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P58^{IPK}, a Plant Ortholog of Double-Stranded RNA-Dependent Protein Kinase PKR Inhibitor, Functions in Viral Pathogenesis

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Summary

P58^{IPK} is a cellular inhibitor of the mammalian double-stranded RNA-activated protein kinase (PKR). Here we provide evidence for the existence of its homolog in plants and its role in viral infection at the organism level. Viral infection of P58^{IPK}-silenced *Nicotiana benthamiana* and *Arabidopsis* knockouts leads to host death. This host cell death is associated with phosphorylation of the α subunit of eukaryotic translation initiation factor (eIF-2α). Loss of P58^{IPK} leads to reduced virus titer, suggesting that wild-type P58^{IPK} protein plays an important role in viral pathogenesis. Although our complementation results using mammalian P58^{IPK} suggest conservation of the P58^{IPK} pathway in plants and animals, its biological significance seems to be different in these two systems. In animals, P58^{IPK} is recruited by the influenza virus to limit PKR-mediated innate antiviral response. In plants, P58^{IPK} is required by viruses for virulence and therefore functions as a susceptibility factor.

Introduction

Eukaryotic hosts defend against viral infection by invoking innate antiviral responses. In plants, one such mechanism involves resistance (*R*) genes that encode proteins required for the recognition of virus-encoded ligands and initiation of defense responses (Dangl and Jones, 2001). The interaction between the *R* gene *N*, encoding a toll-interleukin 1 receptor homology-nucleotide binding site-leucine-rich repeat (TIR-NB-LRR) protein, and TMV is a model system used to study plant innate antiviral defense (Holmes, 1938; Marathe et al., 2002). Expression of the 50 kDa helicase domain of TMV (TMV-p50) in *N*-containing plants is sufficient to induce defense responses (Abbink et al., 1998; Erickson et al., 1999; Padgett et al., 1997). However, the N protein fails to interact directly with TMV-p50 (S.P.D.-K., unpublished data). To understand how the N protein recognizes TMV-p50, we performed a yeast three-hybrid screen to identify intermediates between N and TMV-P50. Interestingly, one of the proteins identified in this screen showed significant homology to bovine P58^{IPK} (BtP58^{IPK}), a tetratricopeptide repeat (TPR)-containing protein. In animal cell cultures, the influenza virus activates BtP58^{IPK} to inhibit cell death mediated by double-stranded RNA (dsRNA)-activated protein kinase (PKR) (Lee et al., 1994; Melville et al., 2000).

In animals, PKR is the first line of interferon-induced innate antiviral defense (Gil and Esteban, 2000). Human PKR has two dsRNA binding (dsRBD) motifs at its N terminus and a serine/threonine kinase domain (KD) at its C terminus. Upon binding viral dsRNA, a replicative intermediate in the life cycle of RNA viruses, PKR dimerizes and transautophosphorylates, resulting in its activation (reviewed in Gil and Esteban, 2000; Gale and Katze, 1998). Activated PKR phosphorylates the α subunit of eukaryotic translation initiation factor (eIF-2α) at serine 51 (S^{51}), leading to a general suppression of protein synthesis, ultimately resulting in cell death (Dever, 1999).

Various counter defense strategies are adopted by animal viruses to suppress PKR-mediated antiviral defense (Gale and Katze, 1998). Many animal viruses encode proteins or RNAs that inhibit PKR function. For example, adenovirus VAI RNAs bind to dsRNA substrates and inhibit PKR; hepatitis C virus (HCV) NS5A protein inhibits dimerization of PKR; and vaccinia virus K3L and HCV E2 proteins act as pseudosubstrates of PKR. A more interesting strategy is used by the influenza virus. It recruits a cellular protein, P58^{IPK}, that inhibits PKR. BtP58^{IPK} directly interacts with PKR and inhibits kinase activity by preventing dimerization (Gale et al., 1996; Tan et al., 1998). The precise role of BtP58^{IPK} at the whole organism level is unclear because knockouts of this gene are unavailable.

Though PKR-like activity has been demonstrated during TMV (Crum et al., 1988) and potato spindle tuber viroid (Hiddinga et al., 1985) infections, direct evidence for PKR-mediated antiviral defense in plants is lacking. Monoclonal antibodies raised against human PKR crossreact with a presumed plant PKR-like protein (pPKR; Langland et al., 1995) and the plant extract containing the putative pPKR protein phosphorylates wheat and mammalian eIF-2α.

We now provide evidence for the existence of a P58^{IPK} pathway in plants. We have identified a plant P58^{IPK} that interacts directly with the helicase proteins of TMV and tobacco etch virus (TEV). The results of silencing P58^{IPK} in *N. benthamiana* and its knockout in *A. thaliana* suggest that P58^{IPK} is required for viral multiplication and pathogenesis. Complementation experiments in plants indicate that plant P58^{IPK} is a functional homolog of bovine P58^{IPK}. We also show that plant P58^{IPK} interacts with mouse PKR in yeast and in vitro. Interestingly, the plant P58^{IPK} can also rescue mouse PKR-induced cell death in yeast. A substantial increase in phosphorylated plant eIF-2α is detected in P58^{IPK}-silenced plants after virus infection. In addition, expression of the nonphosphorylatable eIF-2α(S51A) mutant rescues P58^{IPK}-silenced plants from death induced by viruses. Our data suggest that plant P58^{IPK} plays an important positive role in viral symptom development. The striking conservation of the P58^{IPK} pathway in plants and animals lends further support to the evolution of cellular responses to viruses predating the divergence of these organisms.

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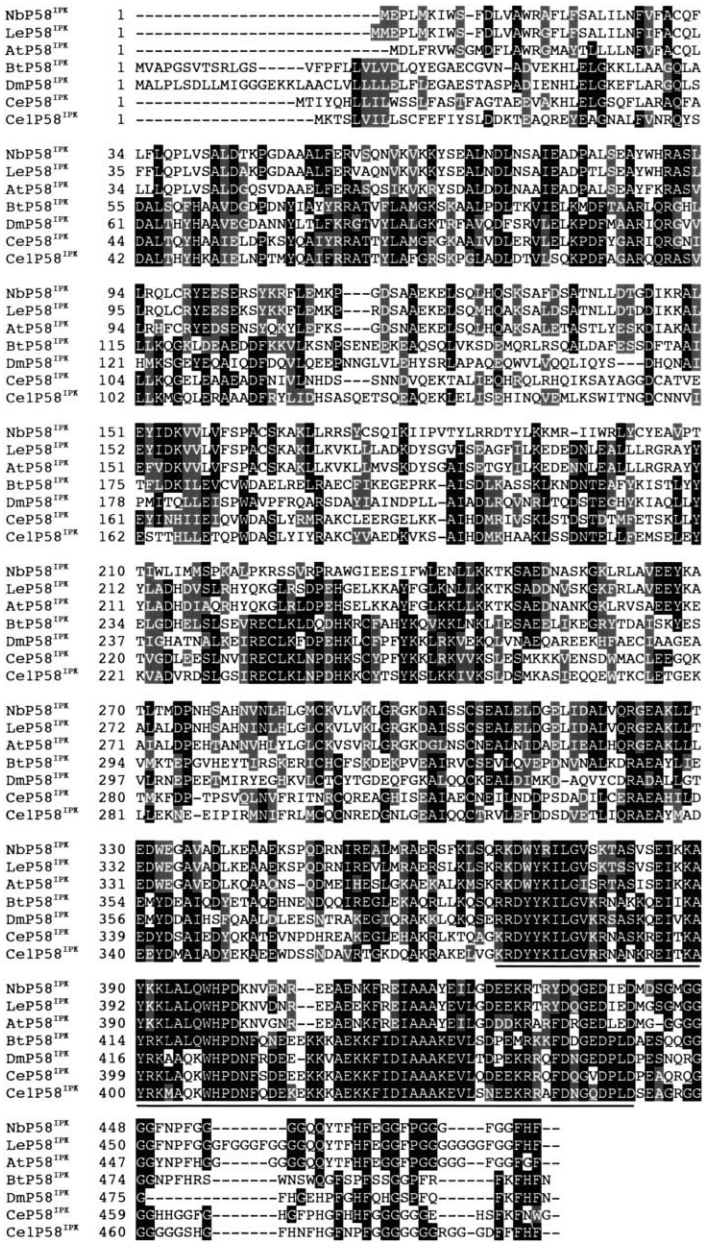

Figure 1. P58^{IPK} Sequences from Different Organisms

Deduced plant P58^{IPK} sequences from *N. benthamiana* (NbP58^{IPK}; AY235682), tomato (LeP58^{IPK}; AY235681), and *Arabidopsis* (AtP58^{IPK}; At5g03160) were compared with P58^{IPK} sequence from bovine (BtP58^{IPK}; AAA17795), *Drosophila* (DmP58^{IPK}; AAL13796), and *C. elegans* (CeP58^{IPK}; AAB42303 and Ce1P58^{IPK}; AAF60776) using the ClustalW program. Identical residues are shaded in black, and similar residues are shaded in gray. The DnaJ domain is underlined.

