

# Adi3 is a Pdk1-interacting AGC kinase that negatively regulates plant cell death

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**Bacterial speck disease in tomato is caused by *Pseudomonas syringae* pv. *tomato*. Resistance to this disease is conferred by the host Pto kinase, which recognizes *P. s. pv. tomato* strains that express the effector AvrPto. We report here that an AvrPto-dependent Pto-interacting protein 3 (Adi3) is a member of the AGC family of protein kinases. In mammals, AGC kinases are regulated by 3-phosphoinositide-dependent protein kinase-1 (Pdk1). We characterized tomato Pdk1 and showed that Pdk1 and Pto phosphorylate Adi3. Gene silencing of *Adi3* in tomato causes MAPKKK $\alpha$ -dependent formation of necrotic lesions. Use of a chemical inhibitor of Pdk1, OSU-03012, also implicates Pdk1 and Adi3 in plant cell death regulation. Adi3 thus appears to function analogously to the mammalian AGC kinase protein kinase B/Akt by negatively regulating cell death via Pdk1 phosphorylation. We speculate that the negative regulatory function of Adi3 might be subverted by interaction with Pto/AvrPto, leading to host cell death that is associated with pathogen attack.**

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## Introduction

Programmed cell death (PCD) is an essential process for development and immune responses in eukaryotic multicellular organisms. In mammalian systems, PCD is used for many processes from digit formation to removal of infected cells (Lam, 2004). In plants, leaf senescence, development of xylem tracheary elements, and host responses to pathogens all involve PCD (Greenberg and Yao, 2004; Lam, 2004). Some characteristics of PCD are shared between animals and plants, such as DNA fragmentation and organelle destruction, whereas others, such as apoptotic body formation and engulfment by neighboring cells, occur only in animals (Lam, 2004).

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Localized PCD occurs in both resistant and susceptible plants during pathogen attack. In the former, PCD termed the hypersensitive response (HR) occurs rapidly (<12 h) upon attempted pathogen infection (Goodman and Novacky, 1994). The HR may inhibit further spread of the pathogen, but its biological significance has been difficult to verify due to the lack of plant mutants affected solely in cell death. In disease-susceptible plants, localized cell death referred to as 'specks', 'spots', or 'blights' appears over the course of many days. Recent studies suggest that these disease symptoms also involve host-mediated PCD (Lincoln *et al.*, 2002; Abramovitch and Martin, 2004; del Pozo *et al.*, 2004). Thus, host-controlled PCD plays a critical role in determining both immunity and disease progression in plants. Despite this significance, relatively few plant genes have been identified that have a demonstrated role in PCD associated with pathogen attack, and upstream components that might regulate these cell death mediators are yet to be identified (Shah *et al.*, 1999; del Pozo *et al.*, 2004; Greenberg and Yao, 2004; Lam, 2004).

In mammals, PCD (apoptosis) has been studied extensively, and many proteins involved in this process are known (Nicholson and Thornberry, 2003). An important central regulator of mammalian apoptosis is protein kinase B (PKB; also known as Akt; Vivanco and Sawyers, 2002). PKB is a member of the AGC family of protein kinases, which often act as downstream effectors of second messengers such as cAMP, cGMP, phospholipids, or Ca<sup>2+</sup>. PKB negatively regulates apoptosis by phosphorylation and inactivation of proapoptotic factors such as BAD and activation of antiapoptotic factors such as CREB and IKK (Vivanco and Sawyers, 2002; Vara *et al.*, 2004).

The activity of mammalian AGC kinases like PKB is regulated through phosphorylation by 3-phosphoinositide-dependent protein kinase-1 (Pdk1). Pdk1 phosphorylates some AGC kinases in a constitutive manner and others upon stimulation (Belham *et al.*, 1999). The physical interaction of Pdk1 with its substrates is mediated through a hydrophobic amino-acid sequence termed the Pdk1-interacting fragment (PIF), found on the AGC kinase. A PIF-binding pocket found on Pdk1 binds the PIF, allowing Pdk1 to phosphorylate and activate the substrate (Biondi, 2004). Once activated, the AGC kinase and Pdk1 disassociate and the PIF is bound by a PIF-binding pocket on the AGC kinase, allowing for stabilization and phosphorylation of downstream substrates (Frödin *et al.*, 2002; Biondi, 2004).

Little is known about the role of AGC kinases in plants. There are at least 39 AGC kinase genes in *Arabidopsis* (Bögge *et al.*, 2003), but the functions of only five of them are known. PINOID (PID) is involved in regulating auxin signals (Christensen *et al.*, 2000; Benjamins *et al.*, 2001), phototropins 1 and 2 (PHOT1 and PHOT2) mediate blue-light signals (Briggs and Christie, 2002), and IRE and AGC2-1/OXII regulate root hair development (Oyama *et al.*, 2002; Anthony *et al.*, 2004; Rentel *et al.*, 2004). AGC2-1/OXII also mediates oxidative burst signals (Rentel *et al.*, 2004).

Bacterial speck disease in tomato is caused by *Pseudomonas syringae* pv. *tomato* (*Pst*). Resistance to *Pst* arises from a 'gene-for-gene' interaction in which the product of the host *Pto* resistance gene, a Ser/Thr protein kinase, physically interacts with the *Pseudomonas* AvrPto protein upon its delivery into the plant cell via the bacterial type III secretion system (Pedley and Martin, 2003). Recognition of AvrPto by Pto elicits an HR-based resistance. This resistance requires another protein, Prf, although its role is unknown (Pedley and Martin, 2003). Several other components have been identified that appear to act in the Pto pathway (Ekengren *et al*, 2003; Pedley and Martin, 2003).

In order to investigate the role of the Pto/AvrPto interaction, we previously used a yeast three-hybrid (Y3H) screen to identify tomato proteins that interact only when both Pto and AvrPto are present (Bogdanove and Martin, 2000). From this screen, we identified AvrPto-dependent Pto-interacting protein 3 (Adi3). We report here the characterization of Adi3, which indicates that it plays a role as a negative regulator of plant cell death and therefore might act analogously to PKB in controlling plant cell death.

## Results

### Adi3 cDNA cloning and protein characterization

Adi3 was identified from a Y3H screen as requiring both Pto and AvrPto for its interaction (Bogdanove and Martin, 2000). The isolated cDNA, which was found to encode a full Ser/Thr protein kinase domain, lacked the 5' end. The full-length Adi3 cDNA was isolated by using a tomato EST database at The Institute for Genomic Research (TIGR; EST *TC102958*) and 5' RACE. The complete Adi3 cDNA has a 471 bp 5' UTR, a 2103 bp coding region, and a 255 bp 3' UTR.

