An ATP analog-sensitive version of the tomato cell death suppressor protein kinase Adi3 for use in substrate identification

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ABSTRACT

Adi3 is a protein kinase from tomato that functions as a cell death suppressor and its substrates are not well defined. As a step toward identifying Adi3 substrates we developed an ATP analog-sensitive version of Adi3 in which the ATP-binding pocket is mutated to allow use of bulky ATP analogs. Met385 was identified as the "gatekeeper" residue and the M385G mutation allows for the use of two bulky ATP analogs. Adi3^{M385G} can also specifically utilize N^6-benzyl-ATP to phosphorylate a known substrate and provides a tool for identifying Adi3 substrates.

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1. Introduction

The genetically encoded process of programmed cell death (PCD) is known to exist in many organisms including multi- and single cell eukaryotes [1,2] and even in some bacteria [3]. PCD is often associated with the successful completion of developmental processes, resistance to pathogens, and responses to abiotic stress [1,4,5]. Despite the importance of PCD, plant genes that regulate PCD have been difficult to identify [1,6,7]. However, in recent years many genes that encode proteins involved in signaling pathways linked to PCD control have been identified [8–15]. But, the pathways associated with these proteins have yet to be fully elucidated.

In tomato, the Ser/Thr protein kinase Adi3 has been identified as a suppressor of PCD and is connected to the control of cell death during the resistance to *Pseudomonas syringae* pv. tomato, the causative agent of bacterial speck disease. Our previous studies have identified portions of the Adi3 signaling pathway including the upstream kinase 3-phosphoinositide-dependent protein kinase-1 (Pdk1), which phosphorylates Adi3 at Ser539, and the mitogen-activated protein kinase MAPKKKα that acts in a downstream parallel pathway [16,17]. We have also identified Adi3 interacting proteins using a yeast two-hybrid screen, but only identified one substrate for Adi3 [18], possibly due to the transient nature of kinase–substrate interactions, which is not ideal for yeast two-hybrid screens [19].

In order to isolate additional Adi3 substrates, we have developed an ATP analog-sensitive (*as-*) form of Adi3 (*as*-Adi3). In this approach, an *as*-kinase is produced by mutating a bulky, or "gatekeeper", amino acid within the ATP-binding pocket to a less bulky amino acid such as Ala or Gly [20,21]. This creates a larger ATP-binding pocket and allows for the specific use of bulky forms of ATP with N^6 substitutions such as N^6-benzyl-ATP or N^6-phenylethyl-ATP [20,21] (Fig. 1). Only the *as*-kinase can utilize the bulky ATP analogs and thus, can be used to specifically identify phosphorylation substrates from cell extracts. The production and use of *as*-kinases have been successfully used on dozens of kinases from yeast and mammalian systems [20,22], but this technology has rarely been used on plant kinases [22–25]. Here we report the development of an *as*-Adi3 and show that it can specifically utilize N^6-benzyl-ATP to phosphorylate a known Adi3 substrate.

2. Materials and methods

2.1. Cloning, expression, and mutagenesis of MBP-Adi3 and v-Src

Cloning of the Adi3 cDNA into pMAL-c2 and expression/purification of protein from *Escherichia coli* for N-terminal maltose binding protein (MBP) translational fusions were previously described [16]. Site-directed mutagenesis was carried out using standard protocols and Pfu Turbo DNA polymerase (Stratagene). The Adi3 S539D and K337Q mutations were previously described [16]. Primers for the M385A and M385G mutations are as follows (mutation sites in bold): For M385A, forward primer 5′-TTCTCATGTTTGGTC**GCA**GAAATATTGTCCTGGA-3′, reverse primer 5′-TCCAGGACAATATTTC**TGC**GACCAAACATGAGAA-3′; for M385G, forward primer 5′TTCTCATGTTTGGTC**GGA**GAATATTGTC