Results

Identification of Plant P58^{IPK}

We performed a three-hybrid screen to identify proteins that might mediate interaction between TMV-p50 and the N protein. From this screen, we obtained a number of candidates that interacted with TMV-p50. Six of these candidates contained a 160–195 amino acid sequence that showed significant homology to the C terminus DnaJ domain of BtP58^{IPK}, a cellular inhibitor of dsRNA activated PKR (Lee et al., 1994).

We cloned the full-length P58^{IPK} cDNA from *N. benthamiana*, *A. thaliana*, and tomato. The predicted amino acid sequences of the plant P58^{IPK} proteins show significant homology to BtP58^{IPK} (Figure 1). At the amino acid level, *N. benthamiana* P58^{IPK} (NbP58^{IPK}) shares 30% identity and 50% similarity with BtP58^{IPK}. In addition, NbP58^{IPK} shares 81% identity and 91% similarity with tomato P58^{IPK} (LeP58^{IPK}), and 69% identity and 80% similarity with *A. thaliana* P58^{IPK} (AtP58^{IPK}). Like BtP58^{IPK}, plant P58^{IPK} proteins also contain nine TPRs arranged in tandem at their N termini and a DnaJ domain at their C termini. P58^{IPK} proteins are also present in the *Drosophila* and *C. elegans* genome databases; however, the function of P58^{IPK} in these species has not been reported.

P58^{IPK} Interacts Directly with the TMV-P50 and TEV-Helicase Proteins

We examined if full-length P58^{IPK} interacted specifically with TMV-p50 or the N protein. The LexA-BD-TMV-P50

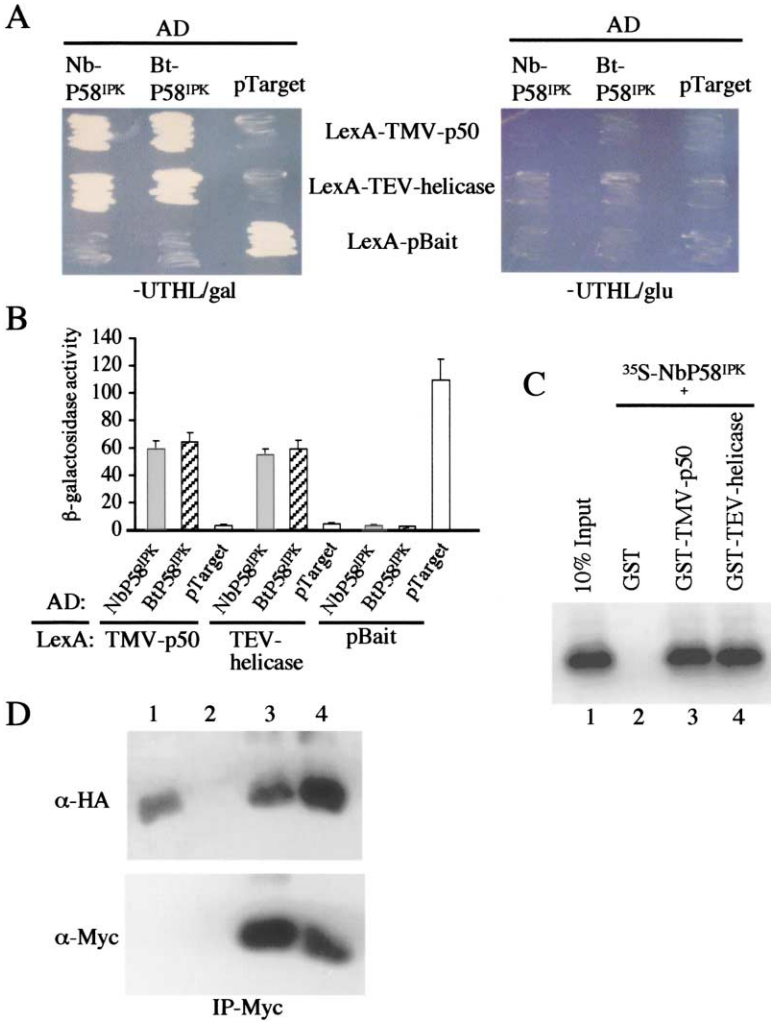

Figure 2. TMV-p50 or TEV-Helicase Interacts with P58IPK

(A) Specific interaction between TMV-p50 or TEV-helicase with NbP58IPK or BtP58IPK was tested using the LexA yeast two-hybrid system. TMV-p50 and TEV-helicase were fused to the LexA DNA binding domain (BD). NbP58IPK and BtP58IPK were fused to the B42 activation domain (AD). LexA-pBait and AD-pTarget fusions served as controls. Various combinations of LexA-BD and AD fusion constructs were introduced into yeast strain EGY48 carrying *LEU2* and *LacZ* reporter genes. Growth of yeast was assessed on selection plates containing galactose (gal) or glucose (glu). Growth of yeast on media containing galactose indicates specific interactions.

(B) Quantification of the β-galactosidase in yeast two-hybrid interactions.

(C) GST pulldown assays between TMV-p50 or TEV-helicase and NbP58IPK. GST-TMV-p50 and GST-TEV-helicase fusion proteins and GST were expressed in *E. coli* and purified using glutathione-sepharose beads. 35S-Met-labeled NbP58IPK was prepared and incubated with 1 μg of GST (lane 2), GST-TMV-p50 (lane 3), or GST-TEV-helicase (lane 4) proteins immobilized on glutathione-sepharose beads. Eluate was analyzed by SDS-PAGE and fluorography. Ten percent of input 35S-Met-labeled NbP58IPK was loaded on the same gel as a control (lane 1).

(D) TMV-p50 and TEV-helicase interact with NbP58IPK in plants. The TMV expression system was used to express MYC-tagged TMV-p50 or TEV-helicase along with HA-tagged NbP58IPK in *N. benthamiana* plants. Protein extracts from transfected leaves producing NbP58IPK-HA together with vector-MYC (lane 2), TMV-p50-MYC (lane 3), or TEV-helicase-MYC (lane 4) were immunoprecipitated using anti-MYC antibody-conjugated sepharose beads after normalizing for protein quantity. Immunoprecipitates were analyzed by SDS PAGE and immunoblot using anti-HA (top panel) or anti-MYC (bottom panel) antibodies. Protein extract derived from TMV expression vector transfected leaves producing NbP58IPK-HA, and vector-MYC was loaded as input control (lane 1).

fusion interacted with the B42 activation domain (AD) fused to NbP58IPK, as indicated by growth of yeast on Leu⁻ plates containing galactose (Figure 2A) and significant levels of β-galactosidase activity (Figure 2B). Thus, N is not required for the interaction of TMV-P50 and P58IPK. In addition, LexA-BD-N does not interact with AD-NbP58IPK (data not shown). Interestingly, LexA-BD-TMV-p50 also interacted with AD-BtP58IPK (Figures 2A and 2B). Neither AD-NbP58IPK nor AD-BtP58IPK interacted with the control bait LexA-BD-pBait fusion proteins.