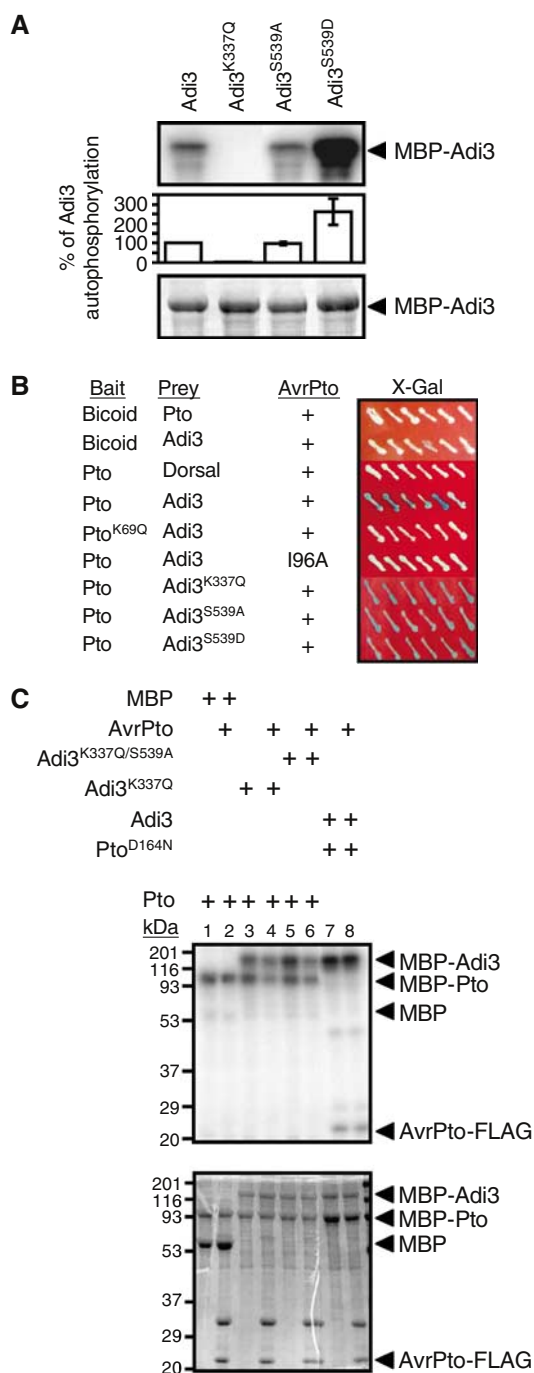
Analysis of Adi3 revealed that it belongs to group VIIIa of plant AGC protein kinases described by Bögre *et al* (2003). Key features of this group include a large amino-acid insertion (74 aa for Adi3) between kinase subdomains VII and VIII (referred to as a T-loop extension), the conserved DFG motif of subdomain VII for Mg<sup>2+</sup> coordination is changed to DFD, and a PIF consensus sequence of FD/ExF (Bögre *et al*, 2003). All other conserved protein kinase residues are present in Adi3. Although the Adi3 T-loop extension is similar to that of the Ndr family of AGC kinases (Tamaskovic *et al*, 2003), phylogenetic analysis indicates that Adi3 does not belong to the Ndr family (Bögre *et al*, 2003).

### Mutations affecting Adi3 autophosphorylation

We examined the possible autophosphorylation activity of MBP-Adi3. MBP-Adi3 showed autophosphorylation activity, whereas MBP-Adi3<sup>K337Q</sup>, which has a substitution at the ATP-binding site, did not (Figure 1A). Adi3 activity was dependent on Mg<sup>2+</sup> and was not observed with Mn<sup>2+</sup> (data not shown). Substitution of S539 for Ala in the activation loop of Adi3 did not affect autophosphorylation (Figure 1A). However, substitution of S539 for Asp greatly increased the level of Adi3 autophosphorylation (Figure 1A), indicating that this protein is constitutively active and that phosphorylation of S539 likely contributes to full Adi3 activity.

### Adi3 interactions in the yeast three-hybrid assay

To gain an insight into the specificity of the Pto/AvrPto/Adi3 interaction, the full-length Adi3 cDNA was tested in the Y3H



**Figure 1** Adi3 autophosphorylation and protein interactions. In (A) and (C), the top panel shows the kinase assay (phosphorimage) and the bottom panel the assay input (Coomassie-stained gel). (A) Adi3 encodes a functional protein kinase. Analysis of kinase-active, -deficient, and constitutively active MBP-Adi3 fusion proteins by *in vitro* autophosphorylation assays is shown. (B) Adi3 interactions in the Y3H assay. The indicated bait and prey constructs were tested in the Y3H assay (Bogdanove and Martin, 2000) for expression of the *lacZ* gene on X-Gal plates (blue = interaction). The *Drosophila* proteins Bicoid and Dorsal were used as negative controls and indicate that the Pto/Adi3/AvrPto interaction is specific. + indicates the presence of AvrPto. (C) Analysis of cross-phosphorylation between Adi3 and Pto. Kinase-deficient MBP-Adi3 proteins were used as substrates for kinase-active MBP-Pto protein with or without AvrPto-FLAG protein. Kinase-deficient MBP-Pto<sup>D164N</sup> protein was used as a substrate for kinase-active MBP-Adi3 with or without AvrPto-FLAG protein. Molecular weight (kDa) markers are indicated.

assay with wild-type and mutant forms of Pto and AvrPto. Substitutions in Pto (K69Q) or AvrPto (I96A) are known to disrupt the Pto/AvrPto yeast two-hybrid (Y2H) interaction although they do not affect protein abundance (Loh and Martin, 1995; Tang *et al*, 1996; Frederick *et al*, 1998). The Pto<sup>K69Q</sup> mutation (subdomain II conserved Lys) also eliminates Pto kinase activity. We found that the Y3H interaction does not occur with Pto<sup>K69Q</sup> or AvrPto<sup>I96A</sup> (Figure 1B). However, the kinase-deficient mutant Adi3<sup>K337Q</sup> as well as the constitutively active protein Adi3<sup>S539D</sup> interacted with Pto/AvrPto similar to wild-type Adi3 (Figure 1B). These data indicate that the kinase-active conformation of Pto, but not that of Adi3, is required for the Pto/AvrPto/Adi3 interaction.

### **Pto phosphorylates Adi3 independently of AvrPto**

The requirement of the kinase-active conformation of Pto for the Y3H interaction suggested that Pto might phosphorylate Adi3. This was tested by using an *in vitro* kinase assay. When MBP-Pto was incubated with the kinase-deficient MBP-Adi3<sup>K337Q</sup> protein, phosphorylation of the kinase-inactive Adi3 protein was observed (Figure 1C, lane 3), which was independent of AvrPto (Figure 1C, lane 4). The ability of Adi3 to utilize Pto as a substrate was also tested. For these assays, Pto<sup>D164N</sup> was used because it is kinase-inactive but, unlike Pto<sup>K69Q</sup>, still interacts with AvrPto in the Y2H system (Rathjen *et al*, 1999). When MBP-Adi3 was incubated with MBP-Pto<sup>D164N</sup>, phosphorylation of Pto<sup>D164N</sup> was not seen regardless of the presence of AvrPto (Figure 1C, lanes 7 and 8). These data demonstrate that Adi3 is a substrate for Pto. The slight reduction in Pto phosphorylation of Adi3 in the presence of AvrPto (Figure 1C, lanes 4 and 6) appears to be nonspecific and due to the additional level of protein in the assay as Pto autophosphorylation level is reduced as well. Additionally, the same effect has been seen with Pdk1 phosphorylation of Adi3 (data not shown).

### **Isolation of the tomato Pdk1 gene and interactions with Adi3**

AGC kinases are commonly regulated by Pdk1 phosphorylation. Thus, we identified a full-length *Pdk1* cDNA in the tomato EST database using the *Arabidopsis Pdk1* sequence (Supplementary Figure S1; Deak *et al*, 1999). Tomato *Pdk1* encodes a protein of 494 aa, with a kinase domain and a pleckstrin homology domain. We found that it is an active protein kinase in autophosphorylation assays by using kinase-active MBP-Pdk1 and kinase-inactive MBP-Pdk1<sup>K77Q</sup> (subdomain II conserved Lys) proteins (Figure 2A). In the Y2H assay, Pdk1 did not interact with Pto or AvrPto, but did interact with Adi3 irrespective of the kinase activity of Adi3 or Pdk1 (Figure 2B).

