco hornworm feeding trial on whole plants.
- (D) Size of larvae recovered at the end of tobacco hornworm feeding trial on whole plants.

The feeding trails on detached leaf or whole plants were each repeated three times with similar results. In each experiment of detached leaf and whole-plant assays, six and 10 newly hatched larvae (~9 to 11 mg each), respectively, were placed on at least five 8-week-old plants of each genotype. Larvae were allowed to feed on the same plant for the duration of the trial. In (A) and (B), the data represent the mean \pm SE from three different experiments. Bars with different letters are significantly different from each other ($P = 0.05$). Analysis of variance and Duncan's multiple range test were performed as described in the legend for Figure 3.

TPK1b Functions Independent of Tomato JA Synthesis and Responses

To establish the relationship between *TPK1b* and genes regulating tomato wound and insect responses, we first determined the *Botrytis* resistance of the tomato mutants *suppressor of prosystemin-mediated response1* (*spr1*; Lee and Howe, 2003), *spr2* (Li et al., 2003), *jasmonate-insensitive1* (*jai1*; Li et al., 2004), *defenseless1* (*def1*; Howe et al., 1996), and *acyl-CoA oxidase* (*acx1*; Li et al., 2005), which are impaired in JA synthesis and wound responses. All of the tomato mutants except *spr1* showed increased susceptibility to *Botrytis* compared with the CasInt-martII wild type (Figure 5A). In the susceptible mutants, the disease lesions were significantly larger than in wild-type plants at 2 DAI (Figures 5A and 5B). In addition, the mutants supported higher levels of *Botrytis* growth as measured by the increased accumulation of *Botrytis ActinA* RNA (Figure 5C). Interestingly, basal resistance of *spr1* against *Botrytis* was comparable to the wild type. *Spr1* is an upstream regulator of JA and systemin responses, and the mutant's basal resistance to *Botrytis* implies the existence of another factor upstream of JA that controls responses to *Botrytis*. Thus, in tomato, JA levels and responses are required for resistance to *Botrytis*, but deficiency in systemin

responses appears to have no effect. However, the *Botrytis* and wound-induced expression of *TPK1b* is largely independent of JA functions defined by *SPR2*, *JAI1*, *DEF1*, and *ACX1* or acts upstream of these genes (Figures 5D and 5E).

TPK1b Mediates Tomato Responses to ET

To identify possible links between the function of *TPK1b* and plant hormones mediating defense and stress responses, we tested *TPK1b* RNAi plants for hormone sensitivity of seed germination or seedling growth. The *TPK1b* RNAi plants showed wild-type responses to abscisic acid and MeJA (see Supplemental Figure 4 online). However, *TPK1b* RNAi seedlings exhibit altered ET responses as measured by the absence of the triple response (Figure 6A). Wild-type tomato seedlings grown in the dark in the presence of ACC displayed a typical triple response, consisting of shortened hypocotyls, reduced root length, and enhanced curvature of the apical hook compared with those grown without ACC (Figures 6A and 6B) (Bleecker et al., 1988). However, in the presence of increasing concentrations of ACC in the dark, *TPK1b* RNAi seedlings retained elongated hypocotyls with no enhancement of the apical hook and no significant reduction in root length, all features of seedlings not responding

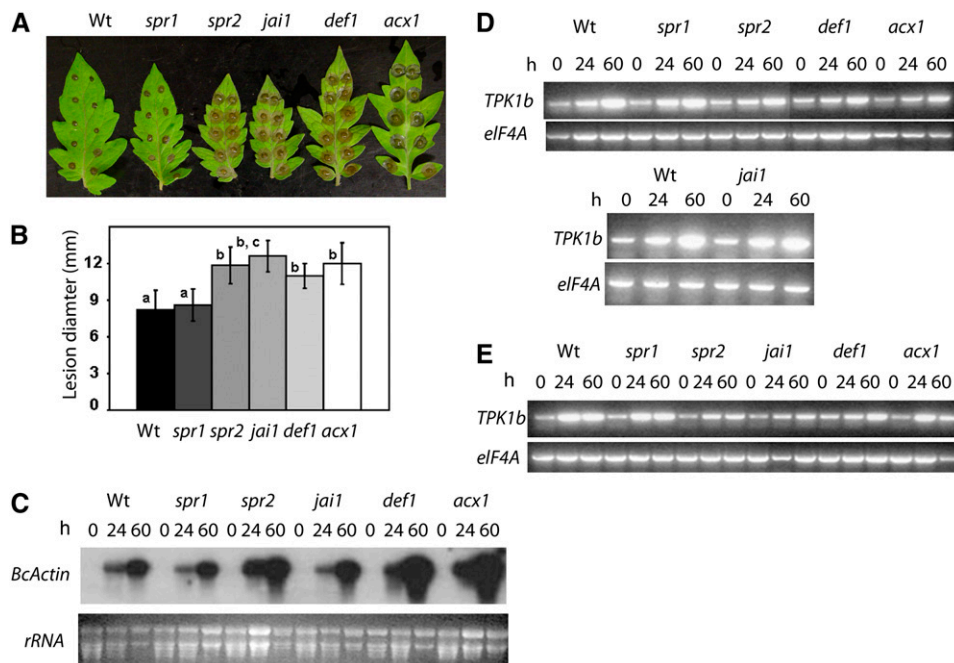


Figure 5. Tomato JA and Wound Response Genes Are Required for *Botrytis* Resistance.

(A) *Botrytis* disease symptoms on leaves of indicated genotypes.

(B) Disease lesion size.

(C) Fungal growth.

(D) *Botrytis*-induced *TPK1b* gene expression.

(E) Wound-induced *TPK1b* gene expression in the tomato JA and/or wound response mutants.

The pictures in **(A)** and lesion sizes in **(B)** are from 3 DAI with *Botrytis* (3×10^5 spores/mL). Fungal growth in **(C)** is based on the accumulation of *Botrytis ActinA* RNA as detected by RNA gel blot hybridization. In **(C)**, total RNA (15 μ g) was loaded per lane. In **(D)** and **(E)**, RT-PCR was performed as described in Methods. The tomato *translation initiation factor* (*eIF4A*) gene was amplified in parallel to demonstrate relative quality and quantity of the cDNA. Experiments were repeated at least three times with similar results. h, h after inoculation.

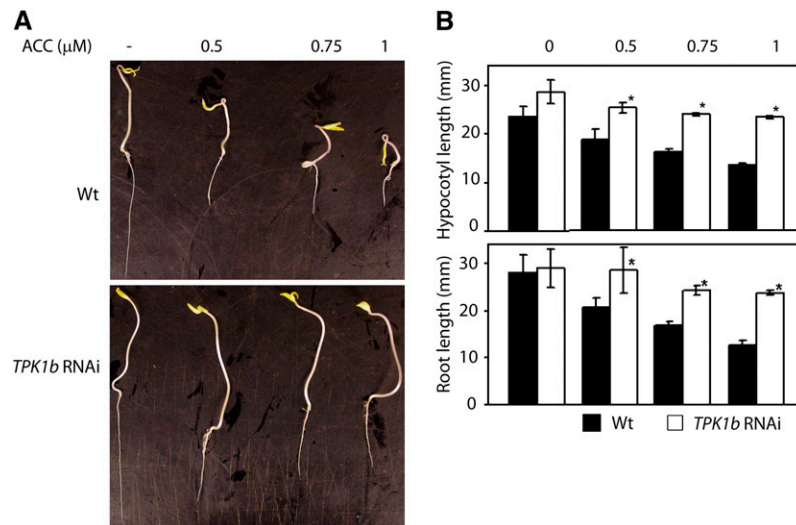


Figure 6. ET Responses of *TPK1b* RNAi and Wild-Type Tomato Seedlings.

(A) The triple response in the wild type but its absence in *TPK1b* RNAi seedlings grown in the dark in the presence of increasing concentrations of ACC. **(B)** Hypocotyl (top) and root (bottom) length of wild-type (black) and *TPK1b* RNAi (white) seedlings grown in the dark at varying concentrations of ACC. The pictures in **(A)** and the measurements in **(B)** were taken at 6 d after plating. Pictures are representative of seedlings of the corresponding treatments. These experiments were repeated at least five times. The asterisk indicates that the data are statistically significant from the corresponding wild-type control ($P = 0.05$).

to ET (Figures 6A and 6B). *TPK1b* RNAi seedlings grown in the dark without ACC have longer hypocotyls with no apical hook and longer roots consistent with the loss of ET response (Klee, 2004). Thus, tomato *TPK1b* has a regulatory function in ET responses of tomato plants.