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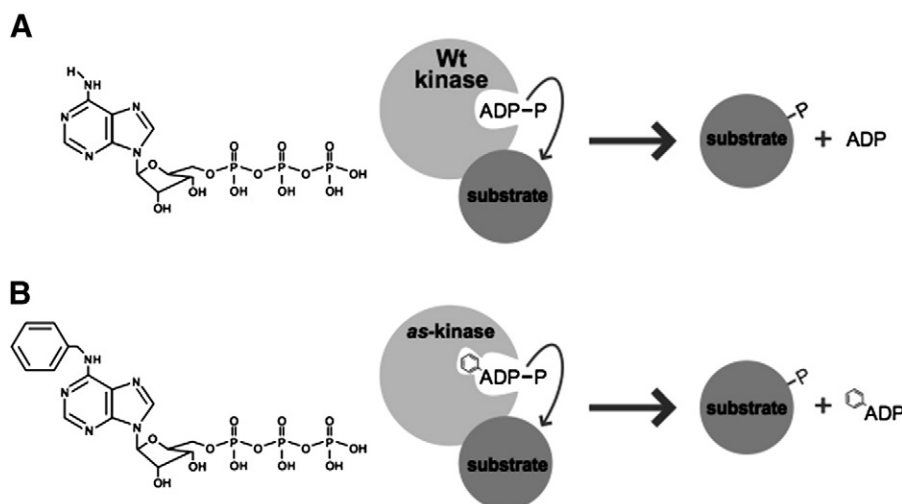*E-mail address:* tpd8@tamu.edu (T.P. Devarenne).

Fig. 1. Schematic of the analog-sensitive kinase technology. (A) A wild-type protein kinase use of the γ-phosphate of ATP to phosphorylate substrates. (B) An *as*-protein kinase use of the γ-phosphate of bulky ATP analogs to phosphorylate substrates.

CTGGA-3′, reverse primer 5′-TCCAGGACAATATTTC**TCC**GACCAAACATGAGAA-3′. v-Src and v-Src[I338G] [26] were cloned into paMAL-c2 and expressed and purified from *E. coli* as with MBP-Adi3.

2.2. Production of γ-[^{32}P]-N^6-benzyl-ATP

Gamma radiolabeled N^6-benzyl-ATP was produced following previously published protocols [23,27,28]. Briefly, nucleoside diphosphate kinase (NDPK) [29] was cloned into pET21b to produce *E. coli* expressed C-terminal 6xHis tagged protein, which was purified using Ni^{+2} resin (Novagen). Eighty μg of NDPK-6xHis was immobilized on a 130 μl Ni^{+2} column followed by addition to the column of 360 pmol (1 mCi at 3000 Ci/mmol) of γ-[^{32}P]ATP diluted in 800 μl of 1x PBS. After washing the column thoroughly with 1x PBS, 8 nmol of non-radiolabeled N^6-benzyl-ADP (BioLog Life Science Institute) was diluted in 32 μl of 1x PBS containing 5 mM MgCl$_2$, added to the column, and incubated at room temp for 5 min. Finally, γ-[^{32}P]-N^6-benzyl-ATP was eluted from the column with a buffer of 100 μl 1x PBS, 5 mM MgCl$_2$ and stored in 10 μl aliquots at −80 °C until used.

2.3. Kinase assays

Adi3 autophosphorylation kinase assays were carried out with 5 μg of purified MBP-Adi3 protein and 0.25 μCi of γ-[^{32}P]ATP or γ-[^{32}P]-N^6-benzyl-ATP per reaction in a volume of 30 μl as previously described [16]. Reactions were stopped by addition of 4x SDS-PAGE sample buffer and samples separated by 10% SDS-PAGE followed by visualization and quantification of incorporated radioactivity using a phosphorimager (Bio-Rad Molecular Imager) and quantification software (Bio-Rad Quantity One). Adi3 phosphorylation of Gal83 was carried out as above with 1 μg MBP-Adi3 protein and 5 μg of MBP-Gal83 as previously described [16]. All kinase assays were carried out two or three independent times. Values shown for phosphorylation levels are from one experiment and are representative of all experiments. v-Src kinase assays were carried out with 3 μg of purified MPB-vSrc protein under conditions previously described [26].