The ability of Pdk1 to phosphorylate Adi3 was tested using MBP-Adi3 proteins as substrates for MBP-Pdk1. The Pdk1 phosphorylation site on AGC kinases is highly conserved (Bögge *et al*, 2003) and corresponds to S539 of Adi3. Mutation of S539 to Ala did not affect Adi3 autophosphorylation (Figure 1A), Adi3 Y3H interaction with Pto/AvrPto (Figure 1B), the ability of Pto to phosphorylate Adi3 (Adi3<sup>K337Q/S539A</sup>; Figure 1C, lane 5), or the Y2H interaction with Pdk1 (Figure 2B). Mutation of S539 to Asp, simulating a phosphorylation event, greatly increases the autophosphorylation activity of Adi3 (Figure 1A), suggesting that phos-

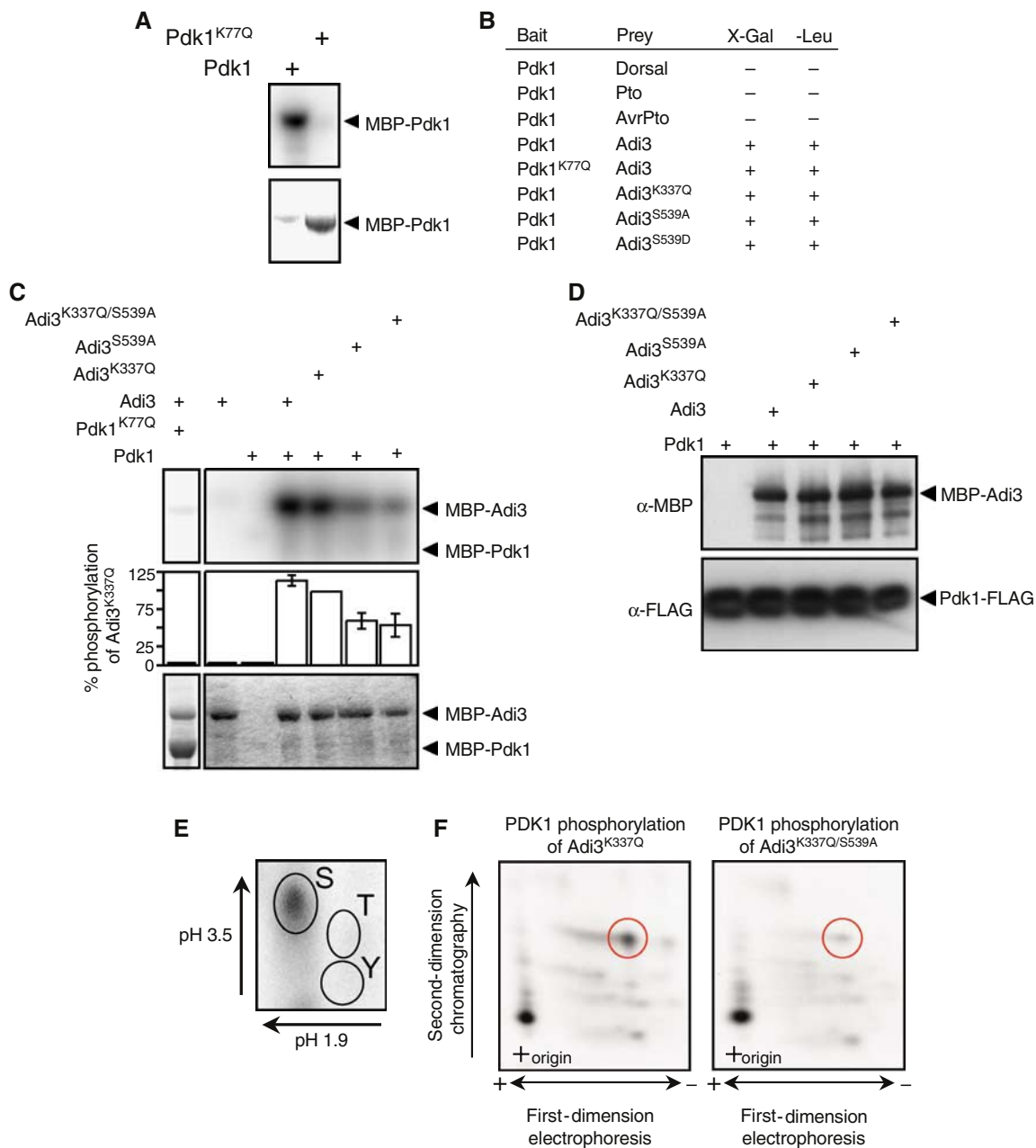
phorylation of S539 by Pdk1 is required for activation of Adi3. MBP-Pdk1 phosphorylated MBP-Adi3 but it phosphorylated MBP-Adi3<sup>S539A</sup> at a much reduced level (Figure 2C). Because MBP-Adi3<sup>S539A</sup> retains autophosphorylation (Figure 1A), MBP-Adi3<sup>K337Q/S539A</sup> kinase-deficient protein was used as a substrate for Pdk1 and was found to be phosphorylated by Pdk1 ~45% less than MBP-Adi3, suggesting the presence of additional Pdk1 phosphorylation sites on Adi3 (Figure 2C). The reduced phosphorylation of the MBP-Adi3 mutant proteins by MBP-Pdk1 is not due to reduced protein–protein interactions, as MBP-Adi3 mutant proteins co-immunoprecipitated *in vitro* with Pdk1-FLAG in equal amounts (Figure 2D). This is also supported by the Pdk1/Adi3 Y2H assay where there was no difference in the interaction of Pdk1 with Adi3<sup>S539A</sup> (Figure 2B).

The likelihood that S539 of Adi3 is a Pdk1 phosphorylation site was further supported by phosphoamino acid and phosphopeptide analysis. MBP-Pdk1 phosphorylation of MBP-Adi3<sup>K337Q</sup> followed by phosphoamino acid analysis indicates that only Ser residues are phosphorylated on Adi3 by Pdk1 (Figure 2E). Phosphorylation of MBP-Adi3<sup>K337Q</sup> by MBP-Pdk1 followed by tryptic digestion and phosphopeptide mapping showed the presence of two major tryptic peptides (Figure 2F, left panel). The same analysis on the MBP-Adi3<sup>K337Q/S539A</sup> protein showed almost complete loss of one tryptic peptide (Figure 2F, right panel, compare red circles between panels). The small amount of radiolabel remaining in this tryptic peptide may be attributable to S537, which is contained in the same tryptic fragment. Taken together, these data indicate that Pdk1 phosphorylates Adi3 at S539 (Figure 2C, E, and F) and this phosphorylation event is required for Adi3 activity (Figure 1A). At least one additional Ser residue on Adi3 is phosphorylated by Pdk1 and remains to be determined.