Since other plant RNA virus helicases function like TMV-p50, we tested if P58IPK interacts with the helicase domain of another plant virus in a yeast two-hybrid assay. Indeed, the tobacco etch virus (TEV) helicase domain interacts with NbP58IPK and BtP58IPK (Figures 2A and 2B).

We performed GST pulldown assays to confirm the interactions of TMV-p50 and TEV-helicase with NbP58IPK. GST-TMV-p50 and GST-TEV-helicase fusion proteins were separately expressed in *Escherichia coli* and mixed with in vitro-translated 35S-radiolabeled NbP58IPK. Analysis of the bound fraction suggests that GST-TMV-p50 and GST-TEV-helicase bind to NbP58IPK (Figure 2C, lanes 3 and 4). No binding was observed between the GST alone control and NbP58IPK (Figure 2C, lane 2).

In vivo pulldown assays were performed to test if NbP58IPK directly interacts with TMV-p50 and TEV-helicase in plants. TMV-p50 and TEV-helicase proteins tagged with MYC epitope and NbP58IPK tagged with hemagglutinin (HA) peptide epitope were expressed using a TMV expression system (Liu et al., 2002a). Protein extracts isolated from leaves transfected with NbP58IPK-HA and either vector-MYC alone, TMV-p50-MYC, or TEV-helicase-MYC were immunoprecipitated using anti-MYC antibodies. The resulting immunoprecipitates were analyzed by immunoblot with anti-HA and anti-MYC antibodies. The immunoprecipitates contained not only TMV-p50-MYC or TEV-helicase-MYC (Figure 2D, bottom panel, lanes 3 and 4), but also NbP58IPK-HA (Figure 2D, top panel, lanes 3 and 4). The vector-MYC control failed to precipitate NbP58IPK-HA (Figure 2D, lane 2). These experiments demonstrate that TMV-p50 and TEV-helicase interact with NbP58IPK in vitro and in vivo.

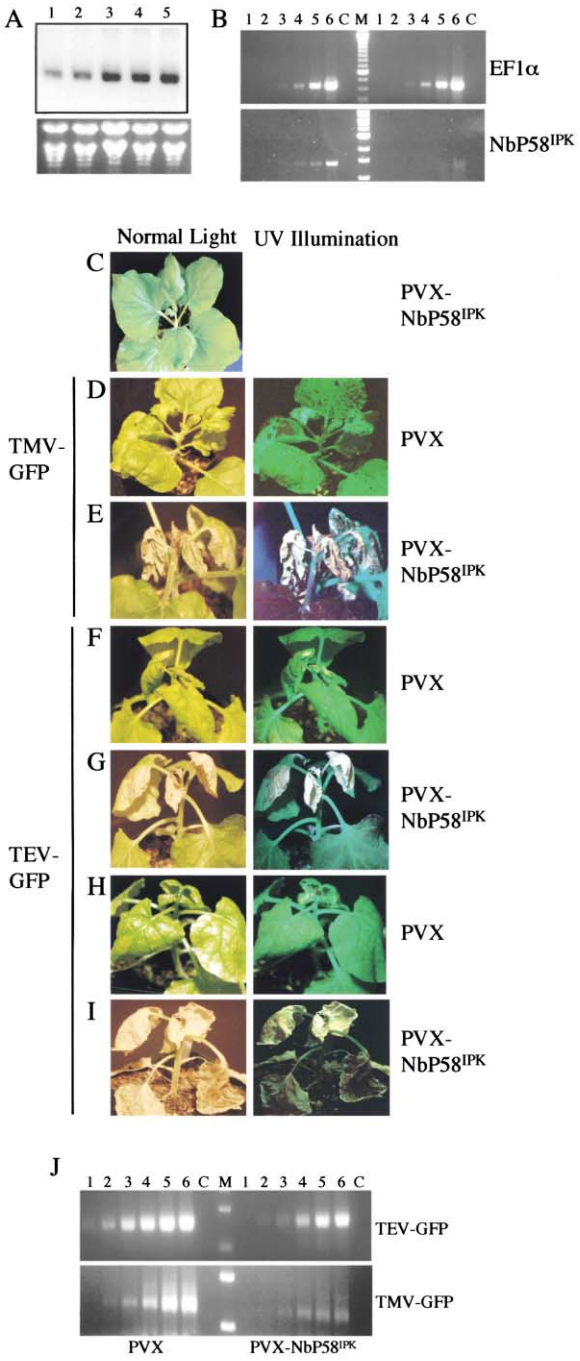

Figure 3. Suppression of NbP58^{IPK} in *N. benthamiana* Leads to Death upon Viral Infection

(A) NbP58^{IPK} expression in *N. benthamiana* plants upon infection with TMV was determined by Northern analysis. Lanes 1–5 correspond to RNA extracted from TMV-infected tissue derived after 0, 0.5, 3, 8, and 18 hr postinfection. The ethidium bromide stained gel shown below the Northern blot indicates equal loading of RNA.

(B) NbP58^{IPK} was silenced in *N. benthamiana* plants using a PVX-VIGS system. Suppression of NbP58^{IPK} expression was confirmed by RT-PCR. First-strand cDNA derived from total RNA from silenced and nonsilenced plants was used for PCR with primers that anneal to NbP58^{IPK} outside the sequence used for silencing. PCR products for EF1α (top panel) and NbP58^{IPK} (bottom panel) derived from nonsilenced (left) and NbP58^{IPK} silenced (right) plants are shown. Lanes 1–6 correspond to products from PCR cycles 20, 23, 26, 29, 32, and 35. Lane C represents the no RT control reaction, in which the RT mix without reverse transcriptase was used as a template. Lane M represents marker.

(C) NbP58^{IPK}-silenced plant.

(D–I) The effect of virus infection on NbP58^{IPK}-silenced plants was assessed. Nonsilenced (D, F, and H) and NbP58^{IPK}-silenced (E, G, and I) *N. benthamiana* plants were challenged with TMV-GFP (D and E) and TEV-GFP (F–I). Photographs were taken 12 (D–G) and 22 (H and I) days after infection under normal light and UV illumination. Suppression of NbP58^{IPK} resulted in the death of upper uninoculated leaves 12 days (E and G) and of the entire plant 22 days (I) after viral infection.

(J) Virus titer in upper leaves was determined 12 days after infection for plants shown in (D)–(G). Total RNA was extracted and RT-PCR was performed using primers that anneal to the TMV MP or the TEV helicase regions. Typical PCR products for TEV-GFP (top panel) and TMV-GFP (bottom panel) derived from nonsilenced (left) and NbP58^{IPK}-silenced (right) plants challenged with viruses. Lanes 1–6 correspond to products from PCR cycles 10, 12, 14, 16, 18, and 20. Lane C represents the control reaction, in which the RT mix without reverse transcriptase was used as a template. Lane M represents marker.

Silencing of NbP58^{IPK} in *N. benthamiana* Leads to Death upon Infection with Plant Viruses

In order to investigate if NbP58^{IPK} expression level changes in response to viral infection, we performed a Northern blot analysis with total RNA isolated from *N. benthamiana* tissue collected 0, 0.5, 3, 8, and 18 hr after TMV infection. We detected a small but definite increase in NbP58^{IPK} transcript levels as early as 3 hr after infection with TMV (Figure 3A).

To examine the biological role of P58^{IPK} in viral pathogenesis, we used virus induced gene silencing (VIGS), a plant RNAi technique. In VIGS, expression of a given gene in a plant host is suppressed by infecting the plant with a recombinant virus vector (VIGS vector) carrying the corresponding gene sequence (Baulcombe, 1999). To suppress P58^{IPK} expression, we cloned the 3′ end of NbP58^{IPK} into a potato virus X (PVX)-based VIGS vector (Ruiz et al., 1998). *Agrobacterium tumefaeciens* cultures containing PVX alone or PVX-NbP58^{IPK} were infiltrated into *N. benthamiana* plants. We confirmed the suppression of NbP58^{IPK} by semiquantitative RT-PCR. In NbP58^{IPK}-silenced plants, P58^{IPK} transcripts were reduced by >79% compared to the PVX-VIGS vector alone infected control (Figure 3B).