3. Results and discussion

3.1. Identification of a target Adi3 gatekeeper residue

The first step in the production an *as*-kinase is the identification of the gatekeeper residue that when mutated will allow for use of bulky ATP analogs. Isoleucine 338 of the Rous sarcoma virus tyrosine

protein kinase v-Src has been identified as the gatekeeper amino acid [26]. This residue was identified by analyzing the structures of two other kinases, PKA and CDK2, to locate the residue(s) closest to the N^6-amine group of ATP; Met120 in PKA and Phe80 in CDK2 (Fig. 2A). Alignment of the v-Src protein sequence with that of PKA and CDK2 identified v-Src Ile338 as a potential mutational target and analysis confirmed that Ile338 mutation allowed for use of N^6-benzyl-ATP [26]. Thus, we aligned Adi3 with PKA, CDK2, and v-Src and identified Met385 of Adi3 as a potential mutational target (Fig. 2A).

3.2. Mutation of Adi3 M385 to Ala allows for use of N^6-benzyl-ATP

Our previous studies have shown that a fusion of Adi3 to (MBP) produces soluble, kinase-active protein that effectively mimics native activity [16,17]. We have also identified Adi3 Lys337 as the invariant Lys in the ATP-binding pocket that coordinates ATP biding, and Ser539 as the upstream activation site on Adi3 [16]. Mutation of Adi3 Lys337 to Gln (Adi3[K337Q]) produces an inactive protein kinase and mutation of Ser539 to Asp (Adi3[S539D]) produces a constitutively active Adi3 [16]. After identification of Adi3 Met385 as a potential gatekeeper amino acid it was mutated to Ala or Gly in the MPB-Adi3, MBP-Adi3[K337Q], and MPB-Adi3[S539D] backgrounds.

First, the ability of the Adi3 *as*-mutants to utilize ATP for autophosphorylation was tested using in vitro kinase assays and γ-[^{32}P]ATP. We also used an MBP tagged version of v-Src[I338G] as a positive control for non-use of ATP. As has been previously reported [26], v-Src[I338G] was not capable of utilizing ATP for autophosphorylation (Fig. 2B, lane 2). As we have seen previously [16], the Adi3[K337Q] protein is autophosphorylation-inactive (Fig. 2B, lane 4) and Adi3[S539D] had greatly increased autophosphorylation over that of wild-type Adi3 (Fig. 2B, compare lanes 3 and 5). The Adi3[M385A] and Adi3[M385G] proteins could both utilize ATP to a low level with Adi3[M385A] being more efficient (Fig. 2B, lanes 7 and 9). Introduction of the M385A and M385G mutations into the Adi3[S539D] background increased the ability of the Adi3[M385A] protein to utilize ATP (Fig. 2B, lane 11), and actually decreased the ability of Adi3[M385G] to use ATP (Fig. 2B, lane 13). The K337Q mutation in any combination with Adi3[M385A] or Adi3[M385G] eliminated the ability of Adi3 to utilize ATP (Fig. 2B, lanes 8, 10, 12, 14). These studies indicate that the Ala or Gly mutations of Met385 decrease, but do not fully eliminate, the ability of Adi3 to utilize ATP. However, the M385G mutation provides a near complete loss of ATP use especially in the Adi3[S539D] background.

