The *Arabidopsis* Protein Kinase PTI1-2 Is Activated by Convergent Phosphatidic Acid and Oxidative Stress Signaling Pathways Downstream of PDK1 and OXI1

Richard G. Anthony §•, Safina Khan ‡, Joana Costa ‡, Maria S. Pais ‡, and László Bögre ‡

From the §School of Biological Sciences, Royal Holloway, University of London, Egham Hill, Egham, Surrey TW20 0EX, United Kingdom, ‡Centre for Plant Sciences, School of Biology, University of Leeds, Leeds LS2 9JT, United Kingdom, and ‡Laboratory for Plant Biotechnology-Instituto de Ciência Aplicada e Tecnologia, Faculdade de Ciências da Universidade de Lisboa, Gampo Grande, 1749-016 Lisboa, Portugal

Arabidopsis PDK1 activity is regulated by binding to the lipid phosphatidic acid (PA) resulting in activation of the oxidative stress-response protein kinase OXI1/AGC2-1. Thus there is an inferred link between lipid signaling and oxidative stress signaling modules. Among a panel of hormones and stresses tested, we found that, in addition to PA, the fungal elicitor xylanase activated PDK1, suggesting that PDK1 has a role in plant pathogen defense mechanisms. The downstream OXI1 was activated by additional stress factors, including PA, H₂O₂, and partially by xylanase. We have isolated an interacting partner of OXI1, a Ser/Thr kinase (PTI1-2), which is downstream of OXI1. Its sequence closely resembles the tomato Pti kinase, which has been implicated in the hypersensitive response, a localized programmed cell death that occurs at the site of pathogen infection. PTI1-2 is activated by the same stresses/elicitors as OXI1 and additionally flagellin. We have used RNA interference to knock down the expression of PDK1 and OXI1 and to study the effects on PTI1-2 activity. We show that specific lipid signaling pathways converge on PTI1-2 via the PDK1-OXI1 axis, whereas H₂O₂ and flagellin signals to OXI1-PTI1-2 via a PDK1-independent pathway. PTI1-2 represents a new downstream component that integrates diverse lipid and reactive oxygen stress signals and functions closely with OXI1.

It is now clear that reactive oxygen species (ROS) play an important signaling role in plants controlling processes such as growth, development, programmed cell death, and responses to biotic and abiotic environmental stimuli (1). Current evidence supports the concept that ROS represent a significant point of convergence between pathways that respond to biotic and abiotic stresses (2). Nevertheless, our current understanding of ROS participation in cross-talk between these pathways is very limited (2). OXI1/AGC2-1 is a protein kinase that was identified as a downstream signaling component to the PDK1 (3-phosphoinositide-dependent protein kinase 1) and as a protein kinase that is required for oxidative burst-mediated signaling in *Arabidopsis*, hereafter referred to as OXI1 (3, 4). OXI1 is critical for at least two very different ROS-mediated processes, basal resistance to *Peronospora parasitica* infection and root hair growth (3, 4). OXI1 is a member of the AGC family of protein kinases, and we have reported previously that OXI1 is activated by PDK1-mediated phosphorylation. In addition PDK1 acts upstream of other AGC kinases and regulates diverse signaling pathways, involved in root hair growth, auxin regulation, and plant cell death (3, 5, 6). PDK1 enzyme activity is regulated by binding the lipid signaling molecule, phosphatidic acid (PA), to its pleckstrin homology (PH) domain (3). PA is produced in response to many different stresses, including abscisic acid (ABA)-induced stomatal closure, pathogen attack, and oxidative stress (7, 8). It is generated via two distinct phospholipase pathways, either directly by phospholipase D (PLD) or the sequential action of phospholipase C (PLC) and diacylglycerol kinase (7). Use of specific inhibitors to PLD and PLC has shown that PDK1 is specifically activated by PLD-generated PA (PA⁺PLD) (3). The oxidative burst is one of the earliest responses of plants to pathogens, transiently produced in forms, including hydrogen peroxide and superoxide (9, 10). ROS produced in this way serve not only as protectants against invading pathogens but also as signals activating further plant defense reactions including the hypersensitive response of infected cells. Similarly the rapid synthesis of either PA⁺PLC or PA⁺PLD has been observed in plant cells when they are exposed to microbial pathogens or elicitors, such as cell wall fragments or highly specific proteins secreted by the pathogen (11, 12). In one study the elicitation of suspension cultured tomato cells triggered formation of PA and diacylglycerol pyrophosphate (11). PA accumulation also occurs as an early response in the Cf-4/Avr4 interaction. The *Cladosporium fulvum* (Cf)-4 gene of tomato confers resistance to the fungus *C. fulvum*, expressing the cor-
responding avirulence (Avr)4 gene (13). The PA increase is likely to be upstream of the oxidative burst because PLC inhibitors blocked the elicitor-induced oxidative burst and because exogenously applied PA induced a partial oxidative burst (13). In general, pathogenic elicitors activate the PLC-diacylglycerol kinase pathway. There are also several reports highlighting the importance of PLD signaling in response to elicitors. Several Arabidopsis PLDs were found to be induced differently in response to Pseudomonas infection (14), and in rice PLD gene expression increased and PLD protein relocalized to the plasma membrane at the site of infection by Xanthomonas oryzae (14). In a suspension of cultured tomato cells both PLD gene expression and enzyme activity were induced in response to the fungal elicitor xylanase (11, 15) but not chitotetrose or bacterially derived flagellin, indicating that different pathways signal the presence of elicitors (11).