The suppression of NbP58^{IPK} had no visible effect on plant growth and development (Figure 3C). NbP58^{IPK}-silenced plants were challenged with TMV or TEV recombinant viruses tagged with green fluorescent protein (GFP). In *N. benthamiana* plants infected with PVX-VIGS vector alone, TMV-GFP and TEV-GFP viruses spread from the inoculated leaf into upper uninoculated leaves as visualized by GFP fluorescence (Figures 3D and 3F). In NbP58^{IPK}-silenced *N. benthamiana* plants, the viruses spread from the inoculated leaves into upper uninoculated leaves as in the nonsilenced control plants. However, the upper leaves of NbP58^{IPK}-silenced plants infected with TMV-GFP or TEV-GFP began dying 12 days after infection (Figures 3E and 3G). By 22 days after infection, all the plants were dead (Figure 3I). However, the PVX-VIGS vector alone control plants did not die after viral infection (Figure 3H). In P58^{IPK}-silenced *N. benthamiana* plants, accumulation of TMV-GFP and TEV-GFP RNA in systemic uninoculated leaves was significantly reduced 12 days after infection as compared to

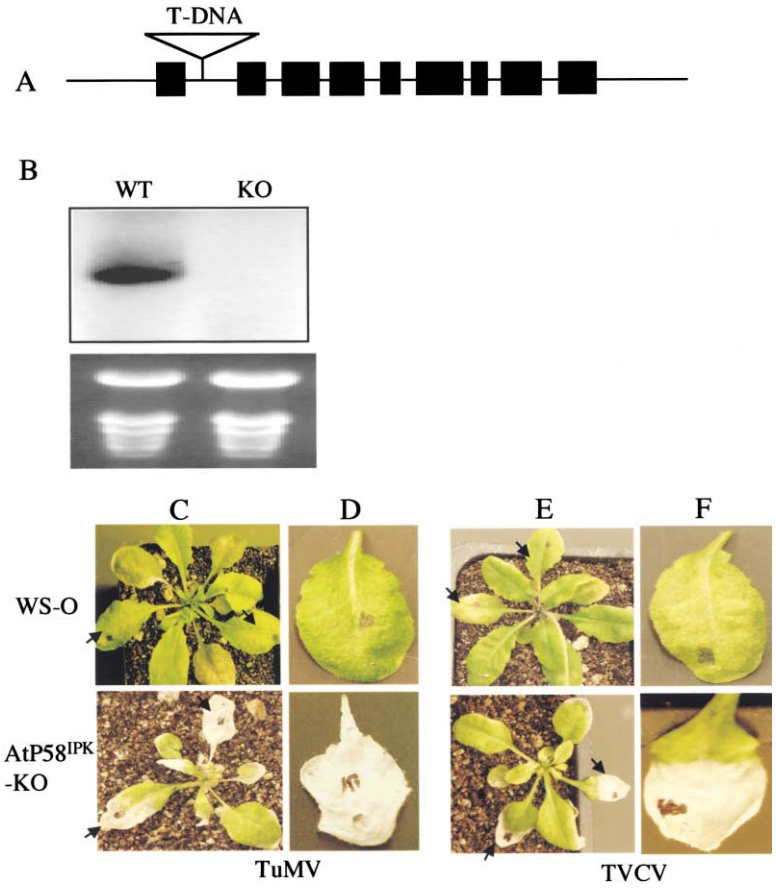

Figure 4. P58IPK Knockout in *Arabidopsis* Leads to Death upon Viral Infection

(A) A schematic representation of a T-DNA insertion in the first intron of P58IPK gene in the ecotype Ws-O.

(B) P58IPK expression in wild-type Ws-O (WT) and knockout (KO) plants was determined by Northern analysis. The ethidium bromide stained gel shows equal loading of RNA.

(C–F) Effect of turnip mosaic virus (TuMV; C and D) and turnip vein clearing virus (TVCV; E and F) infection on wild-type Ws-O (top panel) and P58IPK knockout (lower panel) plants 12 days after infection. Arrows indicate inoculated leaves.

the PVX-VIGS vector alone infected control plants (Figure 3J). This indicates that P58IPK is an essential host component for viral replication and spread.

Since P58IPK interacts directly with TMV-P50, the presumed ligand of *N*-mediated resistance to TMV, we investigated if P58IPK played a role in TMV resistance in *N*-containing plants. P58IPK-silenced *NN* transgenic *N. benthamiana* plants were still completely resistant to TMV (data not shown). This suggests that the *N*-mediated antiviral pathway operates independently of the P58IPK pathway. To test this notion, we challenged P58IPK-silenced *NN* plants with TEV-GFP, a virus not recognized by the *N* gene. These plants died in a manner similar to wild-type P58IPK-silenced *N. benthamiana* plants (data not shown).

P58IPK Knockout *Arabidopsis* Plants Die upon Infection with Viruses

We used a reverse genetics approach to find a knockout mutation in *A. thaliana* P58IPK (AtP58IPK) and identified a line carrying a T-DNA insertion in the first intron of the gene by PCR (Figure 4A). RNA from the homozygous mutant and wild-type plants was extracted and analyzed by Northern blot. Expression of AtP58IPK was not detected in the homozygous mutant plants indicating that we had identified a null mutant (Figure 4B). These plants exhibited normal growth and development (data not shown).

A. thaliana is a host of turnip mosaic virus (TuMV) and turnip vein clearing virus (TVCV). The AtP58IPK null mutant and wild-type *Arabidopsis* plants were challenged with TuMV and TVCV to investigate the effects of viral infection. Wild-type plants infected with these viruses exhibited mild symptoms on the inoculated and uninoculated leaves 10 days after infection (Figures 4C–4F, top panel). In mutant plants infected with these viruses, the inoculated leaf died 12 days after infection (Figures 4C–4F, bottom panel) and the uninoculated leaves died within 18–20 days of infection. These results are consistent with those obtained in P58IPK-silenced *N. benthamiana* plants that suggest that viruses need P58IPK activity to support their replication and spread.

NbP58IPK Is a Functional Homolog of BtP58IPK

The interaction between TMV-p50 and BtP58IPK suggests that NbP58IPK might be a functional homolog of BtP58IPK. To test this hypothesis, *N. benthamiana* plants were coinfiltrated with *Agrobacterium* cultures containing a NbP58IPK-silencing construct (PVX-NbP58IPK) and a BtP58IPK expression construct (PVX-BtP58IPK-HA). RT-PCR analysis confirmed that the level of endogenous NbP58IPK is reduced (Figure 5A) while BtP58IPK is expressed in these plants (Figure 5B). Immunoblot analysis using HA antibody confirmed BtP58IPK expression (data not shown). The NbP58IPK-silenced, BtP58IPK-expressing plants were challenged with TMV-GFP. As with the previous experiments, NbP58IPK-silenced control plants died