In order to test the ability of the *as*-Adi3 mutants to utilize N^6-benzyl-ATP, radiolabeled γ-[^{32}P]-N^6-benzyl-ATP was produced
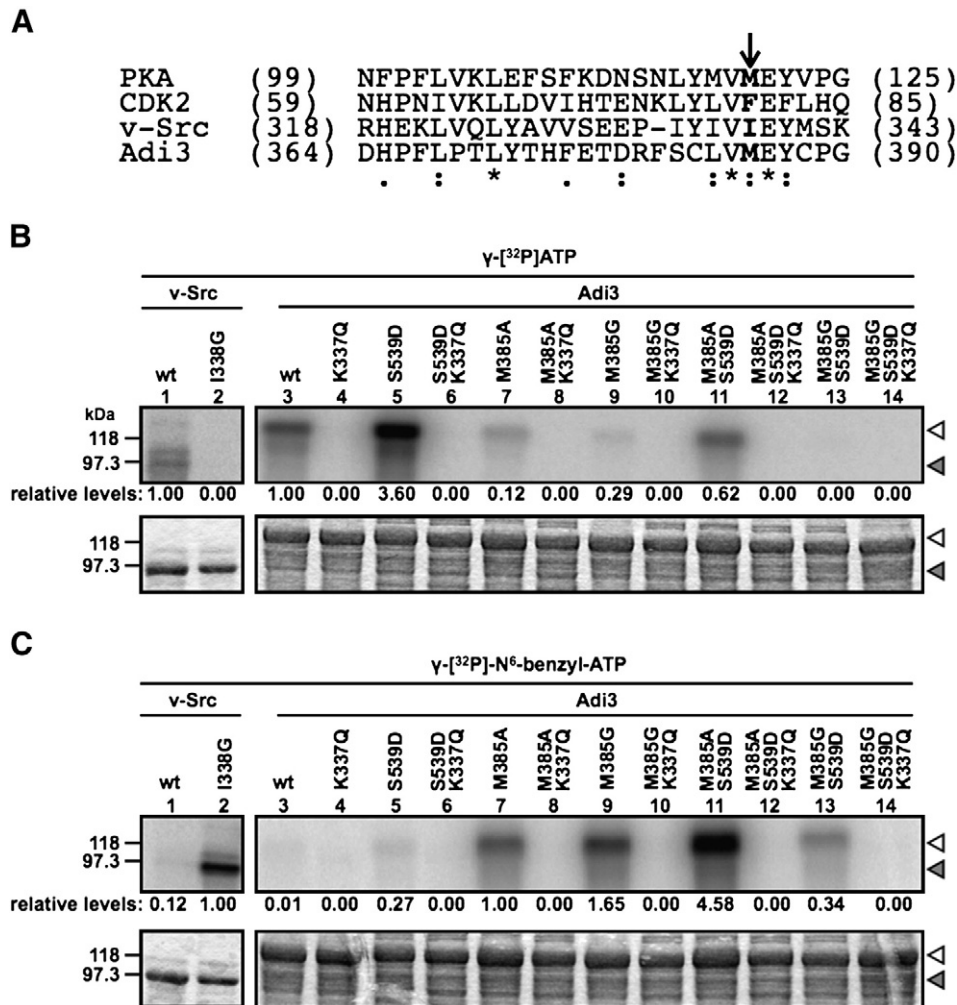
Fig. 2. Mutation of Adi3 Met385 allows use of bulky ATP analogs. (A) Identification of the target Adi3 gatekeeper residue (in bold and indicated with arrow) by alignment with PKA, CDK2, and v-Src. In (B) and (C) the indicated proteins were incubated in an in vitro kinase assay separated by SDS-PAGE, and exposed to a phosphorimager. Top panel, phosphorimage; bottom panel, Coomassie stained gel. Open triangle, MBP-Adi3; gray triangle, MBP-v-Src. (B) Adi3 M385A and M385G mutations limit the use of γ-[^{32}P]ATP in autophosphorylation. (C) Adi3 M385A and M385G mutations can use γ-[^{32}P]-N^6-benzyl-ATP in autophosphorylation.

using NDPK and γ-[^{32}P]ATP to phosphorylate non-labeled N^6-benzyl-ADP. As expected, v-Src^{I338G} efficiently utilized γ-[^{32}P]-N^6-benzyl-ATP while the wild-type v-Src could not (Fig. 2C, lane 1 and 2). Wild-type Adi3 and Adi3^{K337Q} could not utilize γ-[^{32}P]-N^6-benzyl-ATP, while Adi3^{S539D} had a very low ability to use γ-[^{32}P]-N^6-benzyl-ATP (Fig. 2C, lanes 3, 4, and 5). The M385A mutation in the wild-type Adi3 or Adi3^{S539D} backgrounds allowed for use of γ-[^{32}P]-N^6-benzyl-ATP, with Adi3$^{M385A/S539D}$ having the expected increase in activity over Adi3^{M385A} (Fig. 2C, lanes 7 and 11). The M385G mutation in the wild-type Adi3 or Adi3^{S539D} backgrounds allowed for use of γ-[^{32}P]-N^6-benzyl-ATP, however, Adi3$^{M385G/S539D}$ did not have the expected increase in activity as compared to Adi3^{M385G} (Fig. 2C, lanes 9 and 13). These results indicate that mutation of Adi3 Met385 to Ala or Gly enlarges the ATP binding pocket to allow for use of N^6-benzyl-ATP. Since the Adi3^{M385G} protein utilizes ATP less than the Adi3^{M385A} protein, especially in the activated background (Adi3$^{M385G/S539D}$; Fig. 2B), we chose to do follow-up studies on the Adi3^{M385G} and Adi3$^{M385G/S539D}$ proteins. This will be of particular importance in future studies using plant extracts to identify Adi3 substrates.