To further characterize the PDK–OXI1 signaling module and to determine the relationship between lipid signals and ROS signals, we initiated a search for interacting protein partners of OXI1. Here we describe the characterization of a Ser/Thr kinase (PTI1-2) that acts downstream of both PDK1 and OXI1. It is activated in response to PA and xylanase in a PDK1-dependent manner. Furthermore, it is activated by flagellin and H$_2$O$_2$ in an OXI1-dependent but PDK1-independent manner. These results provide evidence that OXI1 and PTI1-2 are capable of integrating both lipid-derived and ROS stress signals and furthermore implicate PDK1 in a plant pathogen defense mechanism.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—The yeast two-hybrid constructs, pAS-AGC2-1 (OXI1), pAS-AGC2-1mut2, pAS-PDK1-1, and pACT-PDK1-1, were generated as described previously (3). Full-length Gateway-compatible versions of Pti1-1, Pti1-2, and Pti1-3 were prepared by PCR from an Arabidopsis cell suspension library, pACT2CS, and cloned into pDONR207 (Invitrogen). The Gateway-compatible vectors GW-pACT and GW-pAS2–1 were generated by converting pACT and pAS-2 (Clontech) by insertion of the gateway reading frame cassette into the Smal site of the original vectors, according to the manufacturer’s protocols. The Pti clones were then recombined into the Gateway-compatible two-hybrid vectors to generate pGW-ACT-PTI1-1, pGW-ACT-PTI1-2, pGW-ACT-PTI1-3, and the corresponding pGW-AS2-1 series. The sequences of the clones were confirmed by sequencing. The Pti (Pto kinase interactor) sequences possess a conserved phosphorylation site in the kinase activation loop (Thr-238) in PTI1-2. To test the role of Thr-238 in the interaction with OXI1, we inserted an alanine to substitute Thr-238 and abolish the phosphorylation site, in both wild-type PTI1-2 and kinase-deficient PTI1-2(K99N). Mutations were generated in the Gateway plasmid pDEST15 (Invitrogen) containing a GST-PTI1-2(K99N/T238A) was generated using PTI1-2(K99N) as a template and the PTI1-2(T238A) primer. The insertion in the gene of the desired mutation and the absence of undesired mutations were confirmed by sequencing. GST-tagged PTI constructs were generated by Gateway recombination into pDEST15 (Invitrogen). The chimera GST-PTI was then subcloned into the plant expression vector pART69 (16). The HA-tagged full-length OXI1, HA-PDK1, GST-PDK1, Myc-PDK1, and the PDK1-RNAi vectors were as described previously (3, 5). PTI1-1, PTI1-2, and PTI1-3 were transferred into the destination vector pGWB15 to generate 3× N-terminal HA-tagged PTI fusion constructs HA-PTI1-1, HA-PTI1-2, and HA-PTI1-3. The OXI1 RNAi hairpin construct was generated by transferring OXI1 into the destination vector pK7GWIWG2 (17).

**Protein Interaction Assays in the Yeast Two-hybrid System—**Saccharomycyes cerevisiae strain AH109 was used to screen an Arabidopsis pACT2 CDNA library (18) with pAS-OXI1 bait clones using methods described previously (19). Resulting colonies were initially assessed for reporter gene activity by nutritional selection (growth on medium lacking leucine, histidine, and tryptophan and containing 5 mM of 3-amino-1,2,4-triazole). Yeast colonies were then transferred to nitrocellulose filters on SD plates with 2 mM 3-amino-1,2,4-triazole. LacZ activity was determined by filter lift analysis, including appropriate positive and negative interaction controls. Putative positives were sorted into groups by diagnostic restriction analysis, and the cDNA inserts were then sequenced at the 5’ and 3’ ends.

**Bioinformatic Analysis**—For phylogenetic studies, the sequences of the PTI kinase family were obtained using NCBI BLAST. The program used for sequence alignment was ClustalW. Phylogenetic analysis was performed using the PHYLIP program with the neighbor-joining method. Numbers at the nodes indicate bootstrap support obtained in 100 replications.

**GST Pulldown Assay and Immunoblotting—**Arabidopsis suspension cells were cotransfected with 10 μg of HA-OXI1 plasmid and 10 μg of GST-PTI construct. 36 h after transfection the cells were lysed in 0.6 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1% (v/v) Triton X-100, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 μM sucrose, 1 μM microcystin-LR, 0.1% (v/v) β-mercaptoethanol, and 1 tablet of protease inhibitor mixture per 50 ml of buffer (Sigma)). The lysates were cleared by centrifugation at 14,000 rpm for 10 min at 4 °C, and 0.4 ml of supernatant was incubated for 2 h at 4 °C with 30 μl of glutathione-Sepharose. The beads were washed twice in lysis buffer containing 0.5 M NaCl, followed by two further washes in lysis buffer. The beads were resuspended in 30 μl of buffer containing 100 mM Tris-HCl, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, and 200 mM dithiothreitol and subjected to SDS-PAGE. The gels were analyzed by immunoblotting with an anti-HA antibody (clone 3F10; Roche Applied Science) and a horseradish peroxidase-conjugated anti-mouse IgG secondary antibody. The horseradish peroxidase reaction was developed with an enhanced chemiluminescence system (Pierce).

**Phosphorylation of PTI by OXI1 in Vitro—**GST-tagged PTI proteins and OXI1 were purified from transfected Arabidopsis protoplasts under a noninduced inactive state, in which the
Stress-induced Activation of PTI1-2

protoplasts exhibited low levels of luciferase activity when transfected with the stress marker GST6-LUC (no more than 10–30,000 light units from 2000 protoplasts).3 Phosphorylation of PTI was tested in a kinase reaction in the presence or absence of OXI1. Purified GST-PTI and OXI1 were resuspended in 30 μl of kinase buffer (20 mM Tris, pH 7.5, 15 mM MgCl2, 1 mM dithiothreitol) containing 50 μM ATP and 10 μCi of [γ-32P]ATP and incubated for 30 min at 30 °C. The reactions were terminated by adding 10 μl of 6× Laemmli loading buffer. The proteins were resolved by SDS-PAGE and visualized by autoradiography.