### **Adi3 Pdk1-interacting fragment regulates interaction with Pdk1 and Adi3 autophosphorylation**

In mammalian systems, the AGC kinase PIF motif regulates the interaction between Pdk1 and AGC kinases (Biondi and Nebreda, 2003; Biondi, 2004). The PIF is a short, hydrophobic amino-acid sequence motif, FxxFS/TF/Y, located close to or at the immediate carboxy-terminus of the protein. A PIF-binding pocket on Pdk1 (Figure 3A) binds the Phe residues and the phosphorylated Ser/Thr residue (Frödin *et al*, 2002). The minimal PIF sequence is defined as FxxF as found in PKA (Biondi, 2004). The Adi3 PIF (FExF) constitutes the last four residues of the protein (698–700; Figure 3A) and appears to be distinct from the PIF of PKA, as another group of plant AGC kinases (VIIIb) have a PIF consensus sequence identical to that of PKA (FxxF; Bögge *et al*, 2003).

The role of the Adi3 PIF motif in regulating interaction with and phosphorylation by Pdk1 was tested by examining MBP-Adi3 PIF mutant proteins as possible substrates for Pdk1. Substitution of Adi3 F697 or F700 to Ala, individually or in combination, significantly reduced Pdk1 phosphorylation of Adi3 (Figure 3B). The E698A substitution also reduced Pdk1 phosphorylation of Adi3, but not to the extent of the F697A and F700A substitutions (Figure 3B). Combining all three substitutions gave the greatest loss of Pdk1 phosphorylation of Adi3 (Figure 3B). To confirm that the Adi3 PIF mutations affect the interaction with Pdk1, Adi3 and Pdk1 were co-immunoprecipitated *in vitro*. The binding of MBP-Adi3 PIF



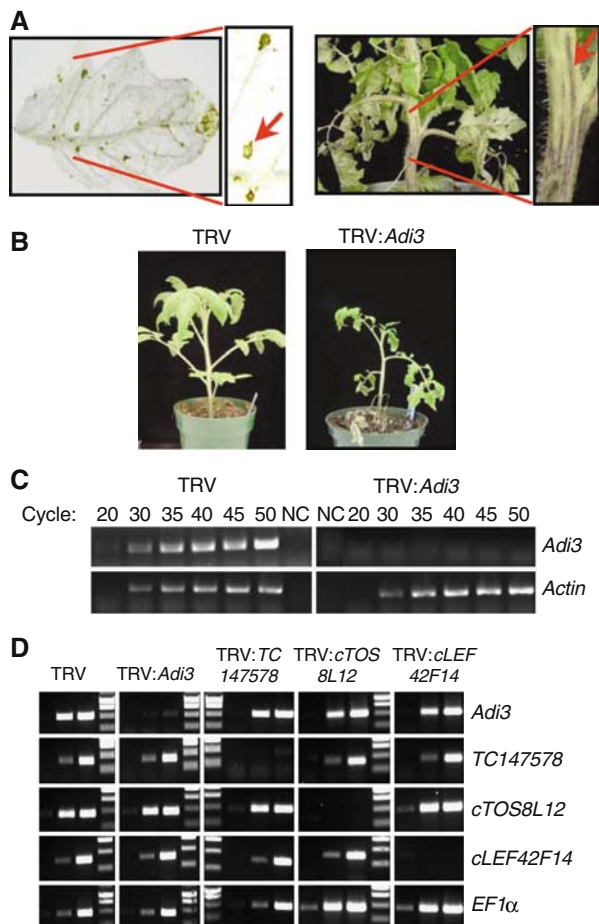
**Figure 2** Pdk1 kinase activity and interaction with Adi3. In (A) and (C), the top panel represents the kinase assay (phosphorimage) and the bottom panel the assay input (Coomassie-stained gel). (A) Tomato Pdk1 is a functional protein kinase. Analysis of kinase-active and -deficient MBP-Pdk1 fusion proteins by *in vitro* autophosphorylation assays is shown. (B) Pdk1 interacts with Adi3 in the Y2H assay. The indicated bait and prey constructs were tested in the Y2H assay for expression of the *lacZ* gene on X-Gal plates or leucine prototrophy (+, interaction; -, no interaction). (C) Pdk1 phosphorylation of Adi3 *in vitro*. MBP-Adi3 proteins were used as substrates for kinase-active MBP-Pdk1 protein. (D) *In vitro* co-immunoprecipitation of Pdk1 and Adi3. MBP-Adi3 and Pdk1-FLAG proteins were co-precipitated using  $\alpha$ -FLAG agarose and analyzed by Western blot. (E) Pdk1 phosphorylates Adi3 on Ser residues. Pdk1 phosphorylated Adi3 was analyzed by phosphoamino acid analysis; autoradiograph is shown. (F) Pdk1 phosphorylates Adi3 at S539 on one of two tryptic peptides. Pdk1 phosphorylated Adi3 was digested with trypsin and analyzed by phosphopeptide analysis; autoradiograph is shown.

mutant proteins with Pdk1-FLAG correlated with the level of phosphorylation of MBP-Aid3 PIF mutant proteins by Pdk1 (Figure 3C), indicating that the Adi3 PIF regulates the interaction with Pdk1.

Mammalian AGC kinases also contain a PIF-binding pocket that binds their own PIF for protein stabilization and autophosphorylation regulation (Frödin *et al*, 2002; Biondi and Nebreda, 2003). Most plant AGC kinases appear to lack a PIF-binding pocket (Bögge *et al*, 2003). Alignment of human and

plant Pdk1 and other mammalian AGC kinase PIF-binding pockets with the corresponding region in Adi3 shows low overall sequence identity; however, most residues predicted to bind the PIF appear to be conserved (Figure 3A). If Adi3 does contain a PIF-binding pocket for interaction with its own PIF, mutation of PIF residues should affect autophosphorylation. This was analyzed using MBP-Adi3 PIF mutant proteins in kinase assays. Mutation of F697, E698, and F700 to Ala, singly or in combination, completely eliminated Adi3 auto-





**Figure 4** Gene silencing of *Adi3* causes localized cell death. (A) *Adi3* silencing causes cell death in leaves and stems. Cell death lesions were photographed 3 weeks after TRV:*Adi3* infection. Arrows point to localized regions of cell death. In the left panel, leaves were cleared in 10% acetic acid, 30% chloroform, 60% EtOH. (B) *Adi3* silencing causes stunting. (C) *Adi3* transcripts are reduced by silencing. Control (TRV) and *Adi3*-silenced (TRV:*Adi3*) tissues were analyzed by RT-PCR for *Adi3* transcripts. Aliquots were removed at the indicated cycles. Actin was the control. NC, negative control. (D) *Adi3* silencing does not affect transcript levels of closely related AGC kinase genes. Tomato plants were silenced for the indicated genes (across top) and analyzed for transcript levels by RT-PCR (right side). *EF1 $\alpha$*  was the control. Lanes show products from cycles 28, 34, and 40 and MW markers.

ment resulted in essentially complete silencing of the *Adi3* gene, as shown by RT-PCR analysis of transcript levels (Figure 4C). Plants silenced for *Adi3* developed spontaneous (pathogen-independent), localized regions of cell death on leaves and stems, and became stunted (Figure 4A and B). This phenotype was independent of Pto or Prf (data not shown), indicating that these proteins act upstream or in concert with *Adi3*. We inoculated TRV:*Adi3*-silenced susceptible tomato plants with *Pst* and observed increased resistance as compared to TRV-only-infected controls (data not shown). However, we do not ascribe much significance to this enhanced resistance because the cell death phenotype that occurred 3 weeks after TRV:*Adi3* infection likely induces general defense responses. In fact, expression of the defense-related genes PR1 and PR3 was induced in *Adi3*-silenced plants (data not shown).