***TPK1b* Regulates ET and *Botrytis*-Induced Defense Gene Expression**

To determine the molecular basis of *TPK1b* function, we studied the expression of tomato defense-related genes. Uninfected wild-type plants show low constitutive expression of *PI-II* but increased expression following *Botrytis* infection (Figure 7A). In *TPK1b* RNAi plants, the *Botrytis*-induced expression of *PI-II* gene was abolished. After wounding, the expression of *PI-II* increased starting at 6 h after wounding with comparable patterns of expression in both wild-type and *TPK1b* RNAi plants. Also, *PI-II* was induced by MeJA in wild-type and *TPK1b* RNAi plants to roughly similar levels (Figure 7B). Thus, *TPK1b* RNAi plants respond normally to JA and wounding, further supporting the hypothesis that *TPK1b* functions independent of JA and wound response pathways. By contrast, *PI-II* gene expression was induced by ACC in wild-type plants, but its induction by ACC was abolished in *TPK1b* RNAi plants, confirming our observation on the altered responses of *TPK1b* RNAi seedlings to ACC (Figure 7B). The *Botrytis* and wound-induced expression of ACC synthase showed similar profiles in *TPK1b* RNAi compared with wild-type plants (Figures 7C and 7D, top rows). Thus, the increased susceptibility of *TPK1b* RNAi plants to *Botrytis* and tobacco hornworm correlates with reduced *PI-II* expression after exogenous ACC application and *Botrytis* infection.

Basal and induced expression of tomato *PR-1*, a salicylic acid (SA)-regulated defense gene, was not altered in response to *Botrytis* in *TPK1b* RNAi plants but showed a slight reduction of expression in response to wounding (Figures 7C and 7D, middle rows). These results indicate that *TPK1b* does not mediate SA responses and that *TPK1b*-mediated *Botrytis* responses have little effect on *Botrytis*-induced *PR-1* gene expression. However, *TPK1b* is required for full wound-induced *PR-1* expression. *Allene oxide synthase 2* (Howe et al., 2000), a late JA-induced gene in tomato, was also induced by *Botrytis* and wounding independent of *TPK1b* (Figure 7E).

***TPK1b* Rescues the Phenotype of the *Arabidopsis* *bik1* Mutant**

TPK1b and *BIK1* are not orthologs, but both are positive regulators of *Botrytis* resistance and share similarities in sequence, structure, and patterns of pathogen-induced gene expression. To determine whether *TPK1b* can functionally substitute for *BIK1*, we generated *Arabidopsis bik1* plants expressing 35S:*TPK1b* (Figure 8A). We also expressed the *TPK1b* gene in the wild-type plants. *Arabidopsis bik1*;35S:*TPK1b* #1 and #2 and *BIK1*:35S:*TPK1b* (35S:*TPK1b*) line #4 were chosen for further experiments due to their high levels of *TPK1b* gene expression. After spray inoculation with *Botrytis*, the *bik1* mutant was highly susceptible (Veronese et al., 2006), but *bik1*;35S:*TPK1b* plants showed levels of resistance comparable to the wild type (Figure 8B). Additionally, the 35S:*TPK1b* (line #4) plants were more resistant to *Botrytis* than the wild-type plants, showing restricted disease symptoms (Figure 8B, top panel). After drop inoculation with *Botrytis*, disease lesions on *bik1* expanded and completely

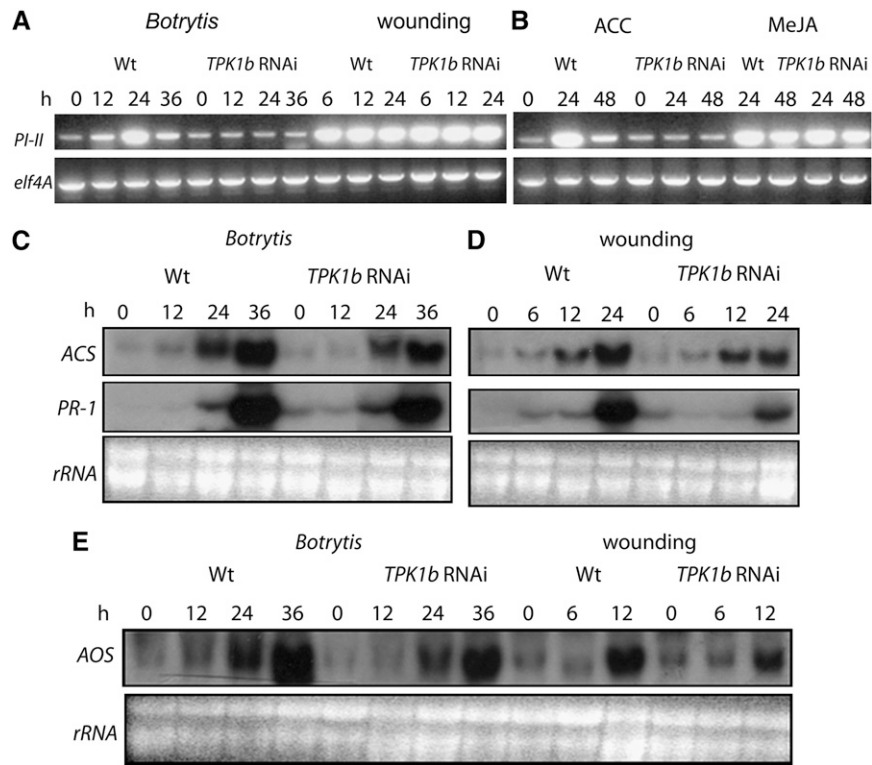


Figure 7. Expression of Tomato Defense-Related Genes in Wild-Type and *TPK1b* RNAi Tomato Plants.

(A) and **(B)** RT-PCR showing the expression of tomato *PI-II* during *Botrytis* infection or mechanical wounding **(A)** and ACC or MeJA treatment **(B)**. **(C)** and **(D)** RNA gel blot showing the expression of tomato *ACC synthase* and *PR-1* during *Botrytis* infection **(C)** and mechanical wounding **(D)**.

(E) Expression of *allene oxide synthase2* during *Botrytis* infection or mechanical wounding.

In **(C)** to **(E)**, total RNA (15 μ g) was loaded per lane, and rRNA is shown as a loading control. Experiments were repeated at least three times with similar results. h, h after *Botrytis* inoculation or wounding treatment; ACS, *ACC synthase*; AOS2, *allene oxide synthase2*.

macerated the entire leaf at 5 DAI (Figure 8B, bottom panels). The 35S:*TPK1b* transgenic plants were resistant with smaller disease lesions and with no significant lesion growth between 3 and 5 DAI. *Botrytis* growth was significantly lower in the 35S:*TPK1b* plants, further confirming the increased *Botrytis* resistance (Figure 8C). However, the increased resistance of 35S:*TPK1b* relative to the *bik1*;35S:*TPK1b* plants could result from higher levels of *TPK1b* expression in the former (Figures 8A and 8B).

Similarly, the *bik1*;35S:*TPK1b* plants rescued the *A. brassicicola* susceptibility of the *bik1* mutant to the wild-type level of resistance (see Supplemental Figure 5 online). As expected, the *bik1* plants were susceptible to *A. brassicicola*, with larger disease lesions and increased pathogen reproduction. The 35S:*TPK1b* transgenic plants show very limited disease symptoms with smaller disease lesions and lower spore counts at 3 and 6 DAI (see Supplemental Figure 5 online). In addition, the shorter primary root length observed in the *bik1* mutant was restored to the wild-type level by 35S:*TPK1b* (see Supplemental Figure 6 online). Thus, ectopic expression of *TPK1b* rescued the *bik1* phenotypes, and *TPK1b* was sufficient to confer increased resistance to two necrotrophic fungi. These data indicate that *TPK1b* and *Arabidopsis BIK1* may perform similar functions in their respective species.