Peptide Kinase Assays—HA-tagged protein kinases were transfected into Arabidopsis protoplasts and expressed for 24 h before harvesting using methodology as described previously (3). Transfected cells were lysed by vortexing frozen samples vigorously for 30 s in 100 μl of extraction buffer (25 mM Tris-Cl, pH 7.8, 10 mM MgCl2, 15 mM EGTA, 75 mM NaCl, 1 mM dithiothreitol, 1 mM NaF, 0.5 mM NaVO3, 15 mM β-glycerophosphate, 15 mM 4-nitrophenyl phosphate bis[tris(hydroxymethyl)aminomethane], 0.1% Tween 20, 0.5 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin), and the HA-epitope-tagged protein kinases were immunoprecipitated as described (20). The following synthetic peptides were used as substrates: OXI1, Kemptide (RRASLG), PDK1, and PIIIptide (REPRLSEEQEMFEDYIADEC). Peptides were added to the kinase assay at a final concentration of 30 μM. The reactions contained 30 μM synthetic peptide, 50 mM Tris-HCl, 7.5, 0.1% β-mercaptoethanol, 0.1 mM EGTA, 1.0 μM microcysteine, 10 mM magnesium acetate, 100 μM [γ-32P]ATP (1000 cpm/pmol) (PerkinElmer Life Sciences). The kinase reaction was incubated for 30 min at room temperature. 18 μl of the mixture was transferred to p81 disks (Whatman) and washed four times in 0.5% (v/v) phosphoric acid solution. The washed disks were transferred to vials containing 5 ml of scintillation mixture, and the activity was measured using a scintillation counter.

MBP Kinase Assays—Cells were lysed by vortexing frozen samples for 30 s in 100 μl of protein extraction buffer as described (21). After centrifugation, 80 μl of protein from the cleared supernatant was preincubated in the presence of 15 μl of protein G-Sepharose beads. The supernatant was immunoprecipitated with 20 μl of protein G beads and 5 μl of HA antibody (clone 3F10; Roche Applied Science) at 4 °C. The beads were washed three times with wash buffer (50 mM Tris, pH 7.4, 250 mM NaCl, 5 mM EGTA, 5 mM EDTA, 0.1% Nonidet P-40, 0.1% Tween 20) and once with kinase buffer. The bound proteins were resuspended in 20 μl of kinase buffer and 1 mM ATP. The kinase reactions were mixed with 10 μg of MBP and 2 μCi of [γ-32P]ATP and incubated for 30 min at room temperature. Phosphorylation of MBP was analyzed by autoradiography. For protein gel blot analysis, equal amounts of total protein extracts from each transfection were separated by SDS-PAGE, transferred to membrane, and probed with either monoclonal HA antibody or c-Myc antibody (clone 9E10; Santa Cruz Biotechnology). The horseradish peroxidase reaction was developed with an enhanced chemiluminescence Western blotting analysis system.

RESULTS

Screening an Arabidopsis Two-hybrid Library for OXI1-interacting Proteins—To identify OXI1-interacting partners, we employed the yeast two-hybrid system. An Arabidopsis thaliana cDNA library prepared from cultured Arabidopsis cells (18) was screened using full-length OXI2 as bait. Approximately 1.1 × 106 transformants were plated on selection plates (synthetic complete minus His, Leu, and Trp). Transformants growing on these plates were screened for β-galactosidase activity using a filter assay (23). Colonies that were ADE2+ / HIS3+ and blue were selected for further analysis. Possible interacting clones were sequenced at the 5′ end and compared with the NCBI data base. 23 cDNA clones encoding OXI2 interacting factors were identified, which were sorted into five groups. One group included three very closely related Ser/Thr protein kinases. A search of the entire NCBI data base showed that the kinases shared 78% (PTI1-1), 72% (PTI1-2), and 78% (PTI1-3) sequence identity to the tomato Pti1 kinase (GenBank™ accession number U28007). Additionally they possess a conserved threonine in the activation loop (PTI1-2, Thr-238), which in tomato is phosphorylated by the upstream kinase Pto. Because of the sequence similarity to tomato Pti1, we designated the proteins as PTI1-1 (At1g06700), PTI1-2 (At2g30740), and PTI1-3 (At3g59350). The PTI proteins did not interact with PDK1, an upstream regulator of OXI1 (Fig. 1A). We also tested the ability of a mutated form of OXI1, which lacks the C-terminal hydrophobic PIF motif, to bind to PTI. It has been proposed that binding the PIF domain is a required step in the conformational changes leading to stimulation of PDK1 activity, and is required for full activation of OXI1 by PDK1 (3). The PTI pro-

3 J. Sheen, personal communication.
proteins were able to interact with mutant OXI1 indicating that the PIF domain is not required for interaction between OXI1 and PTI (Fig. 1A). To confirm the yeast two-hybrid interaction, we performed pulldown studies. The three Pti genes were fused to GST, and the OXI1 kinase was tagged with HA and expressed in Arabidopsis cells. We examined the ability of these fusion proteins to interact with proteins in lysates derived from these cells. Our results showed that OXI1 interacted with the GST-PTI-1, GST-PTI-2, and GST-PTI-3 fusion proteins but not with GST alone. The PDK1-OXI1 interaction was used as a positive control (3). Homology searches of the NCBI data base identified another seven kinases that were closely related to PTI1-1, PTI1-2, and PTI1-3 (Fig. 1C). These kinases share roughly 93% sequence identity in their catalytic domains, with only slight variable sequence homology N- and C-terminal to the catalytic domain. Known plant orthologues can be found in tomato, tobacco, soybean, medicago, rice, and maize. D, comparison of the Pti activation domains. Alignment of the activation domain of the Arabidopsis Pti clones and the tomato protein kinases Pti1 (15). A conserved threonine (Thr233 in tomato and Thr238 in PTI1-2) which is phosphorylated by Pto in tomato is indicated by a caret. A consensus sequence showing the conserved residues is shown.

FIGURE 1. Interaction of OXI1 with PTI1-1, PTI1-2, and PTI1-3. A, yeast two-hybrid interaction assay with wild-type OXI1 or the C-terminal mutated OXI1 fused to the Gal4 DNA-binding domain, and the indicated Pti fused to the Gal4 activation domain. PDK was used as a control for binding to OXI1. The transfected yeast colonies were assessed for reporter gene activity by nutritional selection (growth on medium lacking leucine, histidine, and tryptophan and containing 5 mM 3-amino-1,2,4-triazole). B, Arabidopsis cells were transiently transfected with DNA constructs expressing GST or HA-OXI1 together with the GST-PTI constructs. 36 h post-transfection the cells were lysed. Half of the sample was bound to glutathione-agarose beads to confirm binding by Western blot analysis by using anti-HA antibody (upper panel). The remaining sample was used to check transformation efficiency by blotting for the presence of HA-OXI1 in crude extract (lower panel). The PDK1-OXI1 interaction was used as a control (3). Similar results were obtained in three separate experiments. C, phylogenetic tree of the Arabidopsis Pti family of kinases. The analysis of the Arabidopsis data base revealed 10 protein kinases within the Pti kinase family. These kinases share roughly 93% sequence identity in their catalytic domains, with only slight variable sequence homology N- and C-terminal to the catalytic domain. Known plant orthologues can be found in tomato, tobacco, soybean, medicago, rice, and maize. D, comparison of the Pti activation domains. Alignment of the activation domain of the Arabidopsis Pti clones and the tomato protein kinases Pti1 (15). A conserved threonine (Thr233 in tomato and Thr238 in PTI1-2) which is phosphorylated by Pto in tomato is indicated by a caret. A consensus sequence showing the conserved residues is shown.
Stress-induced Activation of PTI1-2