To check if unintended silencing of *Adi3*-related genes was responsible for the observed localized cell death phenotype,

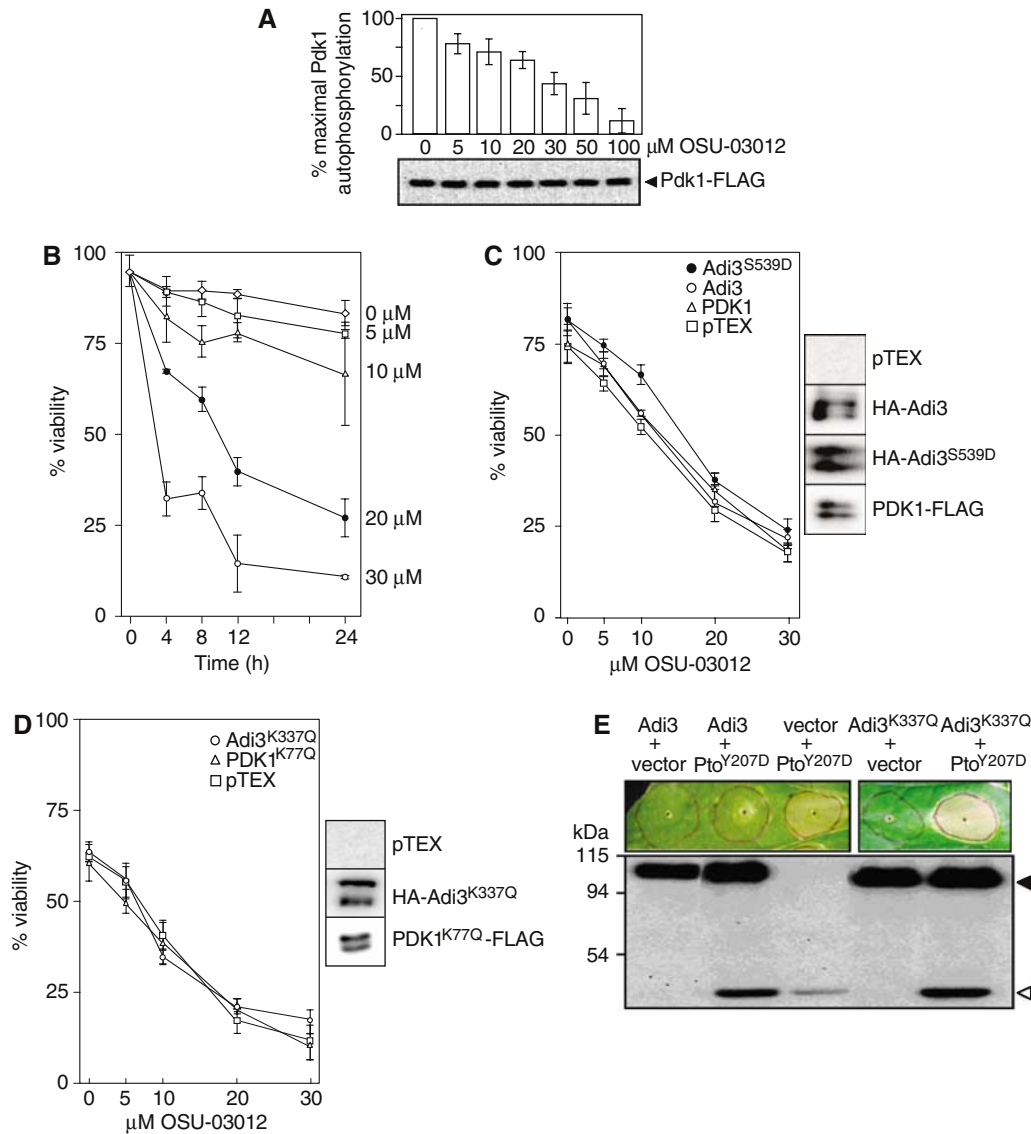
17 genes in the tomato EST database that encode group VIIIa AGC kinases were identified for analysis of expression levels in *Adi3*-silenced plants (note that there are also 17 *Arabidopsis* genes in this group, although we were not able to establish possible orthologs). The DNA sequences were aligned with the *Adi3* sequence and the three genes most similar to *Adi3* were chosen for phenotypic and transcript analysis in silenced plants (i.e., TC147578 (64%; fragment amplified from gDNA), TC144536 (70%; EST *cTOS8L12*), TC138973 (69%; EST *cLEF42F14*)). RT-PCR using gene-specific primers showed that there was remarkable specificity in these silencing experiments—for each gene, only its corresponding transcript was knocked down by VIGS (Figure 4D). Importantly, we found that only *Adi3*, when silenced, caused localized cell death and stunting (Supplementary Figure S2). These experiments suggest that *Adi3* acts as a negative regulator of host cell death and that silencing of *Adi3* releases this inhibition.

### Both *Pdk1* and *Adi3* regulate cell death in plants

In mammalian systems, Pdk1 phosphorylates PKB to promote its function in suppressing PCD (Vivanco and Sawyers, 2002; Vara *et al*, 2004; Zhu *et al*, 2004). Inhibition of Pdk1 does not allow for PKB activation and leads to PCD (Zhu *et al*, 2004). We therefore predicted that inhibition of Pdk1 might lead to PCD in tomato, and in fact, when a *Pdk1* construct designed to silence both tomato *Pdk1* genes (87.3 and 84.6% identical at the cDNA and protein level, respectively; data not shown) was used for VIGS, the plants died (data not shown).

The role of Pdk1 in plant cell death was further investigated using a specific inhibitor of mammalian Pdk1, OSU-03012 (Zhu *et al*, 2004). Tomato Pdk1 autophosphorylation was inhibited by OSU-03012 in a dose-dependent manner (Figure 5A). However, approximately 10 times more inhibitor was needed to achieve similar results seen with mammalian Pdk1 (Zhu *et al*, 2004), suggesting that OSU-03012 may be a less effective inhibitor of plant Pdk1.

OSU-03012 induces cell death in mammalian systems and this response can be reduced by overexpression of constitutively active Pdk1 or PKB (Zhu *et al*, 2004). We found that OSU-03012 reduced viability of tomato protoplasts in a dose-dependent manner over a 24-h period (Figure 5B). The ability of Pdk1, *Adi3*, and constitutively active *Adi3*<sup>S539D</sup> overexpression to reduce OSU-03012-induced cell death was examined in tomato protoplasts. Overexpression of the proteins was confirmed by Western blot (Figure 5C, right panels). *Adi3*<sup>S539D</sup> offered partial yet significant reduction in cell death, whereas Pdk1 and *Adi3* gave marginal cell death reduction in response to OSU-03012 (Figure 5C). Other studies using a constitutively active Pdk1 showed more reduction in cell death (Kulp *et al*, 2004; Zhu *et al*, 2004). Our Pdk1 was not constitutively active and this may explain the low level of protection against OSU-03012. We have yet to identify the residues required to make such a tomato Pdk1 protein. One study using a Pdk1 inhibitor and overexpression of wild-type Pdk1 did show a similar level of protection against the inhibitor as we have seen (Arico *et al*, 2002). Induction of cell death at high levels of OSU-03012 prevented overexpression of any protein from protecting against the inhibitor. Overexpression of kinase-inactive Pdk1<sup>K77Q</sup> and *Adi3*<sup>K337Q</sup> did not protect protoplasts from OSU-03012-induced cell death (Figure 5D). These results are consistent with PDK1