TPK1b Is a Functional Protein Kinase, and Specific Activation Domain Residues Are Important for Kinase Activity and Biological Function

TPK1b is a predicted protein with a large catalytic KD. TPK1b and closely related kinases share particularly high sequence identities in the activation domain with many highly conserved phosphorylatable residues (Figure 9A). Four out of the seven phosphorylatable residues are invariant in RLCKs related to TPK1b. To determine whether TPK1b is a functional protein kinase, we expressed TPK1b as a glutathione S-transferase (GST) fusion protein in *Escherichia coli* and affinity purified the fusion protein using glutathione resins. The purified protein was incubated in a reaction buffer containing (γ - 32 P) with or without Myelin Basic Protein (MBP), an artificial kinase substrate, separated by SDS-PAGE, and autoradiographed. The recombinant TPK1b wild-type protein showed autophosphorylation that was visible as a band of \sim 70 kD corresponding to the combined molecular mass of TPK1b and GST and also phosphorylated MBP, demonstrating that TPK1b is a functional kinase (Figure 9B). GST alone did not show any kinase activity (see Supplemental Figure 7 online).

We also purified TPK1b proteins substituted at selected residues to determine sites that regulate kinase activity and gain

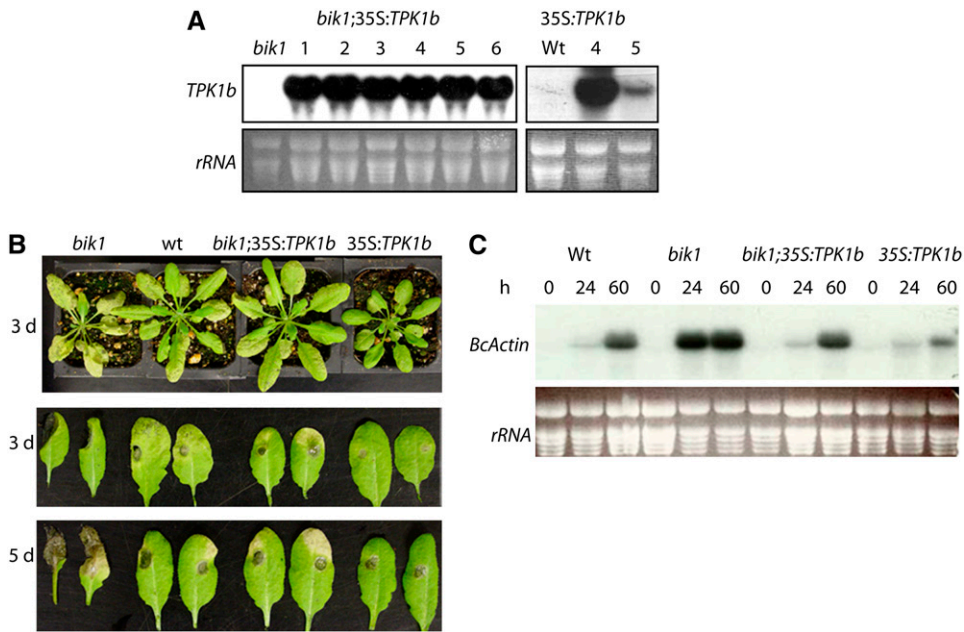


Figure 8. Ectopic Expression of Tomato *TPK1b* Suppresses the *Arabidopsis bik1* Mutant Phenotypes and Confers Increased Resistance to *Botrytis*.

(A) RNA blot showing expression of 35S:*TPK1b* in *bik1* and wild-type plants.

(B) *Botrytis* resistance 3 d after spray-inoculation (top panel) and 3 and 5 d after drop inoculation (bottom panels).

(C) Accumulation of the *Botrytis ActinA* mRNA in spray-inoculated plants.

In **(A)** and **(C)**, total RNA (15 μg) was loaded per lane, and rRNA is shown as a loading control. Experiments were repeated at least three times with similar results. h, h after inoculation; d, days after inoculation.

insight into how TPK1b functions. A total of 10 TPK1b residues were mutated, and the recombinant proteins were purified. Seven phosphorylatable residues, Ser, Thr, and Tyr, within the activation segment of TPK1b and three residues in the N-terminal region of the KD were individually substituted to Ala and assayed for kinase activity (Figure 9B). Coomassie blue staining after electrophoresis shows that the TPK1b recombinant proteins with substitutions were expressed at comparable levels (Figure 9B, bottom panel). However, the various mutants affected autophosphorylation and/or phosphorylation of MBP (Figure 9B). The TPK1b^{K106A} mutation outside the activation domain eliminated kinase activity. The Lys residue at 106 is invariant in all kinases (Hanks and Hunter, 1995). The TPK1b^{T65A} mutation completely eliminated autophosphorylation and significantly reduced phosphorylation of MBP. In the activation segment, mutation of three residues, TPK1b^{T238}, TPK1b^{T243}, and TPK1b^{Y246}, abolished autophosphorylation and phosphorylation of MBP. Thus, consistent with other kinases, three activation segment residues are required for TPK1b kinase activity (Rathjen et al., 1999; Wang et al., 2005). Kinase activity is frequently controlled by the activation domain, defined by the conserved amino acids D₂₂₁FG and APE₂₅₂ in TPK1b (Johnson et al., 1996). In many RD-type RLKs, autophosphorylation of the activation loop is required for general kinase activity. Consistent with this notion, three TPK1b activation segment residues required for autophosphorylation also eliminated phosphorylation of MBP. By contrast, autophosphorylation of N- and C-terminal residues in the KD generates docking sites for

specific downstream substrates and affects catalytic activity toward those substrates (Wang et al., 2005).

We took advantage of suppression of *Arabidopsis bik1* disease susceptibility by 35S:*TPK1b* to determine the biological function of TPK1b substitution mutants in vivo. The site-directed TPK1b mutants above were cloned into a plant expression vector and transformed into *Arabidopsis bik1*. The expression of all *TPK1b* constructs were verified by RNA gel blots (Figure 9C). The untransformed wild-type, *bik1*, *bik1*;35S:*TPK1b*, and the 10 TPK1b substitution lines were compared for disease resistance and changes in growth related traits (Figures 9D and 9E). In the case of the TPK1b^{K106A} mutant, no rescue of the *bik1* disease resistance or growth-related phenotypes were observed. The three mutations in the activation loop (TPK1b^{T238A}, TPK1b^{T243A}, and TPK1b^{Y246A}) that eliminated kinase activity of TPK1b failed to rescue *bik1* susceptibility to *A. brassicicola* and *Botrytis*, indicating these residues are essential for TPK1b signaling function in planta (Figures 9D and 9E). Individual TPK1b mutants, TPK1b^{D212A}, TPK1b^{T230A}, TPK1b^{S234A}, TPK1b^{S237A}, and TPK1b^{Y244A}, had no effect on kinase activity and also suppressed the disease phenotype of *bik1*. Interestingly, the TPK1b^{Y243A} mutations that failed to rescue the disease phenotype partially rescued the *bik1* growth defects. Expression of these constructs resulted in plants that are intermediate between the wild-type and *bik1* plants (Figure 9E; see Supplemental Figure 8 online). The T238A substitution fully restored the growth phenotype of *bik1*, although it was fully susceptible to disease. These data