3.3. Elimination of background use of ATP

Since the Adi3^{M385G} and Adi3$^{M385G/S539D}$ proteins can utilize γ-[^{32}P]ATP to a low level (Fig. 2B), experiments were carried out to determine if use of γ-[^{32}P]ATP by the Adi3^{M385G} and Adi3$^{M385G/S539D}$ proteins can

be blocked through the use of two bulky ATP analogs. For these assays, Adi3 protein was preincubated with non-radiolabeled N^6-benzyl-ATP or N^6-phenylethyl-ATP followed by incubation in a kinase assay with γ-[^{32}P]ATP. If the bulky ATP analogs are efficiently used by the Adi3 proteins, the incorporation of [^{32}P] from γ-[^{32}P]ATP should be eliminated. The results show that bulky ATP analog preincubation did not affect the ability of wild-type Adi3 or Adi3^{S539D} to incorporate [^{32}P] (Fig. 3, lanes 1, 3, 5, and 7), while the preincubation eliminated the background use of γ-[^{32}P]ATP by Adi3^{M385G} and Adi3$^{M385G/S539D}$ (Fig. 3, lanes 2, 4, 6, and 8). This would indicate that the background use of ATP by the as-Adi3 proteins can be eliminated in the presence of the bulky ATP analogs.

3.4. Adi3$^{M385G/S539D}$ can utilize N^6-benzyl-ATP to phosphorylate a known substrate

Finally, we examined if the Adi3$^{M385G/S539D}$ protein could phosphorylate a known Adi3 substrate. Gal83, the β-subunit of the tomato SnRK1 protein complex, has been shown to be phosphorylated by Adi3 at Ser26 [18]. The Adi3$^{M385G/S539D}$ protein was capable of utilizing γ-[^{32}P]-N^6-benzyl-ATP to phosphorylate Gal83 and this phosphorylation was slightly higher than that seen by the non-analog sensitive version Adi3^{S539D} with γ-[^{32}P]ATP (Fig. 4). Phosphorylation of Gal83 by either version of Adi3 was eliminated by the Gal83 Ser26A mutation (Fig. 4). Elimination of Adi3$^{M385G/S539D}$ kinase activity by
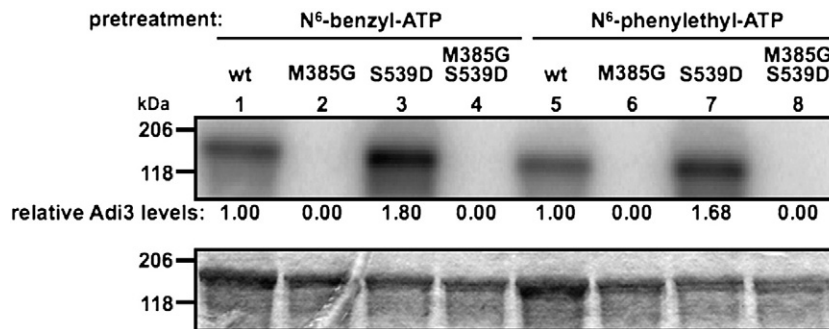
Fig. 3. Background use of ATP by Adi3^{M385G} can be outcompeted by bulky ATP analogs. The indicated MBP-Adi3 proteins were incubated with N^6-benzyl-ATP or N^6-phenylethyl-ATP prior to incubation with γ-[^{32}P]-ATP. Samples were analyzed as in Fig. 2. Top panel, phosphorimage; bottom panel Coomassie stained gel.
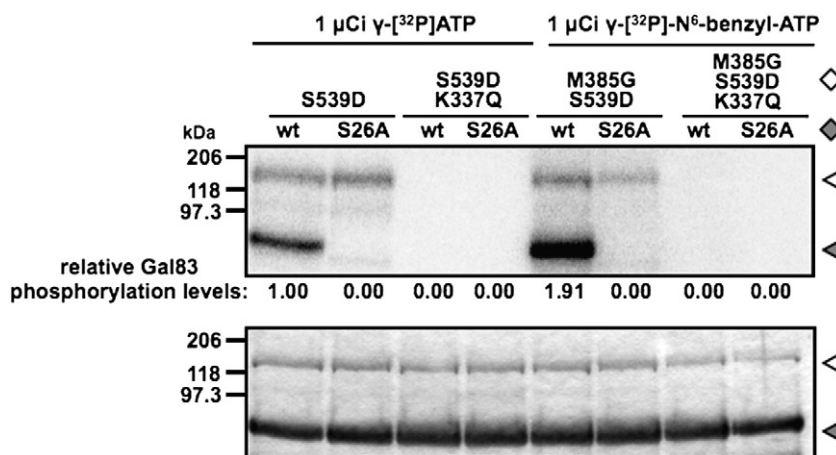