**Figure 5** Pdk1 and Adi3 negatively regulate plant cell death. (A) OSU-03012 inhibition of tomato Pdk1. Top, dose-dependent inhibition of recombinant Pdk1-FLAG autophosphorylation. Bottom,  $\alpha$ -FLAG Western blot showing assay input. (B) Pdk1 inhibitor OSU-03012 induces cell death in plants. Tomato protoplasts were treated with the Pdk1 inhibitor OSU-03012 and cell viability was monitored over 24 h. (C) Attenuation of OSU-03012-induced cell death by overexpression of Pdk1, Adi3, or Adi3<sup>S539D</sup>. Tomato protoplasts overexpressing proteins for 9 h were treated with OSU-03012 for an additional 12 h and cell viability was determined. The right panel shows Western blot detection of overexpressed proteins. (D) Lack of attenuation of OSU-03012-induced cell death by overexpression of kinase-deficient Pdk1<sup>K77Q</sup> or Adi3<sup>K337Q</sup>. The right panel shows Western blot detection of overexpressed proteins. (E) *Agrobacterium*-mediated overexpression of Adi3 can suppress cell death induced by Pto<sup>Y207D</sup> in leaf tissue. The lower panel shows Western blot detection of overexpressed proteins; HA-Adi3, filled triangle; Pto-HA, open triangle.

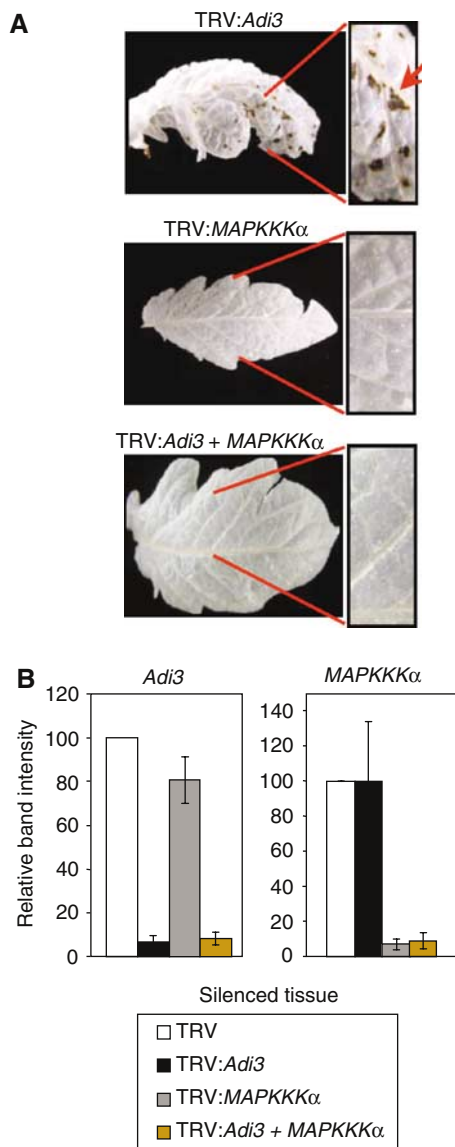
inhibitor studies on mammalian PKB and Pdk1 and suggest that OSU-03012-induced plant cell death is related to the inhibition of Pdk1 activity and that Adi3 function lies downstream of Pdk1 (Arico *et al*, 2002; Kulp *et al*, 2004; Zhu *et al*, 2004).

The ability of Adi3 to suppress cell death in leaves was investigated by using *Agrobacterium*-mediated transient overexpression of Adi3 in the presence of Pto<sup>Y207D</sup>, a constitutively active Pto that produces HR-like cell death in the absence of AvrPto (Rathjen *et al*, 1999). Overexpression of Adi3 suppressed Pto<sup>Y207D</sup>-induced cell death in 28 of 34 trials (Figure 5E). Adi3 kinase activity was required for this cell death suppression because Adi3<sup>K337Q</sup> was not able to suppress Pto<sup>Y207D</sup>-induced cell death in 22 of 25 trials (Figure 5E).

#### **Adi3 silencing-induced cell death is abolished by silencing of a MAPKKK gene known to play a role in pathogen-associated cell death**

To investigate whether cell death lesions formed upon *Adi3* silencing are related to PCD associated with pathogen attack, *Adi3* was co-silenced with *MAPKKK $\alpha$* . The latter gene has recently been shown to regulate PCD associated with both disease susceptibility and Pto/AvrPto-mediated disease resistance (del Pozo *et al*, 2004). *Adi3* and *MAPKKK $\alpha$*  were silenced individually and together in separate tomato plants and the formation of cell death lesions was monitored as silencing progressed. As expected, silencing of *Adi3* alone caused cell death, whereas silencing of *MAPKKK $\alpha$*  alone did not. Significantly, co-silencing of *Adi3/MAPKKK $\alpha$*  completely

eliminated the spontaneous formation of cell death lesions (Figure 6A) and the usual stunting caused by *Adi3* silencing was not observed (Supplementary Figure S3). *Adi3* and *MAPKKK $\alpha$*  transcript levels were not affected when analyzed from *MAPKKK $\alpha$* - and *Adi3*-silenced plants, respectively, and in co-silenced plants both genes were effectively silenced (Figure 6B). Overexpression of *Adi3* in leaf tissue did not affect the Pto/AvrPto induction of a MAPK known to be activated by *MAPKKK $\alpha$*  (Supplementary Figure S4; del Pozo *et al*, 2004). Also, *Adi3* and *MAPKKK $\alpha$*  do not interact in a Y2H assay (data not shown). These data suggest that *Adi3* and *MAPKKK $\alpha$*  act in parallel, interdependent pathways.



**Figure 6** Co-silencing of *Adi3* and *MAPKKK $\alpha$*  abolishes *Adi3* silencing-induced cell death. (A) *Adi3*/*MAPKKK $\alpha$*  co-silencing eliminates cell death caused by *Adi3* silencing. *Adi3* and *MAPKKK $\alpha$*  were silenced in tomato plants. Pictures of *Adi3*/*MAPKKK $\alpha$*  co-silencing represent two independent silencing experiments. Leaves were cleared in 10% acetic acid, 30% chloroform, 60% EtOH. (B) RT-PCR analysis of *Adi3*/*MAPKKK $\alpha$*  co-silencing. Tomato plants were silenced for the indicated gene and analyzed for transcript levels by RT-PCR. Abundance of PCR products from PCR cycle 32 was quantitated by spot densitometry and graphed.

## Discussion

### *Adi3* and *Pdk1* regulate plant cell death

We present several lines of evidence that indicate that *Pdk1*/*Adi3* are part of a general pathway regulating cell death in plants. Loss-of-function analysis of *Adi3* and *Pdk1* using VIGS results in localized cell death and whole plant death, respectively. The *Pdk1* inhibitor OSU-03012 induces cell death in tomato protoplasts that can be attenuated by overexpression of wild-type *Pdk1*, *Adi3*, or a constitutively active *Adi3*. These findings bear striking similarities to observations with mammalian *Pdk1* and *PKB* proteins. *PKB* knockout mice display spontaneous apoptosis in several different tissues, whereas *Pdk1* knockout mice do not survive past embryonic day 9.5 (Chen *et al*, 2001; Lawlor *et al*, 2002). Expression of a kinase-deficient form of *PKB* produces cell death in cerebellar neurons (Dudek *et al*, 1997; Luo *et al*, 2003). In addition, the *Pdk1* inhibitors celecoxib and OSU-03012 induce mammalian cell death, which can be attenuated by the expression of wild-type *Pdk1* and constitutively active forms of *Pdk1* and *PKB* (Arico *et al*, 2002; Kulp *et al*, 2004; Zhu *et al*, 2004). Overexpression of wild-type *PKB* can also reduce cell death in the presence of other apoptosis-inducing conditions (low KCl, minus serum, or plus NMDA; Dudek *et al*, 1997; Luo *et al*, 2003). These convergent observations support a role for *Pdk1* and *Adi3* as negative regulators of plant cell death, analogous to the role of *Pdk1* and *PKB* in mammalian systems. In spite of these functional similarities, *Adi3* and *PKB* have little sequence similarity (21.4% amino-acid identity over the full protein, 34.6% over the kinase domains, data not shown), suggesting that there are important regulatory and functional differences between plant and animal systems.