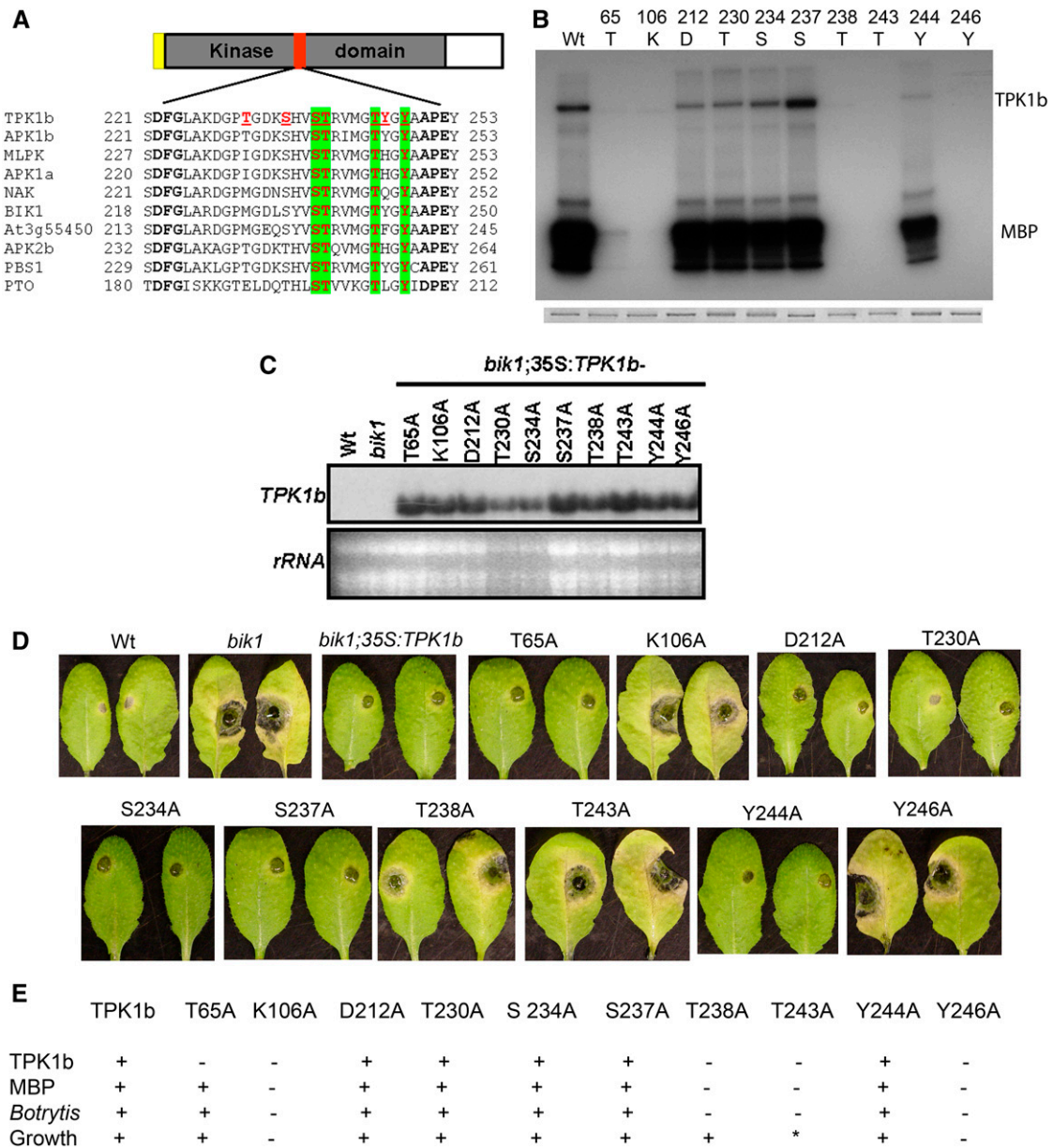


Figure 9. TPK1b Is a Functional Protein Kinase That Localizes to the Plasma Membrane.

(A) Alignment of the activation segment of TPK1b and related kinases.

(B) Autophosphorylation and MBP phosphorylation activities of GST-TPK1b and its mutant derivatives in vitro. Top panel, autoradiogram of the gel; bottom panel, Coomassie blue staining.

(C) RNA blot showing transgenic expression of TPK1b substitution mutants in the *Arabidopsis* *bik1* mutant.

(D) Responses of *bik1* plants expressing TPK1b and its substitution mutants to *A. brassicicola* inoculation.

(E) Summary of kinase activity, *Botrytis* response, and growth-related phenotypes of the *Arabidopsis* *bik1* expressing TPK1b wild type and substitution mutants.

In **(A)**, TPK1b activation segment phosphorylatable residues are in red and underlined. Residues that are phosphorylatable and conserved in TPK1b-related kinases are shaded green. In **(D)**, *A. brassicicola* disease symptoms are from 4 DAI. In **(E)**, the asterisk indicates partial complementation; +, rescues phenotypes or shows auto (TPK1b) or MBP phosphorylation activity; -, fails to rescue the *bik1* growth phenotype or shows no TPK1b or MBP phosphorylation activity.

demonstrate that some residues are essential for disease resistance but are dispensable for growth and that the two functions of TPK1b can be partially uncoupled. Consistent with the recent characterization of *Arabidopsis* BIR1 (Wang et al., 2005), the correlation between the in vitro kinase data and the biological function of TPK1b suggests that in vitro kinase activity is a good indicator for in vivo kinase activity and function. Interestingly, TPK1b^{T65A} abolished kinase activity but suppressed the disease susceptibility of the *bik1* mutant. The residues in the activation loop that are required for TPK1b kinase activity and disease resistance are also invariant in all RLCKs related to TPK1b, possibly indicative of a common regulatory mechanism among these RLCKs (Figure 9A). The TPK1b^{Y246} substitution falls in the region of the activation segment referred to as the P+1 loop, which has been implicated in recognition and binding of protein substrates (Johnson et al., 1996).

The N-Myristoylation Motif Is Required for Disease Resistance Function of TPK1b but Not for Its Association with the Plasma Membrane

To determine the subcellular localization of TPK1b, the full-length TPK1b was translationally fused with green fluorescent protein (GFP), and the chimeric protein was transiently expressed in *Nicotiana benthamiana* leaf tissues through *Agrobacterium* infil-

tration. The TPK1b-GFP fusion protein was observed at the plasma membrane, whereas cells expressing GFP alone (control, top row) exhibited signal around the nucleus and in the cytosol (Figure 10A). We also generated TPK1b^{G2A} constructs where the invariant penultimate Gly residue that is essential for myristoylation was substituted with Ala. The TPK1b^{G2A}-GFP localized to the plasma membrane similar to the wild-type TPK1b-GFP (Figure 10A, bottom), indicating that TPK1b membrane localization is independent of the N-terminal myristoylation motif. N-terminal myristoylation motifs have been implicated in membrane localization, stabilization of protein structure, and protein-protein interactions (Johnson et al., 1994). The N-terminal myristoylation motif is also found in all of the TPK1b-related RLCKs (Kakita et al., 2007; see Supplemental Figure 9 online). BIK1 and MLPK localize to the plasma membrane (Murase et al., 2004; Veronese et al., 2006).

To determine whether the N-myristoylation signal in TPK1b is required for *TPK1b* disease resistance function, we transformed 35S:*TPK1b*^{G2A}-GFP constructs into *bik1*. All of the constructs were expressed based on RNA blot analysis (Figure 10B). However, the expression of 35S:*TPK1b*^{G2A}-GFP failed to complement the *bik1* disease susceptibility (Figure 10C, bottom), although 35S:*TPK1b*-GFP rescued the *A. brassicicola* susceptibility of *bik1* to wild-type levels. 35S:*TPK1b*^{G2A}-GFP also failed to confer increased resistance to *A. brassicicola* when expressed in the

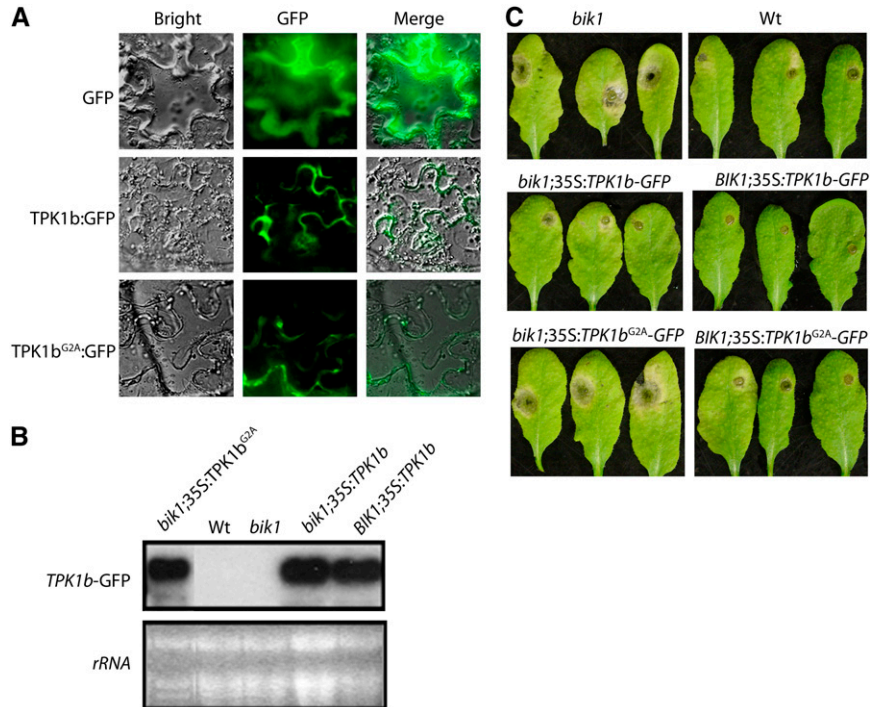


Figure 10. The N-Myristoylation Motif Is Not Required for TPK1b Subcellular Localization but Is Required for Its Disease Resistance Function.

