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PUB40 attenuates *Phytophthora capsici* resistance by destabilizing the MEK2-SIPK/WIPK cascade in *Nicotiana benthamiana*

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Abstract

The mitogen-activated protein kinase (MAPK) cascade MEK2-SIPK/WIPK is essential for immunity in Solanaceae plants. This cascade is tightly controlled to prevent harmful hyperactivation. However, the E3 ubiquitin ligases utilized by plants to reduce MEK2- SIPK/WIPK protein levels remain largely elusive. Here, we confirmed the essential role of *Nicotiana benthamiana* MEK2-SIPK/WIPK in resistance to the oomycete pathogen *Phytophthora capsici*. Using tobacco rattle virus (TRV)-based gene silencing, we screened prevalent plant U-box protein (PUB)-type E3 ligases with Armadillo (ARM) repeats to characterize those involved in *Phytophthora* resistance and MEK2-SIPK/WIPK degradation. We found that *pub40* knockdown mutants exhibited significantly enhanced resistance to *P. capsici*. NbPUB40 was under ubiquitination and proteasomal degradation *in planta*, with two conserved sites (Cys28 and Val41) in the U-box domain being essential for its activity. NbPUB40 was shown to interact with the whole MEK2-SIPK/WIPK cascade and promote their degradation, the ubiquitination levels of which were also notably reduced in the *pub40* mutant. Our results reveal a mechanism in which a PUB E3 ubiquitin ligase negatively regulates plant *P. capsici* resistance by destabilizing the MEK2-SIPK/WIPK cascade.

Keywords *Phytophthora capsici*, E3 ubiquitin ligase, Armadillo repeat, Plant U-box protein, Mitogen-activated protein kinase

Background

Plants have evolved sophisticated immune systems to sense the invasion of phytopathogens (Jones and Dangl. 2006). Cell surface-localized pattern recognition receptors (PRRs) are activated by pathogen-associated molecular patterns (PAMPs), leading to phosphorylation and activation of receptor-like cytoplasmic kinases (RLCKs) and causing multiple immune events (Zhang and Zhang. 2022). Rapid activation of mitogen-activated protein kinase (MAPK or MPK) cascades downstream of the RLCKs is central to the plant immune system (Su et al. 2021). Each of the three layers has multiple members to ensure signaling diversity. Stimulus perception by PRRs activates MAPK kinase kinases (MAPKKKs or MKKKs), MAPKKKs phosphorylate and activate downstream MAPK kinases (MAPKKs, MKKs, or MEKs), which further activates MAPKs through phosphorylation (Group. 2002). Activated MAPKs subsequently phosphorylate substrate proteins, including

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transcription factors, enzymes, and other proteins with distinct functions to induce immune responses (Jonak et al. 2002; Li et al. 2012a; Tsuda and Somssich. 2015; Furlan et al. 2017).

While PAMP-triggered immunity (PTI) induces rapid and instantaneous activation of MAPK cascades to enhance local immune responses, ETI ensures extended and persistent MAPK activation (Zipfel. 2009; Tsuda et al. 2013; Cui et al. 2015). However, hyperactivation of MAPKs is harmful to plants. Ectopic expression of MAPKs, such as NtMEK2^{DD} (a constitutively active mutant of NtMEK2), StMEK1^{DD}, GhMKK6, CA-StMPK7, and NtSIPK often causes excessive hypersensitive response (HR) in plants (Yang et al. 2001; Zhang and Liu. 2001; Kanzaki et al. 2003; Wang et al. 2017; Zhang et al. 2021). Therefore, MAPK signaling needs to be controlled tightly to prevent hyperactivation of defense responses. To this end, MAPK phosphatases dephosphorylate and inactivate MAPKs (Jiang et al. 2017). In potato, the protein tyrosine phosphatase StPTP1a dephosphorylates StMPK4/7 to inhibit resistance to *Phytophthora infestans* (Li et al. 2023a). Cotton microRNA ghr-miR5272a targets and represses the expression of *GhMKK6* to attenuate plant immunity (Wang et al. 2017). In *Arabidopsis thaliana*, MAPK signaling can be inhibited by the competition between MAPKKK proteins YDA and MAPKKK3/5 for binding downstream MKKs (Sun et al. 2018).