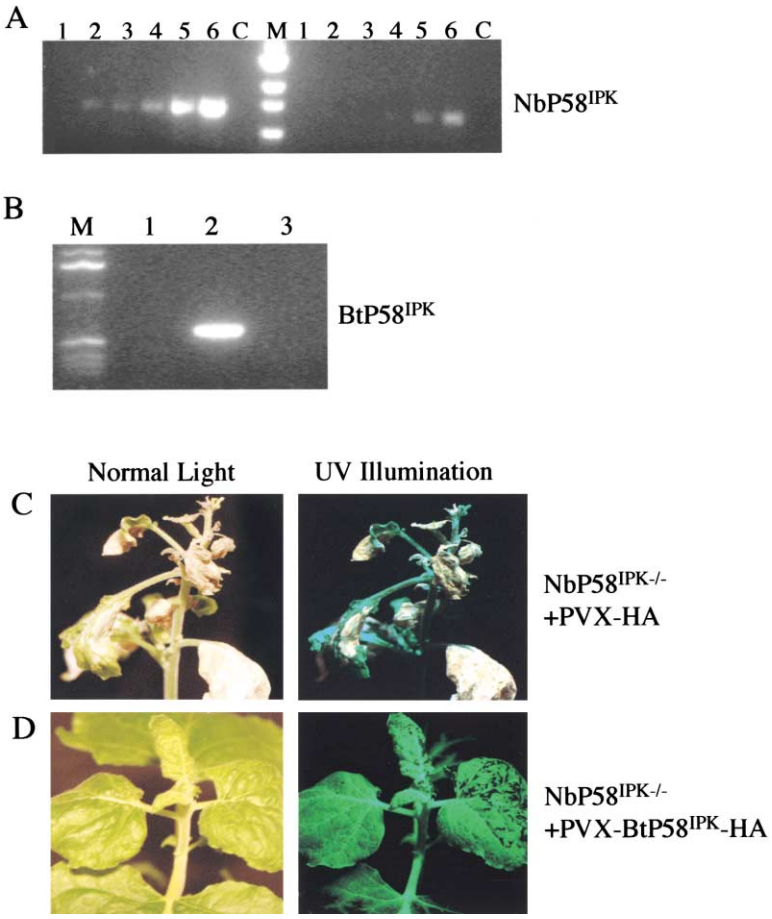

Figure 5. Bovine P58^{IPK} Complements Virus-Induced Death in NbP58^{IPK}-Silenced *N. benthamiana* Plants

Bovine P58^{IPK} (BtP58^{IPK}) was expressed using a PVX expression vector in NbP58^{IPK}-silenced plants.

(A) Suppression of NbP58^{IPK} expression was confirmed by RT-PCR. First-strand cDNA derived from total RNA from silenced (right) and nonsilenced (left) plants was used for PCR with primers that anneal to NbP58^{IPK} outside the sequence used for silencing. Lanes 1–6 correspond to products from PCR cycles 20, 23, 26, 29, 32, and 35. Lane C represents the no RT control reaction, in which the RT mix without reverse transcriptase was used as a template. Lane M represents marker.

(B) Expression of BtP58^{IPK} was confirmed by RT-PCR. Total RNA was extracted and RT-PCR was performed using primers that anneal to the BtP58^{IPK}. Lane 1 represents the no RT control reaction, in which the RT mix without reverse transcriptase was used as a template. Lane 2 represents PCR product of BtP58^{IPK}. Lane 3 represents control reaction in which no template was used. Lane M represents marker.

(C) Effect of TMV-GFP infection on NbP58^{IPK}-silenced and vector-HA expressing plants. Photographs were taken 13 days after infection under normal and UV illumination.

(D) Expression of BtP58^{IPK} in NbP58^{IPK}-silenced plants prevent death induced by TMV-GFP. Photographs were taken 13 days after infection under normal and UV illumination.

13 days after infection with TMV-GFP (Figure 5C). However, NbP58^{IPK}-silenced plants expressing BtP58^{IPK} survived TMV-GFP infection and the virus spread into upper uninoculated leaves (Figure 5D). Thus, NbP58^{IPK} is a functional homolog of BtP58^{IPK}.

eIF-2α Is Phosphorylated in P58^{IPK}-Silenced Plants upon Infection with Viruses

In the mammalian system, PKR is activated upon binding viral dsRNA produced during viral infection (Clemens and Elia, 1997). Activated PKR then phosphorylates eIF-2α at serine 51 (S^{51}), leading to the inhibition of protein synthesis and ultimately cell death (Dever, 1999). During influenza virus infection, the activated P58^{IPK} inhibits PKR's ability to cause eIF-2α phosphorylation (Melville et al., 2000). We hypothesized that a similar mechanism might be causing the death we observed in virus infected P58^{IPK}-silenced *N. benthamiana* and P58^{IPK} knockout *A. thaliana*.

We determined whether eIF-2α is phosphorylated in response to viral infection. Protein was extracted from P58^{IPK}-silenced plants 5, 8, and 12 days after infection with TMV-GFP or mock infection. Immunoblot analysis with anti-eIF-2α[pS^{51}] detected increasing amounts of phosphorylated eIF-2α in silenced plants after infection with TMV-GFP (Figure 6A). In uninfected and mock-infected P58^{IPK}-silenced plants, very little or no phosphorylated eIF-2α was detected (Figure 6A). This suggests that phosphorylation of eIF-2α occurs upon viral infection in the absence of P58^{IPK}. Thus, suppression of protein synthesis may be responsible for the observed cell death in P58^{IPK}-silenced or knockout plants.

Expression of the eIF-2α(S51A) Mutant Prevents Virus-Induced Death in NbP58^{IPK}-Silenced Plants

Since the effect of eIF-2α phosphorylation on protein synthesis in mammalian cells can be rescued by expression of mutant, nonphosphorylatable eIF-2α(S51A) (Donze et al., 1995; Gil et al., 1999), we tested if the mutant can inhibit virus-induced death in P58^{IPK}-silenced plants. We expressed HA-tagged *Arabidopsis* eIF-2α (At-eIF-2α) and mutant eIF-2α(S51A) [At-eIF-2α(S51A)] using a PVX expression system in wild-type and P58^{IPK}-silenced *N. benthamiana* plants. Expression of At-eIF-2α and At-eIF-2α(S51A) was confirmed by RT-PCR (Figure 6B) and Western blot analysis (data not shown). In wild-type plants, the expression of these proteins had no effect on the replication of TMV-GFP and the virus spread (Figures 6C and 6D). P58^{IPK}-silenced plants expressing At-eIF-2α died 9 days after TMV-GFP infection (Figure 6E). However, P58^{IPK}-silenced plants expressing At-eIF-2α(S51A) did not die after infection with TMV-GFP (Figure 6F). Thus, expression of nonphosphorylatable eIF-2α(S51A) can block cell death induced by viruses in the absence of P58^{IPK}.