Fig. 4. Adi3$^{M385G/S539D}$ can use γ-[^{32}P]-N^6-benzyl-ATP to phosphorylate Gal83 but not Gal83^{S26A} in in vitro kinase assays. Samples were analyzed as in Fig. 2. Top panel, phosphorimage; bottom panel, Coomassie stained gel. Open diamond, form of MBP-Adi3 protein used in assay; gray diamond, form of MBP-Gal83 used in assay; open triangle, location of MBP-Adi3; gray triangle, location of MBP-Gal83.

introduction of the K337Q mutation resulted in a loss of Gal83 phosphorylation (Fig. 4).

Our data indicate that mutation of the Adi3 Met385 residue produces an analog-sensitive version of Adi3 and enables the use of N^6-benzyl-ATP for phosphotransfer in both autophosphorylation and trans-phosphorylation. This indicates that the as-Adi3 proteins could be used in the future for the identification of substrates from plant protein extracts through the direct phosphorylation of substrates with γ-[^{32}P]-N^6-benzyl-ATP [21]. Substrates can also be identified through inhibiting phosphorylation with γ-[^{32}P]ATP since as-kinases can also specifically bind ATP analog inhibitors such as 1-NA-PP1 [23,24,30]. For these types of studies it would need to be determined that 1-NA-PP1 can actually inhibit as-Adi3 kinase activity. More importantly, this inhibitor approach requires removal of the endogenous target kinase gene and replacement with the as-kinase [23]. Creating stable knockout lines for specific genes in tomato is not currently possible, but stable plants can be generated with gene silencing constructs to knockdown the expression of target genes. However, this would also target the introduced as-kinase. Additionally, the loss of Adi3 by virus induced gene silencing is lethal [16]. Thus, the as-kinase inhibitor approach appears to hold limited possibilities for identifying Adi3 substrates. Our future studies will focus on using as-Adi3 and the direct phosphorylation approach for identification of Adi3 substrates. It is also important to note that mutation of only Met385 was required for use of N^6-benzyl-ATP by Adi3. This is of importance because in some kinases, mutation of the gatekeeper residue does not allow for use of bulky ATP analogs. These kinases require a second-site mutation to produce a fully active as-kinase [22]. In this sense, Adi3 behaves like the majority of other kinases for which as- versions have been made. These studies

are also of significance since only four plant protein kinases have been mutated to an as-kinase; the tomato protein kinases Pto and MPK3, the tobacco kinase CDPK2, and the *Arabidopsis* kinase CPK1 [22–25]. Our studies indicate that it is possible to use this approach on more plant kinases and this method may be useful for substrate identification of a wider range of plant kinases.

Acknowledgements

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