(A) Subcellular localization of the GFP control, TPK1b-GFP, and TPK1b^{G2A}-GFP fusion proteins in *N. benthamiana* cells.

(B) RNA blot showing transgenic expression of *TPK1b*-GFP and *TPK1b*^{G2A}-GFP.

(C) Resistance of *Arabidopsis* plants expressing *TPK1b* and *TPK1b*^{G2A} to *A. brassicicola*.

The experiments in (A) and (C) were repeated at least three times with similar results.

wild-type plants, as expected for 35S:*TPK1b* (Figure 10C, bottom). Thus, *TPK1b* *N*-myristoylation signal is required for the disease resistance function but not for localization to the plasma membrane.

TPK1b Is Required for Normal Fruit Structure and Seed Set

Tomato *TPK1b* RNAi plants show normal vegetative growth under our growth conditions (see Methods). However, we observed altered fruit structure and reduced number of seeds when we examined six *TPK1b* RNAi transgenic lines with varying levels of *TPK1b* expression (Figures 3A and 11A to 11C). The *TPK1b* RNAi lines produced reduced numbers of seeds in a ratio that correlated with the reduced levels of *TPK1b* gene expression (Figure 11C). The RNAi line #9 has the greatest reduction in *TPK1b* expression, and its fruits were parthenocarpic, containing no developed seeds, contained almost no jelly, and were completely filled with flesh compared with Castlemarill wild-type fruits (Figure 11A). *TPK1b* RNAi line #4 also showed a significant reduction in number of seeds/fruit, which correlated with its level of *TPK1b* gene expression (Figures 11B and 11C). Thus, *TPK1b* is required for normal fruit structure and seed set in tomato, consistent with the role of ET in seed and fruit development (Gillaspy et al., 1993).

DISCUSSION

This study presents data on the function of *TPK1b* in defense against *Botrytis* and the herbivorous insect tobacco hornworm. Reduction of *TPK1b* gene expression resulted in increased susceptibility to *Botrytis*. Ectopic expression of *TPK1b* in *Arabidopsis* conferred increased resistance to *Botrytis* and *A. brassicicola*. These data, coupled with an increased susceptibility of *TPK1b* RNAi plants to insect feeding, suggest a clear physiological role for *TPK1b* in resistance against both necrotrophic fungi and insect pests. Interestingly, *TPK1b* is also required for ET responses of tomato, suggesting that *TPK1b* regulates ET-dependent defense. The impaired disease and insect resistance in *TPK1b* RNAi plants is accompanied by altered ET- and *Botrytis*-induced defense gene expression, further supporting the role of *TPK1b* in ET-mediated defenses. ET integrates plant responses to developmental and environmental signals, including responses to pathogens (Klee, 2004). Our data on the role of *TPK1b* in ET responses and insect resistance corroborate the function of ET as an important regulator of insect and pathogen defense in tomato. *TPK1b* is a functional kinase that localizes to the plasma membrane, suggesting that *TPK1b* acts early in pathogen and insect response pathway and functions in recognition and/or signaling. Our biochemical and genetic data demonstrate an important role for specific *TPK1b* KD residues for in

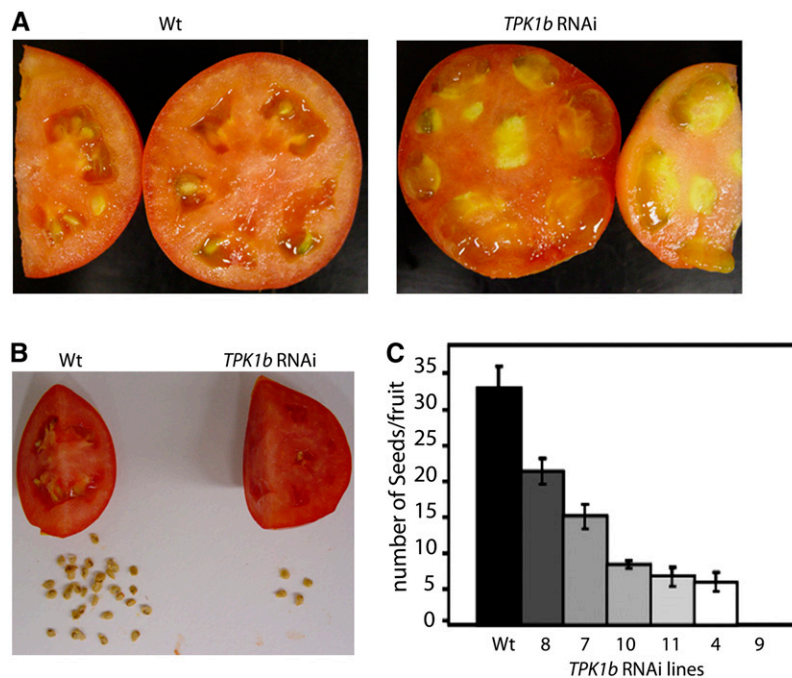


Figure 11. *TPK1b* Is Required for Normal Fruit Structure and Seed Set.

(A) *TPK1b* RNAi plants show defects in fruit structure.

(B) *TPK1b* RNAi plants produce fewer seeds.

(C) A comparison of number of seeds/fruit between the wild type and *TPK1b* RNAi.

Representative fruit tissue and seeds from wild-type and *TPK1b* RNAi plants are shown. In (B), the picture shows part of the fruit and the entire amount of seed recovered from the whole fruit of the corresponding genotype. At least 10 fruits were examined per plant.

planta signaling function. We also present data supporting the function of TPK1b in insect and pathogen defense independent of genes regulating JA biosynthesis and response, which are also required for resistance to *Botrytis*. Thus, plants deploy multiple and overlapping mechanisms of defense against necrotrophic pathogens and chewing insects, despite the disparate strategies of these organisms in deriving nutrition from plants.

Multiple lines of evidence suggest that responses to insects and necrotrophic pathogens are mechanistically linked in tomato (McCormick, 1991; Ryan, 1992). First, *Botrytis* induces wound-like responses, including the expression of the wound response gene *PI-II*, possibly through the actions of OGs released from plant cell walls by the action of fungal endopolygalacturonases (Bishop et al., 1981; Prins et al., 2000). Second, the susceptibility of JA and wound response mutants to *Botrytis* presented in this article and their impaired resistance to tobacco hornworm (*M. sexta*) or spider mites (*Tetranychus urticae*) (Schillmiller and Howe, 2005) demonstrate a clear overlap in the mechanisms of resistance to necrotrophic fungi and chewing insect pests. These overlapping responses are mediated in part by JA levels and signaling (Howe and Jander, 2007). *JAI1*, *SPR2*, *ACX1*, and *DEF1* are required for basal resistance of tomato to *Botrytis*. The *acx1* mutant is JA deficient and lacks local and systemic expression of defensive *PI* genes in response to wounding due to a defect in the first step of β -oxidation in the octadecanoid pathway (Li et al., 2005). *SPR2* is a fatty acid desaturase required for the synthesis of JA and the generation of a systemic wound signal mediating defense gene expression in tomato (Li et al., 2003). The *def1* and *jai1* mutants are impaired in JA responses (Li et al., 2002). *JAI1* is the homolog of *Arabidopsis COI1* (Li et al., 2004); the *coi1* mutant also shows susceptibility to necrotrophic pathogens (Thomma et al., 1998; van Wees et al., 2003). Previous reports on *Botrytis* responses of *def1* were contradictory, with both increased susceptibility and wild-type resistance observed (Audenaert et al., 2002; Diaz et al., 2002). Our data confirm the susceptibility of *def1* based on enhanced disease symptoms and fungal growth. Thus, these genes in the JA pathway are part of the defense against *Botrytis*, and our findings confirm that the *Botrytis* resistance mechanisms regulated by JA are generally conserved between *Arabidopsis* and tomato. The *spr1* mutation abolishes JA accumulation in response to exogenous systemin and reduces JA accumulation in wounded leaves (Lee and Howe, 2003). However, the *spr1* mutant mounts wild-type resistance to *Botrytis*. *SPR1* is an upstream regulator of JA synthesis and systemin signaling (Schillmiller and Howe, 2005), and our data support the hypothesis that an additional factor upstream of JA acts to specify responses to *Botrytis*. Consistent with this, *TPK1b* is expressed normally in *jai1*, *spr1*, *spr2*, *acx1*, and *def1* mutants, which demonstrates that *TPK1b* functions independent of or upstream of the JA and wound response pathways to specify responses to *Botrytis*. This is consistent with previous observations that JA and wound signaling act independent of ET in resistance to *Botrytis* in tomato (Diaz et al., 2002). However, the same authors using tomato plants that have increased or decreased prosystemin expression observed that systemin is important for resistance to *Botrytis*. This appears to contradict our observation in the *spr1* mutant that shows normal resistance to *Botrytis* but is impaired in systemin responses.