PTI1-1 - PTI1-2K99N, T238A

FIGURE 2. Phosphorylation of PTI1-1, -1-2, and -1-3 by OXI1 in vitro. GST-tagged PTI1-1, PTI1-2, and PTI1-3 were purified from transfected Arabidopsis protoplasts under a noninduced inactive state, and its phosphorylation was tested in a kinase reaction in the presence or absence of OXI1 and visualized by autoradiography. Mutated versions of PTI1, either kinase-deficient PTI1-2 protein fused to glutathione-S-transferase (GST-PTI1-2K99N) or mutated at the conserved Thr residue in the activation loop (GST-PTI1-2K99N/T238A) were combined with OXI1 and phosphorylation tested. The sizes of the PTI proteins are as follows: PTI1-1 = 39.8 kDa, PTI1-2 = 40.5 kDa, and PTI1-3 = 40.5 kDa.

when incubated in the absence of OXI1. When the kinases were incubated in the presence of OXI1, both PTI1-1 and PTI1-2 showed an increase in phosphorylation (Fig. 2, lanes 2 and 6). PTI1-3 was only marginally phosphorylated in the presence of OXI1 (Fig. 2, lane 4). We also tested the ability of OXI1 to phosphorylate mutated forms of PTI1-2. OXI1 was able to increase phosphorylation of the kinase-inactive PTI1-2K99N (Fig. 2, lane 7). However, OXI1 did not effect phosphorylation of the mutant protein PTI1-2K99N,T238A mutated at the proposed site of OXI1 phosphorylation (Fig. 2, lane 8). Kinase-inactive PTI1-2K99N,T238A exhibited lower levels of phosphorylation than the wild-type PTI1-2, indicating some autophosphorylation activity (Fig. 2, compare lanes 5 and 9).

PDK1 and OXI1 Activity Is Directly Stimulated by Stress Elicitors—The activity of PDK1 is increased by binding both PA and phosphatidylinositol 4,5-bisphosphate to the PH domain (3). A number of different stresses can induce a rapid PA response; these include osmotic and temperature stress, oxidative stress, and pathogen stress (7). To determine the enzymatic activity of PDK1 and OXI1 following challenges with various stress factors, we performed in vitro kinase assays using synthetic peptides known to be phosphorylated by the kinases (3). For PDK1, we used the PIPtide peptide whose sequence encompasses the PDK1-docking site fused to the PDK1 phosphorylation site on the activation loop of the animal protein kinase B (24). For OXI1, we used the Kemptide peptide (3). As shown in Fig. 3A, exogenously applied PA and xylanase resulted in a rapid transient increase in PDK1 activity. The peak activity following PA treatment occurring 10 min after stimulation was 110% higher than the basal activity measured in untreated dividing cells. Activity induction by xylanase was greater, peaking at 20 min and 170% higher than untreated cells. The bacterial elicitor flagellin and H2O2 did not significantly alter the phosphorylation activity of PDK1 as shown in Fig. 3A. We also tested the effects of chitin (500 nm), chitosan (500 nm), pectin (50 μg/ml), ABA (100 μM), cold stress (4 °C), and the PEP13 peptide elictor (50 nm), but these did not elicit any change in phosphorylation activity (data not shown). PA and H2O2 resulted in a significant increase in enzymatic activity of OXI1, 125 and 70% respectively, but xylanase produced a much smaller increase in phosphorylation activity than PDK1 in this system, i.e. increase activity of 40%. Flagellin (Fig. 3B), chitin, chitosan, pectin, ABA, cold stress, and the PEP13 peptide did not affect enzymatic activity (data not shown).

Xylanase Activation of PDK1 Is Dependent on the PLD Pathway—The enzyme activity of PLD can be manipulated by the specific inhibitor butanol-1, which serves to act as an alternative substrate during a trans-phosphatidylation reaction with the result that PA generation is inhibited (25). Two control isomers are available, butanol-2 and tert-butyl alcohol (25). Similarly PLC activity can be altered using the PLC inhibitor U73122 (active form) and its inactive analogue (U73343) (13). To test whether the increase in PDK1 activity by xylanase was because of endogenous PAPLD, butanol-1 was added together with the xylanase elicitor. Addition of butanol-1 and xylanase did not result in an increase in PDK1 activity above basal levels, whereas butanol-2, the inactive isomer, together with xylanase resulted in activity increases similar to treatments using xylanase alone (Fig. 3C). We also tested the effects of the PLC inhibitor and found that neither active nor the inactive form significantly altered the activity of PDK1 when treated with xylanase (Fig. 3D). To ensure that the active PLC antagonist could reduce PLC activity in the presence of xylanase, we measured PLC activity from microsomal vesicles isolated from protoplasts (26). At a concentration of 10 μM U73122 (active) inhibited the total PLC activity by up to 50% after 20 min (data available on request). The dramatic drop in activity of PDK1 after 20 min of exposure to the active analogue may reflect a rapid degeneration of cellular integrity because of the inhibition of all the PLCs (Fig. 3D). Taken together these results indicate that PDK1 activity increases significantly only when xylanase triggers the PLD pathway and is independent of PA generation via the PLC pathway. The precise isoform(s) of PLD that could be activated by xylanase in Arabidopsis is unknown.