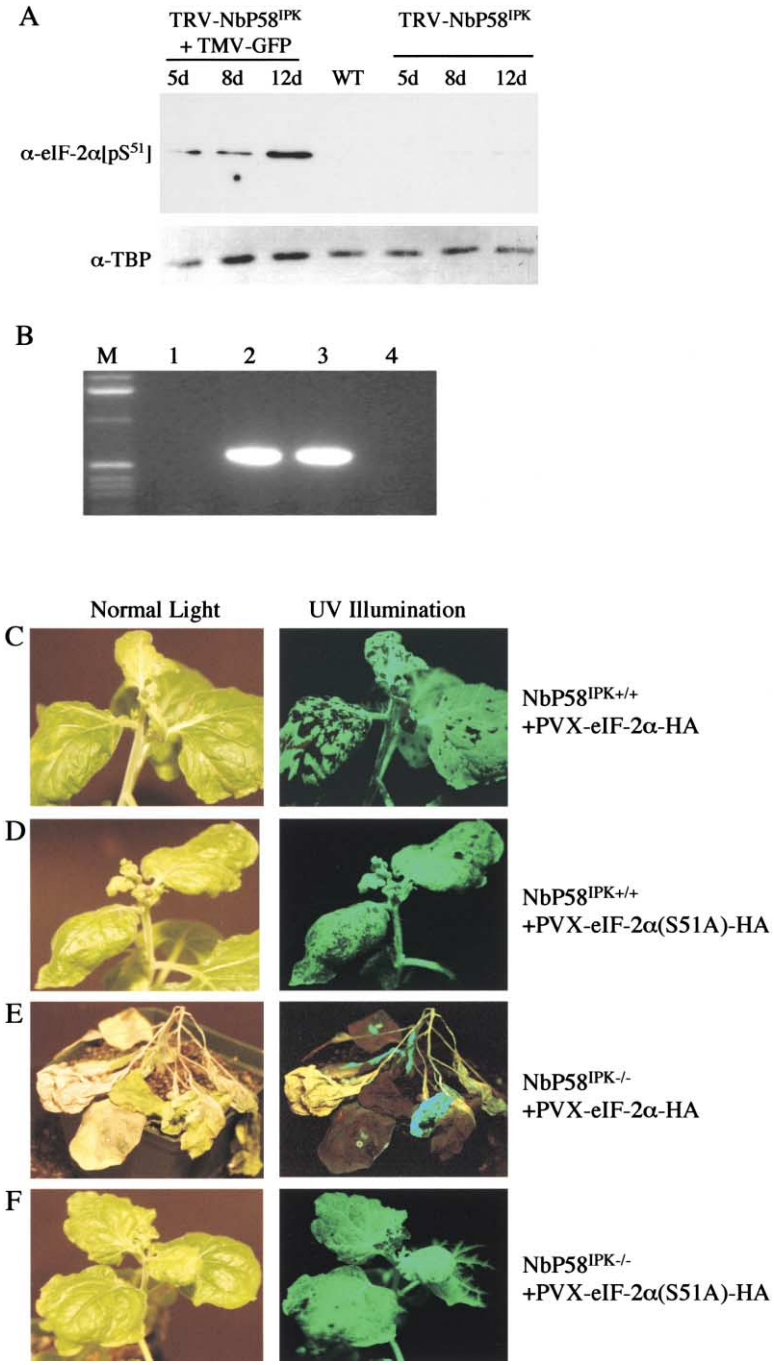

Figure 6. Analysis of eIF-2α Phosphorylation

(A) Tissue from NbP58IPK-silenced plants infected with TMV-GFP (lanes 1–3) and mock-infected NbP58IPK-silenced plants (lanes 5–7) was collected 5, 8, and 12 days after infection; protein extract was prepared and analyzed by immunoblot using antibodies specific for eIF-2α phosphoserine 51 (top panel). An anti-TATA box binding protein (TBP) antibody was used as a loading control (lower panel). WT, protein extract derived from wild-type uninfected tissue.

(B) Wild-type *Arabidopsis* eIF-2α and non-phosphorylatable *Arabidopsis* eIF-2α(S51A) was expressed using a PVX expression vector in nonsilenced and NbP58IPK-silenced plants. RT-PCR was performed to confirm the expression of eIF-2α and eIF-2α(S51A). Total RNA was extracted and RT-PCR was performed using primers that anneal to the eIF2α. Lane 1 represents the no RT control reaction, in which the RT mix without reverse transcriptase was used as a template. Lanes 2 and 3 represent PCR products of eIF2α−HA and eIF2α(S51A)-HA, respectively. Lane 4 represents control reaction in which no template was used. Lane M represents marker.

(C–F) The effect of virus infection on eIF-2α and eIF-2α(S51A) expression was assessed in nonsilenced (C and D) and NbP58IPK-silenced (E and F) plants expressing either At-eIF-2α or At-eIF-2α(S51A) challenged with TMV-GFP. Photographs were taken 12 days after infection under normal and UV illumination.

Plant P58IPK Interacts Directly with Mouse PKR and Rescues PKR-Induced Growth Suppression in Yeast

Mouse PKR (MmPKR) interacts directly with BtP58IPK in yeast two-hybrid assays (Gale et al., 1996). Thus, we wanted to test if plant P58IPK would also interact with MmPKR. We introduced AD-NbP58IPK or AD-BtP58IPK into a yeast strain containing LexA-BD-MmPKR. Both strains grew on Leu⁻ media (Figure 7A). Yeast strains containing either AD-NbP58IPK or AD-BtP58IPK and the control LexA-BD-pBait did not grow on Leu⁻ media (Figure 7A). We confirmed yeast interactions by performing β-galactosidase assays (Figure 7B). We also used in vitro binding assays to study the interaction between plant P58IPK and MmPKR. GST-MmPKR fusion proteins were expressed in *E. coli* and mixed with in vitro-translated ^{35}S-radiolabeled NbP58IPK. Analysis of the bound fraction by SDS-PAGE and autoradiography showed GST-MmPKR bound to NbP58IPK (Figure 7C, lane 3). The GST alone control failed to bind in vitro-translated NbP58IPK (Figure 7C, lane 2). These results demonstrate that NbP58IPK and MmPKR interact directly.

The yeast GCN2 kinase is an eIF-2α kinase related to PKR (Hinnebusch, 1994). Like PKR, GCN2 is regulated

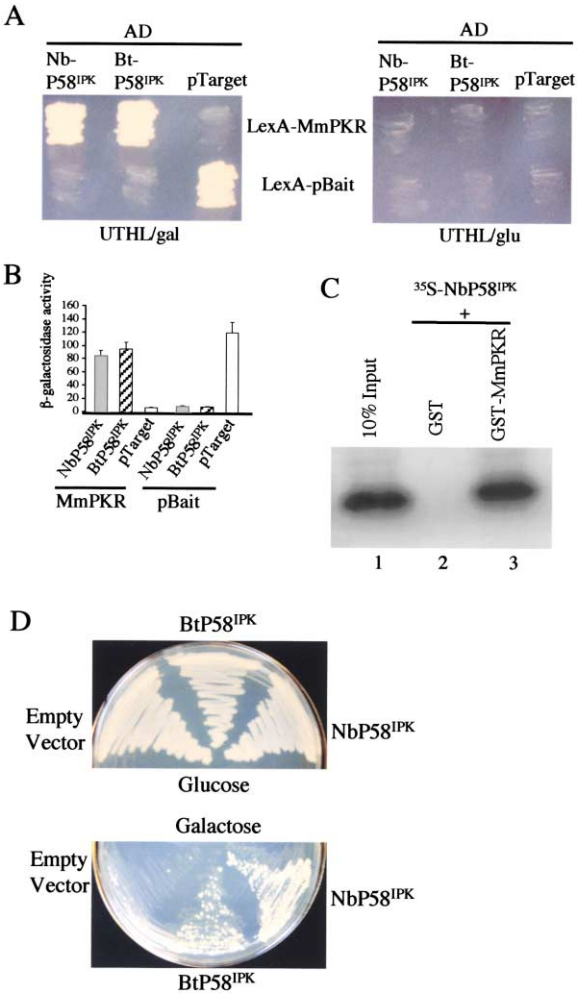

Figure 7. Plant P58^{IPK} Interacts with Mouse PKR and Rescues PKR's Growth Suppression in Yeast

(A) Specific interaction between NbP58^{IPK} and MmPKR was tested using the LexA yeast two-hybrid system. MmPKR was fused to the LexA DNA binding domain (BD) and NbP58^{IPK} and BtP58^{IPK} were separately fused to the B42 activation domain (AD). LexA-pBait and AD-pTarget fusions served as controls. Interaction between various combinations of LexA-BD and AD fusion proteins was tested as described in Figure 2A. Growth of yeast on media containing galactose indicates specific interactions.

(B) Quantification of β-galactosidase in yeast two-hybrid interactions.

(C) GST pulldown assays between NbP58^{IPK} and MmPKR GST-MmPKR fusion protein and GST were expressed in *E. coli* and purified using glutathione-sepharose beads. ^{35}S-Met-labeled NbP58^{IPK} was prepared and incubated with 1 μg of GST or GST-MmPKR immobilized to glutathione-sepharose beads. Eluate was analyzed by SDS-PAGE and fluorography. Ten percent of input ^{35}S-Met-labeled NbP58^{IPK} was loaded on the same gel as control.

(D) The ability of NbP58^{IPK} to rescue MmPKR induced growth suppression in yeast was assessed. Yeast strain H2544 was transformed with constructs expressing NbP58^{IPK} or BtP58^{IPK} under the control of glactose-inducible promoter and empty vector. Transformants were streaked on synthetic minimal media containing 2% glucose (top panel) or 10% galactose and 1% raffinose (bottom panel). Growth of yeast was monitored for 10 days at 30°C.

in response to cellular stress and plays an important role in modulating gene expression in response to environmental stimuli. Wild-type MmPKR has been shown to suppress growth in yeast that have a deletion of *GCN2* (Chong et al., 1992; Dever et al., 1993). The growth suppression caused by MmPKR can be repressed by BtP58^{IPK} (Tan et al., 1998).