### *Adi3* interaction with *Pdk1* shows similarities and differences with mammalian systems

The activity of *PKB* is regulated by the induced phosphorylation of S473 in the PIF motif (FxxFSY; known as the PDK2 site), which allows for interaction with and activation by *Pdk1* (Vara *et al*, 2004). Other AGC kinases such as *S6K* and *SGK* are regulated in a similar manner. However, the interaction of AGC kinases *PRK2* and *PKC $\zeta$*  with *Pdk1* does not appear to be regulated by induced PIF phosphorylation, as they contain an Asp and Glu, respectively, in place of the Ser/Thr in the PIF. These acidic residues appear to mimic the phosphorylated Ser/Thr, as they are absolutely required for *Pdk1* interaction and phosphorylation (Balendran *et al*, 2000).

Our results indicate that *Pdk1* and *Adi3* interact through the *Adi3* PIF. This interaction with *Pdk1* appears to be similar to *PRK2*/*PKC $\zeta$*  and not to the induced PIF phosphorylation of *PKB*, as the *Adi3* PIF (FExF) contains an acidic Glu (E698) instead of a phosphorylatable Ser/Thr. However, *Adi3* residue E698 appears to be only partially required for *Pdk1* interaction, whereas F697 and F700 appear to be more important. Mutation of all three residues to Ala did not completely abolish *Pdk1* interaction, suggesting the involvement of additional amino acids. The only other plant PIF motifs to be analyzed are those of *AGC1-1* (FDxF) and *AGC2-1* (FxxF) (Anthony *et al*, 2004). The Phe residues in these PIFs were shown to be absolutely required for interaction with *Pdk1*, confirming that the need of acidic or phosphorylated residues in this motif is not necessarily required for plant AGC kinases.



The mammalian model for Pdk1/AGC kinase interaction predicts that, after PIF binding, the AGC kinase is phosphorylated by Pdk1, which stabilizes and activates the AGC kinase. This stabilization makes the PIF-binding pocket of the AGC kinase available for binding its own PIF, allowing for release from Pdk1 (Biondi, 2004). In this manner, Pdk1 can only bind an inactive kinase with a free PIF. Our Y2H data do not support this model for Adi3, as the constitutively active Adi3<sup>S539D</sup> protein retains interaction with Pdk1. This suggests that even when activated, the Adi3 PIF is still available for Pdk1 interaction or that phosphorylation of other Adi3 residues contributes to regulation of Pdk1 interaction. We have shown that at least one additional Ser residue in Adi3, in addition to S539, is phosphorylated by Pdk1. Identification and analysis of this residue may show that it is involved in the interaction with Pdk1. Alternatively, other factors distinct from the PIF might regulate the interaction with Pdk1. In support of this idea, some plant AGC kinases such as PHOT1 and PHOT2 contain the conserved Pdk1 phosphorylation site but not a PIF (Bögre *et al*, 2003), suggesting alternate mechanisms for interaction. This represents a potentially significant difference in the underlying mechanism for the interaction of Pdk1 and plant AGC kinases as compared with mammalian systems.

The Adi3 PIF also regulates autophosphorylation (Figure 3C), suggesting the presence of an Adi3 PIF-binding pocket. A recent review suggested that most plant AGC kinases lack a PIF-binding pocket (Bögre *et al*, 2003). Owing to low sequence identity, a PIF-binding pocket is not obvious in the Adi3 sequence (Figure 3A). However, specific residues in mammalian PIF-binding pockets known to interact with PIF residues are conserved in Adi3 (Figure 3A). Of these residues, R357, L383, and F308 appear to be the most important for Adi3 autophosphorylation, suggesting that they are important for self-binding of the PIF. This is in agreement with previous studies, which indicate that L155 and F82 of human Pdk1 (L383 and F308 in Adi3) interact with the first and last Phe, respectively, in the PIF (Biondi *et al*, 2000; Frödin *et al*, 2002). Human Pdk1 R131 (R357 in Adi3) is one of four residues that interact with the phosphorylated Ser/Thr in the PIF (Frödin *et al*, 2002) and is the only residue of the four conserved in Adi3. This difference may be due to the substitution of phosphorylatable Ser/Thr with a Glu (E698) in the Adi3 PIF, as discussed above. Mutation of the other potentially important residues in the Adi3 PIF-binding pocket also affects autophosphorylation (Figure 3E). However, as conformational changes due to these mutations cannot be discounted, a more detailed study is required to determine the true binding nature of these residues to the Adi3 PIF.

In contrast to most mammalian AGC kinases, mutation of the Pdk1 phosphorylation site in Adi3 (S539) to Ala does not abolish autophosphorylation activity (Figure 1A; Parekh *et al*, 2000). This result raises the question as to whether Adi3 is a true substrate of Pdk1. Two observations suggest that it is. First, mutation of Adi3 S539 to Asp greatly enhances its autophosphorylation activity (Figure 1A), indicating that phosphorylation of this site is required for full activity of Adi3. Second, we have shown that S539 is one of at least two Pdk1 phosphorylation sites on Adi3 (Figure 2C and F) and that Adi3 and Pdk1 interact in a PIF-dependent manner (Figure 3A and B). Interestingly, mutation of the Pdk1 phosphorylation site of PKC $\delta$  (T505) to Ala also does not

affect catalytic activity (Stempka *et al*, 1997; Parekh *et al*, 2000) although PKC $\delta$  is known to be a Pdk1 substrate (Le Good *et al*, 1998; Parekh *et al*, 2000).

Our Y2H data suggest that the Adi3/Pdk1 interaction is constitutive (Figure 2B), making it possible that Pdk1 phosphorylation of Adi3 is constitutive. Other AGC kinases, such as conventional PKC family members, are constitutively phosphorylated by Pdk1 but remain in an inactive conformation until interaction with second messengers (Newton, 2003). Adi3 may not be regulated in this manner, as the Adi3<sup>S539D</sup> protein, which mimics Pdk1 phosphorylation, is constitutively active (Figure 1A). Thus, the activity of Pdk1-phosphorylated Adi3 may be regulated through the action of phosphatases.