The functions of a large number of RLCKs in *Arabidopsis* and other species have not been determined. In the *Arabidopsis* genome, there are 46 genes encoding proteins that belong to the RLCK VII subfamily of plant RLCKs that share overall structural and sequence similarity to TPK1b. The TPK1b kinase domain is most closely related to the *Arabidopsis* APK1b, *Brassica* MLPK, and the grapevine predicted protein CAO21648 based on sequence and phylogenetic analysis. *Arabidopsis* APK1a and APK1b are 84% identical and exhibit kinase activities in vitro (Hirayama and Oka, 1992). MLPK shares 76% sequence identity with APK1b and is involved in *Brassica* self-incompatibility (Murase et al., 2004). The T-DNA insertion alleles of APK1b show no fertility or self-incompatibility related phenotypes (Kakita et al., 2007). Whether MLPK and APK1b perform defense functions is not yet known. Interestingly, ectopic expression of *TPK1b* rescues the phenotype of the *Arabidopsis bik1* mutant, indicating that the two kinases perform similar functions in tomato and *Arabidopsis*: both *TPK1b* and *Arabidopsis BIK1* are positive regulators of resistance to *Botrytis*. However, the *TPK1b* RNAi plants show wild-type responses to *P. syringae* in contrast with the *Arabidopsis bik1* mutant, which shows increased resistance. In addition, the role of the two proteins in plant growth and development appears to be distinct. Although *TPK1b* affects fruit development and seed set, it has no apparent effect on root growth and leaf shape. The *Arabidopsis bik1* mutation has significant effect on leaf shape, primary root, and root hair growth (Veronese et al., 2006). These data further corroborate that *TPK1b* and *BIK1* perform similar yet distinct roles in plant defense and other functions. It will also be interesting to determine the function of *Arabidopsis* RLCKs that are in the TPK1b clade and tomato BIK1 to establish the extent of functional redundancy. Mutations in *Arabidopsis BIK1*, *Brassica MLPK*, and *TPK1b* all result in defects in plant fertility, further suggesting that members of this protein family perform similar functions. In tomato, diverse processes control parthenocarpic fruit development (Johnson et al., 1996). The molecular mechanisms underlying TPK1b-mediated seed set need further investigation but may relate to the function of ET in fruit development or the function of TPK1b in self-fertility similar to the function of MLPK (Murase et al., 2004). One intriguing possibility is mechanisms underlying pathogen recognition and response and pollen recognition leading to self-incompatibility may share similar molecular mechanisms. In both interactions, mechanisms of self- and non-self recognition play an important role. Indeed, recently, R proteins were implicated in hybrid-induced necrosis resulting in self-incompatibility (Bomblies et al., 2007).

Based on the altered gene expression in response to ACC and impaired triple response in *TPK1b* RNAi seedlings, *TPK1b* plays a role in ET signaling. The susceptibility of *TPK1b* RNAi plants to tobacco hornworm coupled with the lack of ET responses suggest that *TPK1b*-mediated ET responses are required for defense against pathogens and insect pests. The attenuated *PI-II* gene expression in *TPK1b* RNAi plants is also consistent with the action of ET in parallel with the octadecanoid/wounding pathway for full *PI-II* gene expression (O'Donnell et al., 1996). Mutations causing ET insensitivity in soybean (*Glycine max*) and *Arabidopsis* and inhibition of ET perception in tomato result in increased susceptibility to necrotrophic pathogens (Hoffman

et al., 1999; Thomma et al., 1999; Diaz et al., 2002). ET also functions as a negative regulator of defense against some pathogens (Lund et al., 1998; Ciardi et al., 2000), indicating that its role varies among different pathosystems.

Insect herbivory is known to promote ET emission (Lund et al., 1998; Ciardi et al., 2000), but ET has been implicated as both a negative and positive factor in insect-plant interactions, similar to its role in pathogen defense. In maize (*Zea mays*), ET is required for defense against insect herbivory (Harfouche et al., 2006). The bacterial elicitor harpin induces EIN2-dependent resistance to green peach aphid (*Myzus persicae*) in *Arabidopsis* (Dong et al., 2004). On the other hand, the *Arabidopsis* ET response mutants *hookless1* and *ein2* were more resistant to the generalist insect Egyptian cotton worm (*Spodoptera littoralis*) than wild-type plants, suggesting a negative effect of ET signaling on insect resistance (Bodenhausen and Reymond, 2007). The same mutants show no effect on the specialist herbivore diamondback moth (*Plutella xylostella*) (Stotz et al., 2000). ET suppresses defense against *M. sexta* by lowering the putresine amino transferase transcripts in the root and nicotine levels in the shoot in *Nicotiana attenuate* (Winz and Baldwin, 2001). ET signaling regulates insect oral secretion, which in turn induces nicotine levels and floral longevity, two traits implicated in interaction between *N. attenuate* and *M. sexta* (von Dahl et al., 2007). Our data provide evidence for the role of TPK1b in ET signaling and insect resistance in tomato and differ from some of the previous observations, possibly because the role of ET in different plants may be different, involving diverse traits, some of which contribute directly or indirectly.

TPK1b has two major regions, a conserved catalytic KD and a nonconserved flanking region, that are important for the biochemical activities and biological function of TPK1b. The activation segment of the KD controls kinase activity (Johnson et al., 1996), and phosphorylation of one to three residues in the activation loop of KDs is often required for ligand-induced kinase activation (Wang et al., 2005). In the activation segment of TPK1b, three residues (T238, T243, and Y246) are important for in vitro kinase activity and for disease response signaling in planta. These activation segment residues are highly conserved in TPK1b and related RLCKs and are all located in the C-terminal subregion of the activation segment. How TPK1b gets activated is not yet known but may follow the same general trend observed for other RD-type RLCKs, in which the activation segments play important roles for general kinase activity and biological function (Sessa et al., 2000; Shah et al., 2001; Guo et al., 2004; Yoshida and Parniske, 2005). The activation segment occupies the catalytic cleft of protein kinases and is composed of smaller regulatory elements, including the T-loop, where activating phosphorylation events often occur, and the C-terminal P+1 loop, which plays a role in recognition and binding of protein substrates (Johnson et al., 1996). Comparisons with other protein kinases show that TPK1b Y246 falls in the P+1 loop of the activation segment. The C-terminal region of the activation domain is highly conserved, suggesting that the tertiary structure of TPK1b is important and may be required for interaction with substrates. The TPK1b residues T238 and T243, located in the T-loop region, are critical for TPK1b function most likely due to their role in general kinase activity, consistent with the function of

homologous regions of other kinases. In the activation segment of tomato PTO kinase, S198 (equivalent to TPK1b^{S237}) is required for elicitation of HR (Sessa et al., 2000), T204 (TPK1b^{T243}) is required for recognition specificity (Rathjen et al., 1999), Y205 (TPK1b^{Y244}) plays a subsidiary role in recognition (Frederick et al., 1998), and Y207 (TPK1b^{Y246}) influences binding properties (Rathjen et al., 1999). BAK1 was autophosphorylated in vitro on residues equivalent to TPK1b^{T230A} and TPK1b^{S234A}. Interestingly, BAK1 was recently implicated as a central player in plant immunity and resistance to *Botrytis* (Chinchilla et al., 2007; Heese et al., 2007; Kemmerling et al., 2007). These observations indicate that different activation segment residues play different roles among various RLCKs. RLCKs are predicted to share some common regulatory mechanisms but also have specific mechanisms that control certain physiological processes. Autophosphorylation of less-conserved N-terminal and C-terminal KD regions may help create docking sites for kinase substrates to achieve specificity. Outside the KD, there is significantly less sequence conservation between TPK1b and related RLCKs.