We tested if plant P58^{IPK} can reverse the MmPKR-induced growth suppression in *GCN2* deleted yeast. The yeast strain H2544 lacks GCN2 and contains a single copy of MmPKR under the control of a galactose-inducible promoter. We introduced BtP58^{IPK} or NbP58^{IPK} under a galactose-inducible promoter into H2544. They showed no growth defect when plated on glucose-containing medium (Figure 7D, top panel). The growth of H2544 yeast containing empty vector was suppressed on galactose-containing medium (Figure 7D, bottom panel). However, H2544 yeast containing either NbP58^{IPK} or BtP58^{IPK} were able to grow on galactose-containing medium (Figure 7D, bottom panel). These results demonstrate that, like BtP58^{IPK}, NbP58^{IPK} can also reverse the growth suppressive properties of MmPKR in yeast. This suggests that NbP58^{IPK} is a functional homolog of BtP58^{IPK} and indicates that the P58^{IPK} pathway is conserved between plants and mammals.

Discussion

We have identified a plant ortholog of P58^{IPK}, a cellular inhibitor of PKR in animals. Plant P58^{IPK} was identified as an interactor of the TMV-p50 helicase domain in a yeast three-hybrid analysis. This interaction was further confirmed by in vitro and in vivo immunoprecipitation experiments. We show that NbP58^{IPK} also interacts with the helicase domain of another plant virus, TEV. Interestingly, the helicase domains from TMV and TEV also interact with the BtP58^{IPK} in yeast and in vitro. At least one copy of a P58^{IPK} gene has been identified in many animal genomes (Lee et al., 1994). Our analysis of *A. thaliana* and *N. benthamiana* suggests that, in plants, P58^{IPK} is also a single copy gene (data not shown). Both plant and animal P58^{IPK} contain nine TPR domains arranged in tandem and a C terminus region that shows homology to the J domain of the DnaJ protein family (Kelley, 1998). TPR motifs and J domains both mediate protein-protein interactions and are found in proteins known to participate in a wide array of biological activities (Lamb et al., 1995). For example, TPR motif-containing proteins can act as scaffolding in the assembly of multiprotein complexes (Ponting and Phillips, 1996), and J domain proteins can function as chaperones to regulate protein folding, transport, and secretion and also in stress response signaling (Kelley, 1998).

The TMV-p50 helicase plays an important role in TMV pathogenesis. It is part of the replication complex involved in unwinding double-stranded RNA regions during viral replication and in cell-to-cell movement of TMV (Hirashima and Watanabe, 2001). In addition to its role in the viral life cycle, TMV-p50 is also the presumed ligand for the TMV resistance gene *N* (Abbink et al., 1998; Erickson et al., 1999; Padgett et al., 1997). Since TMV-p50 interacted with NbP58^{IPK}, we investigated the biological function of NbP58^{IPK} by silencing this gene in

both wild-type *N. benthamiana* and transgenic *N. benthamiana* plants carrying the *N* gene. We found that silencing of NbP58IPK had no effect on *N*-mediated resistance to TMV in *NN* plants. This is consistent with the fact that TMV-p50 interacts directly with P58IPK in the absence of *N*. However, silencing of NbP58IPK caused massive cell death in wild-type *N. benthamiana* plants when challenged with TMV-GFP and TEV-GFP viruses. In addition, cell death was observed in P58IPK knockout *Arabidopsis* plants upon infection with viruses. TMV and TEV infection of control wild-type *N. benthamiana* and *Arabidopsis* plants does not induce death. These results imply that plant P58IPK protein is required for development of viral symptoms.

Our complementation experiments show that death induced by viruses in P58IPK-silenced plants could be rescued by expression of BtP58IPK. This points to a well-conserved P58IPK signaling pathway between plants and animals. However, the biological outcome of the pathway for these two systems seems completely different. In animals, the interferon (IFN)-induced antiviral response is the first line of defense against viral infections (Katze et al., 2002). PKR is one of the key components of the IFN-mediated innate antiviral response. Activation of PKR leads to phosphorylation of eIF-2α, resulting in the cessation of protein synthesis and eventually host cell death. During influenza virus infection, P58IPK is recruited to neutralize PKR-mediated cell death. This prevents release of proinflammatory factors, resulting in slow clearance of virus from the host. Therefore, P58IPK in animals plays a key role in regulation of the hosts' innate immune response. On the other hand, plants do not invoke cell death as part of their innate immune responses to TMV and TEV infection. However, loss of P58IPK in plants leads to death of the plant upon infection with viruses, indicating that the wild-type P58IPK protein functions to limit cell death and promote viral infection. Therefore, plant P58IPK functions as a virulence factor that is required for normal development of viral disease symptoms. This accounts for the decreased virus titer observed in P58IPK-silenced plants. Thus, plant viruses seem to depend on P58IPK for successful infection without cell death. Unlike animal P58IPK, plant P58IPK does not participate in the regulation of host innate immune response. It will be interesting to see if loss of P58IPK in animals also leads to reduced viral growth and hence if it also functions in viral virulence.

Why does the loss of P58IPK lead to death of the plant upon viral infection? In mammalian cells, PKR and the endoplasmic reticulum (ER) resident kinase PERK/PEK are activated upon viral infection and ER stress, respectively, and cause the phosphorylation of eIF-2α. This results in the inhibition of protein synthesis and ultimately cell death (Dever, 1999; Harding et al., 1999). Three lines of evidence suggest that the death we observed in NbP58IPK-silenced plants was due to the activity of a PKR- or PERK-like kinase, which is relieved of its P58IPK inhibition. First, we found increased levels of phosphorylated eIF-2α upon viral infection in the NbP58IPK-silenced plants. Second, when we expressed a nonphosphorylatable eIF-2α(S51A) mutant in P58IPK-silenced plants, it suppressed the virus-induced cell death when compared to plants expressing eIF-2α. Third, like BtP58IPK, NbP58IPK can also reverse the growth-suppressive phenotype induced by MmPKR in yeast through a direct interaction. In addition, death induced by viruses in P58IPK-silenced plants could be rescued by expression of BtP58IPK. This therefore, points to the existence of a previously unidentified eIF-2α phosphorylation pathway mediated by a PKR- or PERK-like kinases in plants.

A putative PKR-like protein (Langland et al., 1995) was identified in a plant extract using monoclonal antibodies raised against human PKR. However, using mouse and human PKR sequences the search of the *Arabidopsis*, rice, and tomato databanks did not recover any proteins that showed high similarity to mammalian PKR. However, we were able to identify genes with significant similarity to the dsRBD of PKR as well as Ser/Thr kinases that show significant similarity to the KD of PKR. It is possible that in plants two different proteins, one possessing a dsRBD and another possessing a KD, may act together to perform the same function as mammalian PKR.

Mammalian P58IPK is associated with the ER (Yan et al., 2002) and interacts with PERK (Gale et al., 2002). Mammalian P58IPK expression is induced during ER stress (Scheuner et al., 2001; Yan et al., 2002). In addition, PKR in mammalian cells is associated with the rough ER membrane through binding to the 60S ribosomal subunit (Kaufman, 1999). We hypothesize that, like mammalian P58IPK, plant P58IPK may be regulating PKR- or PERK-like kinases during virus infection or ER stress. However, extensive database searches failed to identify a PERK-like kinase in plants. Interestingly, the ER stress response element (ERSE) (Roy and Lee, 1999) sequence is present in the AtP58IPK promoter between -244 and -225. Moreover, most plant viruses replicate on cellular membranes (Reichel and Beachy, 1999), and both TMV and TEV replicase proteins associate with the ER (Heinlein et al., 1998; Schaad et al., 1997). TMV and TEV have also been shown to alter the ER network in infected plant cells. Therefore, it is possible that the helicase domains of plant viral replicases interact directly with P58IPK to regulate the PKR or PERK pathway, promoting viral replication in the ER. Alternatively, P58IPK may function to regulate ER stress caused by virus replication.