PTI1-2 Activity Increases in Response to Stress Signals—Next, we tested whether the external addition of stress elicitors to Arabidopsis cells could alter the activity of the PTI kinases. We first tested different synthetic peptides, including crosstide, LKBtide, and Kemptide, to see if they could be phosphorylated by PTI. However, we were unable to detect any phosphorylation activity (data not shown). We therefore tested the ability of the PTI proteins to phosphorylate the substrate MBP in response to either PA or phosphatidylinositol 4,5-bisphosphate treatment. Classic transient signal kinetics were observed for PTI1-2 in response to PA, producing an increase as early as 2 min, peaking at 10 min, and rapidly declining to basal level after 40 min (Fig. 4A). PTI1-2 was unresponsive to phosphatidylinositol 4,5-bisphosphate, the only other lipid that has been shown to activate PDK1 (3) (data not shown). After optimizing the assay, we tested the effects of PA, xylanase, flagellin, and H2O2 on all three PTI kinases (Fig. 4B). PA and xylanase strongly increased the activity of PTI1-2. Flagellin and H2O2 also increased the activity but to a noticeably lesser extent. The PTI1-1 and PTI1-3 kinases did not phosphorylate MBP when challenged with any of the stress factors tested (Fig. 4B). We then tested to see whether H2O2 and flagellin signaling to PTI1-2 is dependent on either PLD or PLC using the phospholipase inhibitors. Activity of PTI1-2 in response to H2O2 and flagellin was found to be the same for both the active and inac-
Stress-induced Activation of PTI1-2

PDK1 and OXI1 Are Upstream Activators of PTI1-2—To address the question whether PDK1 or OXI1 mediates the activation of PTI1-2, the activity of PTI1-2 was monitored in cells, in which the expression of either PDK1 or OXI1 was inhibited using RNAi technology. The PDK1-RNAi construct was designed using the inverted hairpin-loop approach as described previously (3). The efficiency of RNAi gene silencing was measured with the help of a cotransfected Myc-Pdk1 reporter to a nondetectable level (Fig. 5) as reported previously (3). The expression of OXI1 was also ablated using an OXI1-RNAi construct (see “Experimental Procedures”). The expression levels were monitored in the same way as for PDK1 by coexpression of Myc-OXI1. The effect of PA, xylanase, flagellin, and H$_2$O$_2$ on PTI1-2 activity was assessed in control cells, PDK-1 ablated cells, and OXI1 ablated cells. The results show that RNAi-directed knockdown of PDK1 expression reduces the activity of PTI1-2 in response to both PA and xylanase to near basal levels. However, PDK1 ablation did not reduce the activity of PTI1-2 when challenged with H$_2$O$_2$ or flagellin (Fig. 5, A, D, G, and J, compare lanes 4 and 8). In contrast, when OXI1 expression was knocked down PTI1-2 activity was reduced to basal levels for all treatments tested (Fig. 5, A, D, G, and J, compare lanes 4 and 6). These results indicate that the signaling pathways for flagellin and H$_2$O$_2$ converge on OXI1/PTI1-2 independently to xylanase/PA signaling.

PTI1-2 Activates Oxidative Stress-inducible Promoters in Arabidopsis Protoplasts—One of the mechanisms contributing to oxidative stress-induced stress and pathogen tolerance is the activation of detoxification and protection/defense gene expression. For example, Arabidopsis plants respond to oxidative stress with an increase in production of antioxidant enzymes, including glutathione S-transferases (GSTs), peroxidases, superoxide dismutases, and catalases, as well as the activation of protective genes encoding heat shock proteins (HSPs) and pathogenesis-related proteins (27). We used three well characterized Arabidopsis stress-responsive promoters, GST6 (28), HSPI18.2 (29), and RD29 (30), which are fused to the luciferase reporter and which are activated by oxidative stress, heat shock, and ABA/drought/cold, respectively. Activation of these constructs has been optimized in Arabidopsis protoplasts (27). The constructs were transfected into protoplasts and were tested for their responses to PTI1-2 overexpression either alone or in the presence of OXI1. We found that PTI1-2 could substitute for the effects of H$_2$O$_2$ with the GST6 promoter giving a 5-fold increase over the control. The activity increased nearly 9-fold in the presence of PTI1-2 and OXI1.
precipitated, and kinase assayed as above. The assay mixture was separated by SDS-PAGE, and MBP kinase activity was assayed as described under "Experimental Procedures." The assay mixture was separated by SDS-PAGE, and MBP phosphorylation was visualized by autoradiography.

Neither PTI1-1 nor PTI1-3 could activate the GST6 promoter (data not shown). Furthermore the kinaseinactive mutant PTI1-2K99N and the PTI1-2T238A mutant were unable to increase promoter activity. PTI1-2 partially activated the HSP18.2 promoter producing a 3-fold increase when co-overexpressed with OXI1. This was much lower than the levels observed after addition of H2O2. The promoter RD29A was not activated either by PTI1-2 or H2O2. The RD29A promoter is functional in the transfected protoplasts, because ABA can induce the promoter (data not shown and see Ref. 27). The H2O2 treatment also had no significant effects on the constitutive triple response promoter activity, which served as an internal control, or on the CaMV35S promoter activity (Fig. 6).

**MPK6 Is Activated by Xylanase in a PDK1-dependent Manner**—Previous studies have shown that OXI1 expression is required for activation of MPK3 and MPK6 (4). Furthermore, MAPKs have been shown to be activated by PA, and many are implicated in plant pathogen defense, for example MPK6 (31, 32). We therefore performed kinase activity experiments using MPK6 to determine the involvement of PDK1 (Fig. 7). MPK6 activity is efficiently monitored by using MBP. Our results clearly demonstrated that MPK6 was activated by both xylanase and flagellin, confirming previous observations (22). Furthermore, the activity kinetics of xylanase-induced activation of MPK6 closely mirrored those observed with the activation of PDK1 (Fig. 7). However, when the expression of PDK1 was ablated using RNAi, only xylanase activation of MPK6 was shown to be PDK1-dependent, whereas flagellin activation of MPK6 was PDK1-independent (Fig. 7). This indicates that MPK6 can be activated by a fungal elicitor and a bacterial elicitor via different signaling pathways. Interestingly, we were unable to demonstrate a similar response for MPK3 in this assay (data not shown).