Outside the activation segment, the single amino acid mutation TPK1b^{T65A} was dispensable for disease resistance function, although it eliminated autophosphorylation and severely reduced phosphorylation of MBP. The equivalent residue in PTO is the main autophosphorylation site and is required for HR (Sessa et al., 2000). Currently, we have no plausible explanation for the apparent lack of correlation between the kinase activity and disease resistance function of TPK1b Thr-65. In contrast with TPK1b Thr-65, Lys-106 is important for TPK1b phosphorylation activities and biological function. This residue is the ATP binding site and is frequently mutated to generate kinase dead mutants, suggesting that it may be important for maintaining the tertiary structure of the kinase molecules and thus influence interaction with other proteins (Hanks and Hunter, 1995). Three residues in the TPK1b activation segment are required for autophosphorylation. Some of these residues are likely to be TPK1b autophosphorylation sites. Protein kinases that require autophosphorylation of the activation segment for kinase activity are of the RD type, consistent with TPK1b being an RD-type protein kinase. In sum, specific TPK1b residues that affect tertiary structure and phosphorylation sites are required for function by interfering with kinase activation and interaction with other proteins.

Substitution of the penultimate Gly, a residue essential for myristylation in other proteins, did not affect TPK1b localization but affected its disease resistance function. Consistent with this, the myristylation motif was required for Fen kinase-mediated fenthion sensitivity (Rommens et al., 1995). Myristylation was also required for salt tolerance function of SOS3 but not for its membrane association (Ishitani et al., 2000). It is possible that TPK1b is not myristylated but TPK1b N-terminal conserved sequence affects TPK1b function through an unknown mechanism. Thus, the penultimate Gly residue in TPK1b is required for disease resistance possibly through a myristylation-independent mechanism, such as conferring structural stability or protein-protein interactions.

TPK1b functions early in pathogen and insect response pathways. Future research should focus on the regulation of in vivo kinase activity of TPK1b and its impact on disease resistance and early events in defense against insects and pathogens.

METHODS

Plant Growth

Tomato (*Solanum lycopersicum*) cultivars CastlemarIII and Micro-Tom were grown in plastic pots containing compost soil mix in a greenhouse with a photoperiod extended to 15 h under fluorescent lights (160 W mol⁻¹ m⁻² s⁻¹) at a temperature of 24 ± 4°C. The tomato mutants *spr1*, *spr2*, *def1*, *acx1*, and *jai1* were from Gregg Howe (Michigan State University). All plants were fertilized twice weekly.

Fungal Culture and Disease Assays

The *Botrytis cinerea* strain BO5-10 and *Alternaria brassicicola* strain MUCL 20297 were used for disease assays. Fungal culture and preparation of conidial spore suspension were as described previously (Abu-Qamar et al., 2006). *Botrytis* disease assays were done on whole plants or detached leaves by spray or drop inoculation of a conidial suspension on *Arabidopsis thaliana* (2.5 × 10⁵ spores/mL) and tomato (5 × 10⁵ spores/mL) unless stated otherwise in the Results.

Bacterial Disease Assay

Bacterial disease assays were done essentially as described (Mengiste et al., 2003). Leaves of 6-week-old tomato plants were infiltrated with suspensions (OD₆₀₀ = 0.001 in 10 mM MgCl₂) of the bacterial strain *Pseudomonas syringae* pv *tomato* DC3000 (Generous gift of Greg Martin, Cornell University). To determine bacterial growth, leaf discs from infected leaves were collected at 0, 2, and 4 DAI. Each experiment for bacterial growth assay was performed in three replicates. At each time point, two leaf discs were collected from wild-type and *TPK1b* RNAi plants for each replicate. Leaf discs of the same size were made using a hole puncher, and bacterial titer per leaf area was determined.

Insect Feeding Trials

Eggs of tobacco hornworm (*Manduca sexta*) and an artificial diet for the larvae were purchased from Carolina Biological Supply Company. Eggs were hatched by incubation at 26°C as recommended by the supplier. Hatched larvae were kept on the artificial diet for 3 d before transfer to whole tomato plants or detached leaves. The average larval weight at the beginning of the feeding trial was ~9 to 11 mg.

Generating Constructs for Expression in Tomato and Arabidopsis Transformation

To generate 35S:*TPK1b* constructs, full-length cDNA was amplified by PCR from the RACE-Ready cDNA with primers *TPK1b*/LP (5'-TCCCG-CGGCTAATGGGGATATGTTTGAGTG-3', the *Sac*II site is underlined) and *TPK1b*/RP (5'-CGCGGATCCTAATTATTTAGCGTAAGGGGGAG-3', the *Bam*HI site is underlined). Restriction-digested PCR products were cloned into a modified version of the binary vector pCambia1200 behind the cauliflower mosaic virus 35S promoters between the *Sac*II and *Bam*HI sites. *Agrobacterium tumefaciens* strain GV3101 was used for transformation. Hygromycin-resistant homozygous plants were identified from progenies of primary transformants, and lines that express *TPK1b* cDNA were identified. Transgenic tomato lines were selected on hygromycin and homozygous transgenic lines identified from selfed progenies of the subsequent generations and used for further experiments.

To generate a *TPK1b* RNAi construct, 250 bp from the 3' end (162 bp) and the 3' untranslated region (88 bp) of *TPK1b* were amplified by PCR from the RACE-Ready cDNA with primers *TPK1b*RNAi-LP (5'-GCAC-TAGTCCATGGGAAATGGAGCAACTTTAT-3', the *Spe*I and *Nco*I sites are

underlined) and *TPK1b*RNAi-RP (5'-CGGGATCCGGCGGCCATCA-GCCTCAACTACTTAT-3', *Bam*HI and *Asc*I sites are underlined). The inverted repeat is assembled directly in the binary vector by a two-step cloning process using the introduced restriction enzyme sites. In the first cloning step, the PCR product is cleaved at the inner restriction sites, *Nco*I and *Asc*I, and ligated to *Nco*I and *Asc*I sites in pGSA1165 (<http://www.chromdb.org/info/plasmids/pGSA1165.html>). For the second cloning step, the plasmid resulting from the first cloning step serves as a template for a second amplification using the original set of primers. The resulting PCR product is cleaved with *Bam*HI and *Spe*I and inserted into the *Bam*HI- and *Spe*I-cleaved template plasmid. This second ligation inserts the PCR product in inverted orientation with respect to first cloned fragment, yielding an inverted repeat separated by the β-glucuronidase fragment.

Plant Transformation and Regeneration

In *Arabidopsis*, *Agrobacterium* strain GV3101 was used for transformation through the floral dip method (Clough and Bent, 1998). Hygromycin-resistant homozygous T2 plants were identified from progenies of primary transformants and selfed to produce the T3 homozygous lines.

Tomato transformation was done as described with some modification (Howe et al., 1996). For tomato transformation, *S. lycopersicum* cv CastlemarIII and Micro-Tom were used for RNAi and overexpression experiments, respectively. Seeds were surface-sterilized by immersion for 2 min in 70% ethanol and 30 min in 35% commercial bleach solution (5.25% [w/v] sodium hypochlorite) plus 0.1% Tween 20. Treated seeds were then rinsed five to six times with sterile deionized-distilled water, and 12 seeds per Magenta box were placed on germination medium (GM) (4.3 g L⁻¹ Murashige and Skoog [MS] salts, 30 g L⁻¹ sucrose, 1 mL L⁻¹ Nitsch and Nitsch's Vitamin mix, and 0.8% agar). Seedlings were grown at 25°C and 70% relative humidity for 10 d under fluorescent light (90 μmol m⁻² s⁻¹), 8 h dark and 16 h light. Cotyledon explants of 10- to 14-d-old seedlings were cut off, and one to four holes were made on the cotyledon pieces to increase *Agrobacterium* transformation efficiency. Cotyledon pieces were placed upside down in 90 × 15-mm Petri dishes containing preculture medium (PM = GM + 3 mg L⁻¹ 2,4-D) and incubated for 24 h at 25°C.

A single colony from *Agrobacterium* strain LBA4404 containing desired construct was grown in YEP media (10 g L⁻¹ yeast extract, 10 g L⁻¹ peptone, and 5 g L⁻¹ NaCl) and 50 mL L⁻¹ rifampicin with appropriate antibiotics (40 mg L⁻¹ chloramphenicol) and 300 μM acetosyringone at 28°C to OD₆₀₀ = 0.5 to 0.8. The culture was centrifuged at low speed for 5 min and diluted by liquid 2% MSO (4.3 g L⁻¹ MS salts, 100 mg L⁻¹ myo-inositol, 0.4 mg L⁻¹ thiamine-HCl, and 20 g L⁻¹ sucrose). Tomato cotyledon explants were removed from the PM plates and transferred to the bacterial suspension for 30 min and then placed on cocultivation culture medium (PM + 150 μM acetosyringone) for 2 d (24 h in dark, 24 h in light). After 2 d, the infected tissue was transferred to shoot induction and regeneration medium containing 2 mg L⁻¹ zeatin with 300 mg L⁻¹ timentin and 40 mg L⁻¹ hygromycin or 50 mg L⁻¹ kanamycin. Explants were subcultured into fresh medium containing 1 mg L⁻¹ zeatin and 1 mg L⁻¹ GA every 2 weeks. After 2 to 4 weeks, initial calli were transferred (with shoot primordia) into regeneration (GM) including desired antibiotics. The 2- to 4-cm-long excised shoots were transferred from calli to rooting media containing 2 mg L⁻¹ indole butyric acid and desired antibiotics, and 10- to 15-cm plants were transferred to the greenhouse after 4 to 6 weeks.