There remain many questions regarding the role of plant P58IPK in general stress responses and regulation of important metabolic processes. Further, the study of P58IPK is necessary for dissecting the biological role of eIF-2α phosphorylation in plant growth and development. This work identifies a plant P58IPK pathway and also provides, to our knowledge, the first evidence at the organism level for the role of P58IPK in viral virulence. The P58IPK pathway is surprisingly well conserved in higher organisms and hints at a broader evolutionary conservation of viral survival mechanisms in eukaryotic hosts.

Experimental Procedures

Plasmids

Bait construct GAL10:LexA-BD-TMV-p50::GAL1:N used for three-hybrid screen was generated by cloning LexA-BD-TMV-p50 in front of the GAL10 promoter and *N* in front of the GAL1 promoter in pESC-HIS (Stratagene). LexA-BD fusion vectors were generated by cloning

the 50 kDa C-terminal domain of the TMV 126 kDa replicase (amino acids [aa] 676–1116), TEV helicase domain (aa 1267–1529), and mouse PKR into pTBS1 (Tessa Burch-Smith and S.P.D.-K., unpublished data). AD fusion constructs were generated by cloning full-length NbP58^{IPK} and BtP58^{IPK} into pJG4-5 (Finley and Brent, 1996). The library screened was generated in pJG4-5 as described in Liu et al. (2002a). To express proteins in yeast, full-length NbP58^{IPK} and BtP58^{IPK} were cloned downstream of the GAL1 promoter in pESC-HIS (Stratagene). To express GST fusion proteins in *E. coli*, the TMV-p50, the TEV helicase, and mouse PKR were amplified by PCR and recombined into pDEST15 using GATEWAY system (Invitrogen). To synthesize ^{35}S-Met-labeled in vitro translation products, PCR-amplified product of full-length NbP58^{IPK} was recombined into pDEST14 using GATEWAY system. To express proteins in planta, PCR products of NbP58^{IPK} with 2×HA tag, TMV-p50 with 2×MYC tag, and TEV helicase with 2×MYC tag were recombined into pYL257 (Liu et al., 2002a) using GATEWAY system. pPVX-BtP58^{IPK}-2×HA, pPVX-At-eIF2α-2×HA, and pPVX-At-eIF2α(S51A)-2×HA were created by recombining PCR products into pYL254 (Liu et al., 2002a) using GATEWAY system. To silence NbP58^{IPK}, a cDNA fragment of *N. benthamiana* P58^{IPK} (bases 833–1420) was amplified from a cDNA clone and inserted into pPVX (Liu et al., 2002a). The T-DNA vector containing GFP-tagged TEV was generated using T7-TEV vector (Schaad et al., 1997) in a pBin19-based vector that contains duplicated CaMV 35S promoter and NOS terminator. The T-DNA vector containing TMV-GFP was described in Liu et al. (2002b). Full length *N. benthamiana*, tomato, and *Arabidopsis* P58^{IPK} were generated by RT-PCR using SMART cDNA amplification kit (CLONTECH) and cloned into TOPO vector (Invitrogen).

Yeast Two- and Three-Hybrid Interaction Assays

The yeast three-hybrid screen was performed by following the protocol described for the two-hybrid screen (Finley and Brent, 1996). Approximately, 5 × 10^6 transformants were screened. β-galactosidase assay was performed on three independent yeast colonies by using the yeast β-galactosidase assay (Pierce, IL). The average from three experiments were used for the analysis.

Yeast Growth Suppression Assay

Expression plasmids containing GAL1::NbP58^{IPK} and GAL1::BtP58^{IPK} and the empty vector pESC-HIS were introduced into the yeast strain H2544 (*MATα, ura3-52, leu2-3, leu2-112, trp1-Δ63, gcn2Δ, <GAL-PKR,LEU2>1 @ leu2*). Transformed yeast strains were plated on to 2% glucose containing His$^-$Leu$^-$ plates. The transformants were streaked on to His$^-$Leu$^-$ plates containing either 2% glucose or 10% galactose and 1% raffinose and were incubated at 30°C. Yeast growth was monitored for 8 days.

GST Pulldown Assays

GST-TMV-p50, GST-TEV helicase, and GST-MmPKR fusion proteins were produced in BL21 codon plus cells (Stratagene) and affinity purified using glutathione-sepharose beads. Approximately 1 μg of purified GST fusion proteins and GST were used to pulldown ^{35}S-Met-labeled in vitro-translated (TNT; Promega) NbP58^{IPK} as described in (Liu et al., 2002a).

Coimmunoprecipitation with Plant Protein Extracts

Total protein from nontransfected and transfected leaves was extracted using the protocol describe in (Liu et al., 2002a). Immunoprecipitations were performed using anti-MYC antibody affinity matrix (CRP, Berkeley, CA) at 4°C overnight. After washing four times with PBS solution, immune complexes were analyzed by SDS-PAGE and immunoblotting using anti-MYC and anti-HA antibodies and were detected using WestPico SuperSignal-enhanced chemiluminescence (Pierce, IL).

Isolation of *Arabidopsis* P58^{IPK} Knockout Mutant and Virus Infection Assay

The AtP58^{IPK} knockout mutant was obtained by PCR screening *Arabidopsis* functional genomics consortium (AFGC) T-DNA insertion lines using primers specific for the AtP58^{IPK} gene and the T-DNA (http://www.biotech.wisc.edu/*Arabidopsis*). The T-DNA insertion site was determined by DNA sequencing.

Three-week-old AtP58^{IPK} knockout and wild-type plants were infected with TuMV and TVCV on two opposite leaves per plant. Symptoms were monitored for 20 days after infection.

eIF-2α Phosphorylation Assay

Total protein was extracted and separated by standard SDS-PAGE and transferred to PVDF membrane by following the protocol described in Liu et al. (2002a). Immunoblots were probed with anti-eIF-2α[pS^{51}] phosphospecific antibodies (Biosource International, CA) and detected using WestPico SuperSignal-enhanced chemiluminescence.

VIGS Assay, Virus Infection, GFP Imaging, and RT-PCR Analysis

VIGS assay was performed as described in Liu et al. (2002b). *A. tumefaciens* GV2260 containing pPVX or pPVX derivative plasmids were infiltrated into the lower leaves of four-leaf-stage plants using a 1 ml needleless syringe. Eight days after infiltration, the upper leaves of plants were infected with TMV-GFP or TEV-GFP virus. In the overexpression experiments, silencing and overexpression constructs were coinfiltrated. Each silencing experiment was repeated at least three times, and each experiment included at least four independent plants. GFP imaging was performed using UV illumination, and photographs were taken using an Olympus Camedia E10 digital camera.

To analyze the silencing effect on endogenous mRNA expression level, total RNA was extracted from leaves derived from silenced plants and treated using the Message Clean Kit (Genhunter, Houston, TX) to remove DNA contamination. This RNA (1 μg) was used to synthesize the first-strand cDNA. Samples from each reaction (1 μl) were used in a 30 μl PCR mixture containing the cDNA template, Taq DNA polymerase, and primers that anneal outside the region of the VIGS.

To detect the presence of TMV, TEV, or PVX derivatives, total RNA extracted from upper uninoculated leaves was used to generate the first-strand cDNA using a primer that anneals to the 3′ end of TMV movement protein, TEV helicase domain and BtP58^{IPK} or AteIF-2α, respectively. These first strands were used in PCR reactions. The resulting PCR products were analyzed by agarose gel electrophoresis.

Total RNA Isolation and Northern Analysis

Total RNA from AtP58^{IPK} knockout and wild-type plants was isolated from three-week-old plants. Ten micrograms of total RNA was loaded per lane and resolved on a formaldehyde-agarose gel and transferred to hybond-N nylon membrane (Amersham) following manufacturer's instructions. Blots were hybridized with AtP58^{IPK} cDNA probe at 42°C for 12 hr in a 50% formamide containing sodium phosphate buffer. Blots were washed twice at 65°C using 0.1× SSC/0.1% SDS solution for 15 min and exposed to X-ray film for 22 hr.

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