**DISCUSSION**

Considerable progress has been made recently toward understanding the regulatory functions of both oxidative stress and PA signals in plants. However much less is known about the cellular cross-talk between these different modes of signaling. It is now clear that production of PA is mediated by families of multiple enzymes that regulate the timing, location, amount, and molecular species of PA. A well characterized target of signaling PA is the PDK1-OXI1 module, which is also implicated in oxidative stress signaling. Although PDK1 is activated by exogenously applied PA, little is known about the upstream signals that generate PA in vivo, how PA and ROS signaling are integrated in this module, or the target proteins for OXI1. To identify potential downstream components of PA signaling in plants and to understand the role of PDK1-OXI1 signaling in ROS signaling, we initiated a two-hybrid analysis using OXI1 as bait. We identified three Ser/Thr kinases that share close homology to the tomato Pti kinase. Tomato Pti1 can physically interact with the Ser/Thr kinase Pto, which in turn confers resistance to Pseudomonas syringae pv. tomato strains that express the avirulence gene avrPto in tomato (33–35). The Pto kinase mediates a signaling pathway leading to the oxidative burst in tomato. The role of the Pti1-Pto interaction is unclear, although one report has demonstrated that in tobacco Pti1 is able to enhance the hypersensitive response to P. syringae pv. tomato strains (33). In Arabidopsis PTI1-2 belongs to a small family of 10 closely related kinases (Fig. 1C). These possess a highly conserved motif in the kinase catalytic domain with a conserved Thr. In tomato this threonine is phosphorylated by Pto. OXI1 was able to phosphorylate both PTI1-1 and PTI1-2 in vitro; however, we were unable to detect significant phosphorylation of PTI1-3 in vitro under the conditions tested. We cannot preclude the possibility that OXI1 is able to interact and activate PTI1-3 in vivo. Furthermore, the data show that PTI1-2 has a low basal level of autophosphorylation,

---

**FIGURE 4. Induction of PTI kinase activities in Arabidopsis cell culture.** A, time course of PTI1-2 activity after treatment with PA. Samples were taken at different times before and after treatment of an Arabidopsis suspension culture with 20 μM PA. PTI1-2 was immunoprecipitated from 200 μg of crude cell extracts, and MBP kinase activity was assayed as described under "Experimental Procedures." The assay mixture was separated by SDS-PAGE, and MBP phosphorylation was visualized by autoradiography. B, immunokinase assay of the PTI1 kinases after treatment of cells with either water, PA, xylanase, flg22, or H2O2. Samples were removed at 0 and 10 min after treatment, immunoprecipitated, and kinase assayed as above.
which does not increase following OXI1-induced transphosphorylation. Under these conditions the mechanism of activation of PTI1-2 by OXI1 more closely resembles the trans-activation mechanism of OXI1 by PDK1, in which autophosphorylation is not required (3), as opposed to other examples involving AGC kinases such as PID regulation by PDK1 in which autophosphorylation is a required step in PID activation (5). Further investigations are required to see if OXI1 targets other PTI family members. These may either represent redundant substrates or possibly signify divergence of different signaling pathways.

To identify upstream signals that regulate the PDK1-OXI signaling pathway, we tested different stress factors, including temperature, ABA, ROS, and fungal or bacterial pathogenic elicitors for their ability to activate either PDK1 or OXI1. From the elicitors tested, only PA and xylanase activated PDK1, whereas PA, xylanase, and H$_2$O$_2$ activated OXI1. We did not observe any direct activation of OXI1 by flagellin in this particular assay. We focused our attentions on xylanase activation of PDK1. The downstream mode of action of fungal pathogenic elicitors is poorly understood. Xylanase elicits the accumulation of PLD$\beta$1 mRNA in plants (15). Interestingly chitin does not increase the activity of PLD$\beta$1, and van der Luit et al. (11) showed that xylanase activated PLD, whereas chitin did not. Thus, although all the elicitors tested (chitin, xylanase, and flagellin) generated PA via a PLC pathway, only xylanase generated PAPLD. We tested the ability of xylanase to activate PDK1 in the absence of PLD using the specific inhibitor butanol-1. Our results showed that PDK1 could only be activated by xylanase in the presence of PLD and not PLC. This is consistent with our observation that this signaling pathway only transmits signals in response to the proteinaceous fungal elicitor xylanase but not polysaccharide-based fungal elicitors or the bacterial elicitors tested, such as flagellin, which do not generate PAPLD. We therefore propose that xylanase results in the activation of a specific PLD isoform(s) generating localized PAPLD at the plasma membrane. The changes in PA concentration at the plasma membrane could result in localized activation of PDK1 (Fig. 8).

Another early event in the plants response to pathogens is cytoskeletal rearrangement. The reorganization of the cortical microtubules, in synchrony with rearrangement of the actin network, allows the cell to remodel the cell structure.
process is instrumental in achieving structural responses to pathogens. For example, cellular redeployment results in the formation of an apoplastic barrier to repel pathogen ingress.

PLD has recently gained prominence as a central player in microtubule-signaling events (36–38). In plants, a PLD isoform can bind microtubules and is proposed to represent the microtubule-plasma membrane linker protein (38). The model proposes that PLD activation leads to microtubule dissociation simultaneously producing PA, which then functions as a second messenger to regulate protein kinases, such as PDK1, small G proteins, and phosphatidylinositol-4-phosphate 5-kinase (7, 39). A dual consequence of PLD activation and PA production is the activation of PDK1 and the subsequent release of microtubules to allow microtubule rearrangement, uniting cytoskeletal reorganization and signal transduction in a single linked mechanism. Localization experiments with the plant PDK1 should provide clues as to the precise mechanism by which PLD-PA-PDK1 functions.