DNA and RNA Extractions, cDNA Synthesis, and RT-PCR

For DNA blots, genomic DNA was extracted from tomato leaves according to the method described (Dellaporta et al., 1983). Total RNA from tomato and *Arabidopsis* was extracted from tissues frozen in liquid nitrogen as described (Lagrimini et al., 1987). RNA was separated on

1.2% formaldehyde agarose gels. The gels were then blotted onto Hybond N⁺ nylon membranes (Amersham Pharmacia Biotech). Probes were labeled with ³²P by random priming using a commercial kit (Sigma-Aldrich). Hybridization of probe and subsequent washings were performed as described (Church and Gilbert, 1984).

For RT-PCR, cDNA was synthesized from both control and treated samples using equal amounts of total RNA (2 µg), AMV reverse transcriptase (Promega), and oligo (dT₁₅) primers according to standard protocols. The PCR was performed for 35 cycles using 2.5 µL cDNA as a template and specific primer pairs (94°C 30 s, 52°C 30 s, 72°C 1 min). The tomato *translation initiation factor* (*eIF4A*) gene was amplified as a control to demonstrate relative quantity of the cDNA. The amplified products were separated on 1.5% agarose gels and visualized under UV light after staining with ethidium bromide. The following primer pairs were used for RT-PCR: TPK1b forward (5'-ATGGGGATATGTTTGGAGTGCTAGAA-3'), reverse (5'-GAACGTGTTCTCGTCGATCCACCCT-3'); PI-II forward (5'-ATGGC-TGTTCAAGGAAGTTAATTTTG-3'), reverse (5'-TCACATTACAGGGTACATATTTGCCTTG-3'); *B. cinerea Actin A* forward (5'-ACTCATATGTTG-GAGATGAAGCGCAA-3'), reverse (5'-AATGTTACCATACAAATCCTTAC-GGACA-3'); *eIF4A* forward (5'-CAAATCTTTCAGATCTTTCTTGAC-3') and reverse (5'-GTGCAAGCTCACGGGTTGGTGCAAG-3').

VIGS

The *TPK1b* silencing was done using the TRV vector system essentially as described (Liu et al., 2002).

Subcellular Localization

For the subcellular localization, the *TPK1b* coding sequence was translationally fused into an N-terminal GFP fusion vector. *TPK1b-LP* or *TPK1b-G2A-LP* coding sequence was amplified using the primers 5'-GGATCCATGGG(C)GATATGTTTGGAGTGCTAGA-3' (C was PCR mutated to replace Gly with Ala) and *TPK1b-RP* 5'-TCTAGATTTAGCG-TAAGGGGAGAGCAGA-3' (the *Bam*HI and *Xba*I sites are underlined, respectively). The correct amplification was verified by sequencing.

Expression and Purification of Recombinant Proteins and in Vitro Kinase Assay

The open reading frame of *TPK1b* was cloned into GST fusion protein expression vector pGEX4T-1 (Pharmacia). Expression of GST-fusion protein and affinity purification were performed as described previously (Cardinale et al., 2002). The protein concentration of the recombinant proteins were determined with the Bio-Rad detection system using BSA as a standard. Kinase reactions were performed in 15 µL of kinase buffer (20 mM HEPES, pH 7.4, 10 mM MgCl₂, 5 mM EGTA, and 1 mM DTT) containing 5 µg of GST-fusion protein, 5 µg of myelin basic protein, 0.1 mM ATP, and 2 µCi [³²P]ATP. The protein kinase reactions were performed at room temperature for 30 min, and the reactions were stopped by adding 4× SDS loading buffer. The phosphorylation of MBP was analyzed by autoradiography after separation on 12.5% SDS-PAGE.

Site-Directed Mutagenesis

Mutagenesis of *TPK1b* residues was performed in the plasmid pGEX4T, containing GST-*TPK1b* fusion protein. Site-specific mutations were introduced into *TPK1b* using the Quickchange kit from Stratagene, according to the manufacturer's protocols. The presence of the desired mutations was confirmed by sequencing.

Phylogenetic Analysis

The KD amino acid sequences were used for building the phylogenetic tree comparing *TPK1b* and related kinases. Sequences were aligned

using ClustalW (Thompson et al., 1994) with default gap penalties, and the alignment was manually adjusted where necessary. Mean character distances were used to construct a rooted neighbor-joining phylogeny (Saitou and Nei, 1987) from the PHYLIP version 3.67 package (Felsenstein, 1993). Statistical support of the branches was tested with 1000 bootstrap resamples. The tomato PTO and PBS1 kinase were used as outgroups. These two genes are divergent from the other members of the TPK1b-related proteins

Seedling ET Response Assays

Tomato seeds were surface sterilized with 70% ethanol for 2 min and 30 min in 35% commercial bleach solution (5.25% [w/v] sodium hypochlorite) plus 0.1% Tween 20, rinsed in sterile water, and then plated on appropriate media and incubated for 6 d in the dark. In vitro, tomato seeds were cultured on MS medium and 0.8% agar with or without ACC (A0430; Sigma-Aldrich) as described.

Induction Treatments

Induction treatments were performed by spraying 4-week-old soil-grown tomato plants with 5 × 10⁵ *Botrytis* spores/mL, 100 µM SA (Sigma-Aldrich), 100 µM abscisic acid (Sigma-Aldrich), 100 µM ACC (Sigma-Aldrich), 100 µM paraquat (methyl viologen; Sigma-Aldrich), and 100 µM MeJA (Sigma-Aldrich). In the wounding experiment, the main veins of apical leaflets of compound leaves were wounded with dented forceps.

Accession Numbers

Sequence data for the genes described in this study or genes that were used in the phylogenetic analysis can be found in the GenBank/EMBL data libraries under the following accession numbers: *TPK1b* (GenBank accession number EU555286), CAO21648, BIK1 (At2g39660), NAK (At5g02290), PBS1 (At5g13160), APK1b (At2g28930), APK1a (At1g07570), APK2b (At2g02800), MPLKe (BAD12263), PTO (A49332), *Arabidopsis* putative protein kinase (At3g55450), Le ACIK1 (AF332960), and ARSK1 (ROOT-SPECIFIC KINASE1, At2G26290).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Suppression of *TPK1b* through Virus-Induced Gene Silencing Causes Susceptibility to *Botrytis*.

Supplemental Figure 2. *TPK1b* RNAi Plants Show No Altered Responses to the Bacterial Pathogen *P. syringae* pv *Tomato*.

Supplemental Figure 3. Feeding Trial on Detached Leaves Showing Reduced Resistance of *TPK1b* RNAi Plants to Tobacco Hornworm (*Manduca sexta*).

Supplemental Figure 4. *TPK1b* RNAi Tomato Plants Do Not Show Any Altered Responses to the Plant Hormones Abscisic Acid and Methyl-Jasmonate.

Supplemental Figure 5. Tomato *TPK1b* Suppresses the *Arabidopsis bik1* Susceptibility to *A. brassicicola*.

Supplemental Figure 6. 35S:*TPK1b* Rescues the Short Root Growth of *Arabidopsis bik1* Mutant to Wild-Type Levels.

Supplemental Figure 7. The Recombinant GST-*TPK1b* Fusion Protein Autophosphorylates and Phosphorylates the Myelin Basic Protein in Vitro.

Supplemental Figure 8. 35S:*TPK1b*^{T243A} Partially Rescues the Growth-Related Phenotypes of the *Arabidopsis bik1* Mutant.

Supplemental Figure 9. Alignment of N-Terminal Amino Acid Sequences of TPK1b and Closely Related Proteins.

Supplemental Data Set 1. Multiple Sequence Alignment (PHYLIP) Used as Input for the Phylogenetic Tree Presented in Figure 2C.

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