MEK2-SIPK/WIPK is a well-known MAPK immunity cascade in Solanaceae plants (Yang et al. 2001). MEK2 shares the highest similarity with *Arabidopsis* MKK4 and MKK5, and SIPK and WIPK are orthologs of *Arabidopsis* MPK6 and MPK3, respectively (Asai et al. 2002). *N*-gene-mediated TMV resistance is compromised by the silencing of *MEK2*, *SIPK*, or *WIPK* in tobacco plants. Likewise, *N. benthamiana* MEK2 and SIPK participate in brassinosteroid (BR)-induced TMV resistance (Jin et al. 2003; Deng et al. 2016). *Solanum lycopersicum* MEK2-SIPK/WIPK cascade acts downstream of SlMAPKKKε, which is essential for resistance to Gram-negative bacterial pathogens (Melech-Bonfil and Sessa. 2010). Pathogens have targeted MEK2-SIPK/WIPK due to their importance in plant resistance. Although the regulation of the MEK2-SIPK/WIPK cascade by *Phytophthora* effectors is unknown, many other pathogens secrete a series of effectors to disturb MEK2-SIPK/WIPK activation. *Xanthomonas* employs at least five effectors (XopE1, XopM, XopQ, AvrBs1, and AvrXv4) to inhibit MEK2^{DD}-mediated cell death (Teper et al. 2015), with XopQ also suppressing SIPK-induced cell death (Teper et al. 2014). CSEP0139 and CSEP0192 are fungi *Blumeria graminis* f. sp. *hordei* (*Bgh*) effectors that inhibit cell death induced by MEK2^{DD} (Li et al. 2021). However, it remains unclear how plants regulate the MEK2-SIPK/WIPK cascade.

The ubiquitin-proteasome degradation system attenuates plant immune responses via an efficient negative feedback loop (Trenner et al. 2022). Ubiquitin is attached to substrates with E3 ubiquitin ligases (Chen and Hellmann. 2013), which are divided into four structural groups: RING (Really Interesting New Gene), HECT (Homologous to E6AP C-Terminus), U-box, and CRL (Cullin-RING Ligases) (Vierstra. 2009; Zhou and Zeng. 2017). KEEP ON GOING (KEG), an *Arabidopsis* RING-type E3 ubiquitin ligase ubiquitinates MKK4 and MKK5 to attenuate immunity (Gao et al. 2021).

Plant U-box proteins (PUBs) with C-terminal Armadillo (ARM) repeats are the most abundant U-box E3 ligases in plants (Patterson. 2002). ARM-containing PUBs ubiquitinate and degrade immune components, especially protein kinases (Trujillo. 2018). For example, *Arabidopsis* PUB13 targets include the flg22 receptor FLS2 (FLAGELLIN-SENSING2), the chitin receptor LYK5 (LYSM-CONTAINING RECEPTOR-LIKE KINASE 5), and the phytosulfokine receptor PSKR1 (PHYTOSULFOKINE RECEPTOR 1) (Lu et al. 2011; Liao et al. 2017; Hu et al. 2023). Rice SPL11, a PUB13 ortholog, ubiquitinates a S-domain receptor-like kinase SDS2 (SPL11 cell-death suppressor 2) (Fan et al. 2018). PUB25 and PUB26 negatively regulate immunity by ubiquitinating the unphosphorylated (non-activated) RLCK BIK1 (BOTRYTIS-INDUCED KINASE 1) to facilitate its proteasomal degradation (Wang et al. 2018b).

Given the importance of MAPK signaling in plant immunity and the significance of PUBs in destabilizing MAPKs, we screened *Nicotiana benthamiana* PUBs using tobacco rattle virus (TRV)-based gene silencing and identified PUB40 as an attenuator of plant resistance to *Phytophthora capsici*. We further demonstrated that NbPUB40-mediated resistance attenuation is achieved by interacting with and destabilizing the MEK2-SIPK/WIPK (wound-induced protein kinase) cascade, which is an essential MAPK signaling pathway for Solanaceae plant immunity (Mase et al. 2012).