In addition to having the potential to initiate microtubule rearrangement, Arabidopsis PLDα1 and its product PA have been shown to trigger the oxidative burst in both Arabidopsis leaves and tobacco cells (40). It occurs within minutes of elicitor perception, requires Ca2+, and is readily blocked by NADPH oxidase inhibitors and mutants (40). It is thought to kill the invading pathogen, but ROS could also be involved in reinforcing the plant cell wall and in signaling. The production of extracellular ROS in plants is thought to be similar to that in mammalian macrophages, where a functional NADPH oxidase complex is reconstituted by recruitment of different subunits to the plasma membrane. In mammalian cells this recruitment is regulated by a PA-dependent protein kinase (41). In contrast to generation of ROS by PLDα, the PLDδ isoform appears to be downstream of ROS. In Arabidopsis PLDδ is activated by H2O2, and the resulting PA leads to a decrease in H2O2-promoted

FIGURE 6. PTI1-2 activates oxidative stress-inducible gene expression. Arabidopsis protoplasts were cotransfected with one of the following reporter constructs: A, GST6-LUC (GST6); B, HSP18.2-LUC (HSP18.2); or C, RD29A-LUC (RD29A) and either wild-type PTI1-2 (PTI1-2), or WT PTI1-2 with OXI1. The mutated versions of PTI1-2, PTI1-2<sup>K89N</sup> or PTI1-2<sup>T238A</sup> were also tested with the promoters. The control kinase construct was the catalytic domain of CTR1 (ΔCTR1). Vector DNA was used as a control. The transduced protoplasts were incubated for 12 h to allow kinase expression before water or 200 μM H2O2 was added to induce the GST6 and HSP18.2 promoters. The cells were incubated for another 3 h before the promoter activities were measured. Data are the results of triplicate samples and three independent experiments.

FIGURE 7. Xylanase activation of MPK6 is PDK1-dependent. Arabidopsis protoplasts were transfected with HA-MPK6 and Myc-PDK1 either with or without PDK1-RNAi. 24 h after transfection cells were treated either with xylanase or flg22 over a 40-min time course. Kinase activity assays were performed using the MBP substrate. Expression levels of HA-MPK6 were determined by blotting with the HA antibody. Efficiency of the PDK1-RNA interference was determined by the protein levels of the Myc-PDK1 coexpressed within the same cells as the HA-MPK6 and PDK1-RNAi.
programmed cell death (42). In general it appears that the PLDδ pathway protects plants against ROS-induced cell death.

Downstream of PDK1 and OXI1, the PTI1-2 protein is activated in response to PA, xylanase, flagellin, and H$_2$O$_2$. RNA interference experiments showed that PTI1-2 activation by exogenously applied PA, xylanase induced PA, and H$_2$O$_2$ requires OXI1 expression (Fig. 5). However, only PA signaling to PTI1-2 requires PDK1 expression. Membrane-generated signaling PA has the potential to activate targets such as PDK1 and NADPH oxidase simultaneously. This could represent an upstream point of signal divergence, leading to activation of multiple diverse stress signaling modules. In this model activated PDK1 phosphorylates OXI1, which is also regulated by ROS such as H$_2$O$_2$ and cellulase. PTI1-2 in cooperation with OXI1 is able to integrate convergent PA and ROS signals from independent pathways (Fig. 8).

We have also shown that kinase active PTI1-2 is able to increase the expression of ROS promoters indicating that PTI1-2 functions in specific ROS signaling pathways. It is possible that PTI1-2 also targets more specific pathogen promoters. Future studies involving characterization of the PTI1-2

T-DNA knock-out mutant should provide information on the downstream factors targeted by PTI1-2. One possibility is the involvement of MAPK cascades. A central role for the involvement of MAPKs in the onset of pathogen defense is now firmly established (43). Studies have shown Oxi1 null mutants are impaired in the activation of the MAPKs MPK3 and MPK6 upon oxidative stress, suggesting that OXI1 functions downstream of ROS but upstream of the MAPK module (4). In Arabidopsis, MPK3, MPK4, and MPK6 are all activated by bacterial and fungal pathogen-associated molecular patterns and ROS (22, 27, 44). To demonstrate the potential involvement of PDK1 in OXI1-MPK signaling, we ablated the expression of PDK1 and found that activation of MPK6 by xylanase was clearly PDK1-dependent (Fig. 7). It is likely that MAPK signaling cascades function downstream of OXI1 and PTI1-2, resulting in the eventual activation of pathogenesis-related genes in response to different signals; however, MAPK cascades may also play a role upstream and have been implicated in the activation of the NADPH oxidase genes (45).

The results presented in this study provide evidence that PDK1 is involved in pathogen/stress response but is independent of the ROS signaling that can activate OXI1 and its interacting partner PTI1-2. Further investigations are required to unravel the factors and particular mechanisms connecting PLD enzyme activity to PDK function and the downstream targets of OXI1/PTI1-2, such as MAPK cascades.

Acknowledgments—We are very grateful to Professor Jen Sheen for providing the LUC-GST6, LUC-HSP18.2, and LUC-RD29 plasmids. We thank Dr. Jeff Velten for the Renilla luciferase plasmid, Dr. Teun Munnik for providing the PLC inhibitor and inactive isoform, Dr. Scott Peck for providing chitin and pectic fragments, and Professor Tsuyoshi Nakagawa for the G WB15 vector.