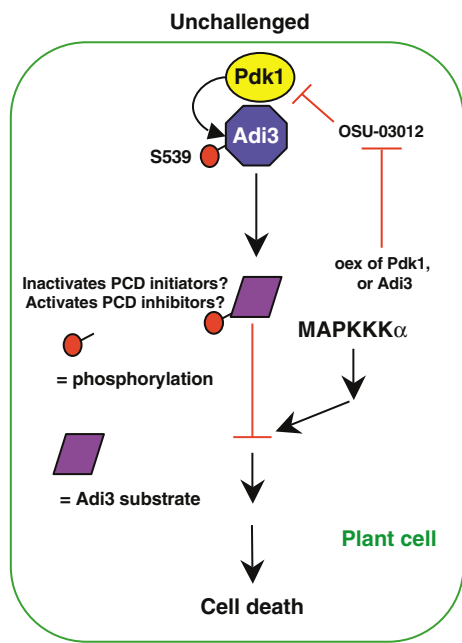
### **Adi3 function may be subverted for induction of cell death in response to pathogen attack**

Four observations suggest that loss of Adi3 function contributes to cell death associated with the Pto/AvrPto-mediated host response. First, Adi3 interacts specifically in the Y3H system only when functional forms of both AvrPto and Pto are present. Second, Pto phosphorylates Adi3. Third, suppression of *Adi3* gene expression causes formation of spontaneous necrotic lesions on plant leaves; similar localized cell death develops during the defense response (the HR) and disease progression. Fourth, co-silencing of *MAPKKK $\alpha$*  with *Adi3* suppresses Adi3-related cell death, indicating that this MAPKKK plays a role downstream of Adi3; MAPKKK $\alpha$  is known to be a positive regulator of cell death associated with *Pst*-related immunity and disease (del Pozo *et al*, 2004).

AGC kinases are central regulators of many eukaryotic cellular processes and it is perhaps not surprising that these proteins are targeted for manipulation by pathogens. In mammals, type III effector proteins YopH from *Yersinia enterocolitica* and ExoS and OdDHL from *Pseudomonas aeruginosa* inhibit activation of PKB, presumably for induction of apoptosis (Henriksson *et al*, 2000; Sauvonnnet *et al*, 2002; Li *et al*, 2004). In addition, the *Y. enterocolitica* effector protein YopM forms a complex with AGC kinases PRK2 and RSK, although the function of this interaction is not yet known (McDonald *et al*, 2003). Our discovery that the *Pseudomonas* type III effector AvrPto interacts with Adi3/Pto suggests that pathogens may also target plant AGC kinases to manipulate PCD in a manner similar to the targeting of PKB by mammalian bacterial pathogens. Considering that PKB regulates cell death in response to many different stimuli (Luo *et al*, 2003), it would not be surprising for Adi3 to be involved in cell death control during both disease and resistance, given its apparent upstream regulatory position.

### **A model for Adi3 control of plant cell death**

Based on our results and by analogy with Pdk1/PKB, we propose a model that suggests that in unchallenged plant cells, Adi3 is phosphorylated and activated by Pdk1 (Figure 7). This phosphorylation would activate the negative regulatory function of Adi3 and inhibit PCD through the activation of PCD inhibitors or the inactivation of PCD initiators. PKB acts similarly by phosphorylating substrates ranging from the apoptotic inhibitors NF- $\kappa$ B, CREB, and IKK to proapoptotic proteins such as BAD, caspase-9, and the Forkhead family transcription factor FKHR (Vivanco and Sawyers, 2002). However, the Adi3 recognition site in poten-



**Figure 7** Model for Pdk1/Adi3 cell death regulation. Negative regulation of plant cell death is initiated by Pdk1 phosphorylation of Adi3 at S539. Adi3 may then activate/inactivate PCD inhibitors/initiators by phosphorylation, preventing cell death through a parallel MAPKKK $\alpha$  pathway. OSU-03012-induced cell death can be overcome by overexpression of Pdk1 or the constitutively active Adi3<sup>S539D</sup>.

tial substrates may be different from that of PKB, as Adi3 is not capable of phosphorylating an artificial PKB substrate (data not shown). The mechanisms regulating the Pdk1/Adi3 pathway, and the identity and role of Adi3 substrates, are unknown and will be important goals for future research.

Our co-silencing and MAPK assay results suggest that Adi3 acts in parallel to MAPKKK $\alpha$  to inhibit a MAPK cascade. This role is consistent with evidence from both mammalian and plant systems that AGC kinases signal through MAPK cascades. In mammalian cells, EGF activation of the Raf-1/MEK1,2 cascade has been shown to signal through PKC $\zeta$  in a Pdk1-dependent manner, with PKC $\zeta$  acting upstream of Raf-1 (Corbit *et al*, 2000, 2003). Other studies contradict PKC $\zeta$  involvement, showing that Pdk1 directly phosphorylates MEK1 and MEK2 (Sato *et al*, 2004). *Arabidopsis* AGC kinase OX11/AGC2-1 signaling utilizes MPK3 and MPK6 to mediate oxidative burst signals generated during pathogen defense and cellular development (Rentel *et al*, 2004). Further study is needed to determine the extent to which regulation of the Adi3 pathway might be a master switch controlling various MAPK-controlled host cell death pathways.

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## Materials and methods

### Adi3 and Pdk1 cDNA cloning

The full-length *Adi3* cDNA was cloned using a combination of screening the tomato EST database with the 776-bp *Adi3* Y3H cDNA fragment (Bogdanove and Martin, 2000) and 5' RACE. More specifics on *Adi3* cDNA cloning can be found in Supplementary data.

Tomato *Pdk1* was identified by searching the tomato EST database (www.tigr.org) using the *Arabidopsis Pdk1* sequence (Deak *et al*, 1999). More specifics on *Pdk1* cDNA cloning can be found in Supplementary data.

### Yeast two- and three-hybrid assays

Y2H and Y3H assays were carried out as previously reported (Bogdanove and Martin, 2000) and detailed in Supplementary data using the pEG202 vector for bait constructs and the pJG4-5 vector for prey constructs.

### Protein expression, kinase assays, and in vitro co-immunoprecipitation

MBP translational fusion proteins were produced using the pMAL-c2 vector (New England Biolabs) and FLAG translational fusion proteins were produced using the pFLAG-CTC vector (Sigma). *In vitro* kinase assays were carried out on MBP translational fusion proteins. MBP-Adi3 and Pdk1-FLAG proteins were co-immunoprecipitated using  $\alpha$ -FLAG M2 agarose. Specifics can be found in Supplementary data.

### Phosphoamino acid analysis and phosphopeptide mapping

Phosphoamino acid analysis and phosphopeptide mapping were carried out as described in Supplementary data.

### Virus-induced gene silencing

Silencing was carried out using the pTRV1 and pTRV2 vectors with cloning by the Gateway system (Invitrogen) as described (Ekengren *et al*, 2003). Gene fragments used for VIGS are described in Supplementary data.

### OSU-03012 inhibition of Pdk1 and treatment of protoplast

Tomato protoplast isolation was performed as described in Supplementary data using 3-week-old Rio Grande-PtoR leaves. OSU-03012 was obtained from Dr Ching-Shih Chen at The Ohio State University and dissolved in DMSO. Protoplast treatment and transformation are described in Supplementary data.

### Agrobacterium-mediated transient expression

*Agrobacterium tumefaciens* strain GV2260 was used for *Agrobacterium*-mediated transient expression in *Nicotiana benthamiana* leaves as reported (del Pozo *et al*, 2004). HA-Adi3 (0.5 OD<sub>600</sub>) and Pto<sup>Y207D</sup>-HA (0.05 OD<sub>600</sub>) were infiltrated at a final density of 0.55 OD<sub>600</sub> for expression from the constitutive 35S CaMV promoter in the binary vector pCambia (HA-Adi3) or pBTEX (Pto<sup>Y207D</sup>-HA).

### Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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