Results

Identification of *N. benthamiana* ARM PUBs repressing resistance to *P. capsici*

A total of 47 ARM PUBs were identified in *N. benthamiana* using hidden Markov model-based profiles (Fig. 1a) (Kourelis et al. 2019). A maximum likelihood tree was constructed using these NbPUBs and 41 *Arabidopsis* ARM PUBs (Mudgil et al. 2004). Since *N. benthamiana* is an allotetraploid, many NbPUBs with high similarity occurred in pairs in the phylogenetic tree (Fig. 1b). Accordingly, we constructed 28 TRV gene silencing constructs (TRV:*P1–28*) targeting 47 ARM NbPUBs (Fig. 1b and Additional file 1: Table S1). TRV:*P22*-infiltrated *N.*

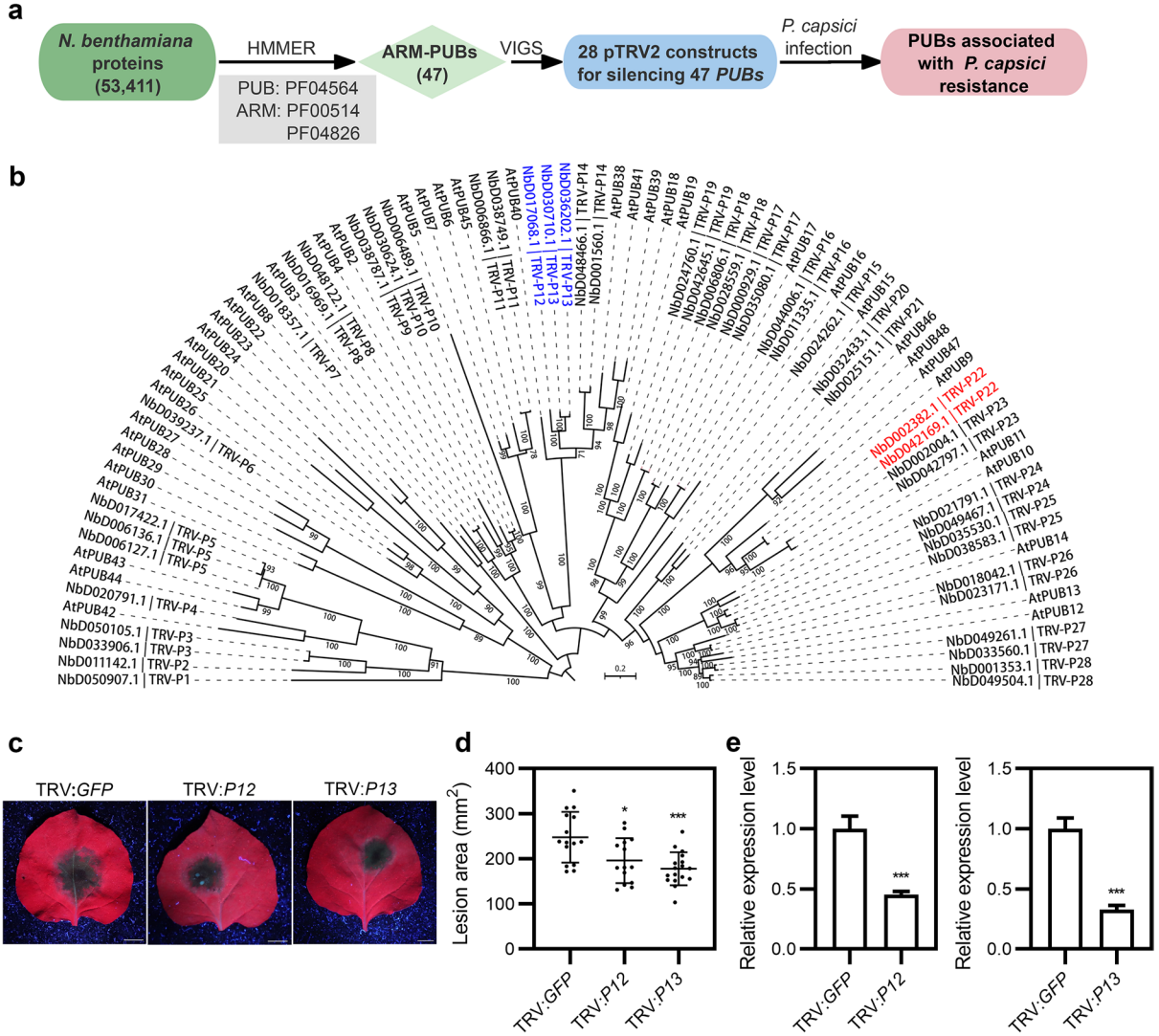

Fig. 1 Identification of *Nicotiana benthamiana* ARM PUBs repressing plant immunity. **a** The screening pipeline for ARM PUBs repressing *P. capsici* resistance using TRV-based gene silencing. **b** Phylogenetic analysis of 47 *N. benthamiana* and 41 *Arabidopsis* ARM PUBs. A maximum likelihood tree was constructed using IQ-TREE 2 (evolutionary model: JTT + F + I + G4). The blue font represents *NbPUB40* and *NbPUB40-like*. The red font represents the two genes silenced by TRV:*P22*. Their silencing activated plant autoimmunity. **c, d** Silencing of *NbPUB40* homologs induced by TRV:*P12* or TRV:*P13* inhibited *P. capsici* infection. TRV:*GFP* was used as a negative control for all the assays. TRV:*P12/13*-treated leaves were inoculated with *P. capsici* zoospores and photographed after 36–48 h under UV light. Seven leaves were counted in each experiment, and the experiment was repeated three times. Lesion areas were measured by ImageJ. Error bars indicate ± SD (Dunnett's post hoc test, * $P < 0.05$, *** $P < 0.001$). **e** Relative transcript accumulation levels of *NbPUB40* in TRV:*GFP*- and TRV:*P12*-treated plants, the expression level of *NbPUB40-like* in TRV:*GFP*- and TRV:*P13*-treated plants. Error bars indicate ± SD (Student's *t*-test, *** $P < 0.001$)