REFERENCES

1. Apel, K., and Hirt, H. (2004) Annu. Rev. Plant Biol. 55, 373–399
2. Fujita, M., Fujita, Y., Noutoshi, Y., Takahashi, F., Narusaka, Y., Yamaguchi-Shinozaki, K., and Shinozaki, K. (2006) Curr. Opin. Plant Biol. 9, 436–442
3. Anthony, R. G., Henriques, R., Helfer, A., Meszaros, T., Rios, G., Testerink, C., Munnik, T., Deak, M., Koncz, C., and Bogre, L. (2004) EMBO J. 23, 572–581
4. Rentel, M. C., Lecourieux, D., Ouaked, F., Usher, S. L., Petersen, L., Okamoto, H., Knight, H., Peck, S. C., Grierson, C. S., Hirt, H., and Knight, M. R. (2004) Nature 427, 858–861
5. Zegzouti, H., Anthony, R. G., Jahchan, N., Bogre, L., and Christensen, S. K. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 6404–6409
6. Devarre, T. P., Ekengren, S. K., Pedley, K. F., and Martin, G. B. (2006) EMBO J. 25, 255–265
7. Testerink, C., and Munnik, T. (2005) Trends Plant Sci. 10, 368–375
8. Wang, X., Deviaah, S. P., Zhang, W., and Welti, R. (2006) Prog Lipid. Res. 45, 250–278
9. Torres, M. A., Jones, J. D., and Dangl, J. L. (2005) Nat. Genet. 37, 1130–1134
10. Davies, D. R., Bindschedler, L. V., Strickland, T. S., and Bolwell, G. P. (2006) J. Exp. Bot. 57, 1817–1827
11. van der Luit, A. H., Piatti, T., van Doorn, A., Musgrave, A., Felix, G., Boller, T., and Munnik, T. (2000) Plant Physiol. 123, 1507–1516
12. Yamaguchi, T., Minami, E., Ueki, J., and Shibuya, N. (2005) Plant Cell Physiol. 46, 579–587
13. de Jong, C. F., Laxalt, A. M., Bargmann, B. O., de Wit, P. J., Joosten, M. H.,
and Munnik, T. (2004) Plant J. 39, 1–12
14. de Torres Zabelaqq, M., Fernandez-Delmondo, I., Niittyyla, T., Sanchez, P., and Grant, M. (2002) Mol. Plant-Microbe Interact. 15, 808–816
15. Laxalt, A. M., ter Riet, B., Verdonk, J. C., Parigi, L., Tameling, W. I., Vossen, J., Haring, M., Musgrave, A., and Munnik, T. (2001) Plant J. 26, 237–247
16. Topfer, R., Matzeit, V., Gronenborn, B., Schell, J., and Steinbiss, H. H. (1987) Nucleic Acids. Res. 15, 5890
17. Karimi, M., De Meyer, B., and Hilson, P. (2005) Trends Plant Sci. 10, 103–105
18. Nemeth, K., Salchert, K., Bhalerao, R., Koncz-Kalman, Z., Stankovic-Stangeland, B., Bako, L., Mathur, J., Okresz, L., Stabel, S., Geigenberger, P., Stitt, M., Redei, G. P., Schell, J., and Koncz, C. (1998) Genes Dev. 12, 3059–3073
19. Allwood, E. G., Anthony, R. G., Smertenko, A. P., Reichelt, S., Drobak, B. K., Doonan, J. H., Weeds, A. G., and Hussey, P. J. (2002) Plant Cell 14, 2915–2927
20. Munnik, T., Ligterink, W., Meskiene, I. I., Calderini, O., Beyerly, J., Musgrave, A., and Hirt, H. (1999) Plant J. 20, 381–388
21. Meskiene, I., Baudouin, E., Schweighofer, A., Liwosz, A., Jonak, C., Rodriguez, P. L., Jelinek, H., and Hirt, H. (2003) J. Biol. Chem. 278, 18945–18952
22. Nohse, T. S., Peck, S. C., Hirt, H., and Boller, T. (2000) J. Biol. Chem. 275, 7521–7526
23. Schneider, S., Buchert, M., and Hovens, C. M. (1996) BioTechniques 20, 960–962
24. Biondi, R. M., Cheung, P. C., Casamayor, A., Deak, M., Currie, R. A., and Alessi, D. R. (2000) EMBO J. 19, 979–988
25. Munnik, T., Arisz, S. A., De Vrije, T., and Musgrave, A. (1995) Plant Cell 7, 2197–2210
26. Melin, P. M., Pical, C., Jergil, B., and Sommarin, M. (1992) Biochim. Biophys. Acta 1123, 163–169
27. Kottuk, Y., Chiu, W. L., Tena, G., and Sheen, J. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 2940–2945
28. Chen, W., Chao, G., and Singh, K. B. (1996) Plant J. 10, 955–966
29. Takahashi, T., and Komeda, Y. (1989) Mol. Gen. Genet. 219, 365–372
30. Ishitani, M., Xiong, L., Stevenson, B., and Zhu, J. K. (1997) Plant Cell 9, 1935–1949
31. Asai, T., Tena, G., Plotnikova, J., Willmann, M. R., Chiu, W. L., Gomez-Gomez, L., Boller, T., Ausubel, F. M., and Sheen, J. (2002) Nature 415, 977–983
32. Menke, F. L., van Pelt, J. A., Pieterse, C. M., and Klessig, D. F. (2004) Plant Cell 16, 897–907
33. Zhou, J., Loh, Y. T., Bressan, R. A., and Martin, G. B. (1995) Cell 83, 925–935
34. Loh, Y. T., and Martin, G. B. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 4181–4184
35. Martin, G. B., Brommonschenkel, S. H., Chunwongse, J., Frary, A., Ganal, M. W., Spivey, R., Wu, T., Earle, E. D., and Tanksley, S. D. (1993) Science 262, 1432–1436
36. Gardiner, J. C., Harper, J. D., Weerakoon, N. D., Collings, D. A., Ritchie, S., Gilroy, S., Cyr, R. J., and Marc, J. (2001) Plant Cell 13, 2143–2158
37. Gardiner, J., Collings, D. A., Harper, J. D., and Marc, J. (2003) Plant Cell Physiol. 44, 687–696
38. Dhoukhe, P., Laxalt, A. M., Goedhart, J., Gadella, T. W., and Munnik, T. (2003) Plant Cell 15, 2666–2679
39. Testerink, C., Dekker, H. L., Lim, Z. Y., Johns, M. K., Holmes, A. B., Koster, C. G., Kitakakski, N. T., and Munnik, T. (2004) Plant J. 39, 527–536
40. Sang, Y., Cui, D., and Wang, X. (2001) Plant Physiol. 126, 1449–1458
41. Regier, D. S., Waite, K. A., Wallin, R., and McPhail, L. C. (1999) J. Biol. Chem. 274, 36601–36608
42. Zhang, W., Wang, C., Qin, C., Wood, T., Olafsdottir, G., Welti, R., and Wang, X. (2003) Plant Cell 15, 2285–2295
43. Nakagami, H., Pitzschke, A., and Hirt, H. (2005) Trends Plant Sci. 10, 339–346
44. Desikan, R., Reynolds, A., Hancock, J. T., and Neill, S. J. (1998) Biochem. J. 330, 115–120
45. Yoshioka, H., Numata, N., Nakajima, K., Katou, S., Kawakita, K., Rowland, O., Jones, J. D., and Doke, N. (2003) Plant Cell 15, 706–718