benthamiana plants exhibited notable growth defect (Additional file 2: Figure S1a) and reduced *P22* expression (Additional file 2: Figure S1b), spontaneous cell death spreading from leaf veins (Additional file 2: Figure S1a) as detected by trypan blue staining (Additional file 2: Figure S1c), increased H_2O_2 accumulation shown by 3,3'-diaminobenzidine tetrahydrochloride (DAB) staining (Additional file 2: Figure S1d), and highly induced expression of pathogenesis-related genes *PR1* and *PR2* (Additional file 2: Figure S1e). These results demonstrate that the two *NbPUBs* (*NbD002382* and *NbD042169*) silenced by TRV:*P22* may negatively regulate plant immunity, which is consistent with a previous report that *Arabidopsis* PUB13 suppresses defense responses including cell death (Li et al. 2012b).

Compared to TRV:*GFP*-treated control plants, no visible phenotype was induced by the other 27 constructs one week after infiltration. To test their involvement in

Phytophthora resistance, we inoculated TRV-treated plants with *P. capsici* zoospores 18 days after infiltration (Additional file 2: Figure S2). Lesions caused by *P. capsici* were significantly inhibited by TRV:*P12* targeting *NbD017068* (*NbPUB40*) or TRV:*P13* targeting *NbD030710*/*NbD036202* (*NbPUB40-like*) (Fig. 1c, d). RT-qPCR analysis confirmed that *P12* was effectively silenced in TRV:*P12*-treated plants, and *P13* was silenced in TRV:*P13*-treated plants (Fig. 1e). We then generated at least three heterozygous *pub40-like* mutants and two homozygous *pub40* mutant lines (*pub40-1* and *pub40-2*) via CRISPR/Cas9-mediated genome editing. The respective 1-bp insertion and 154-bp deletion in *pub40-1* and *pub40-2* lead to premature termination of NbPUB40 (Fig. 2a and Additional file 2: Figure S3), which did not cause an obvious difference in growth phenotypes

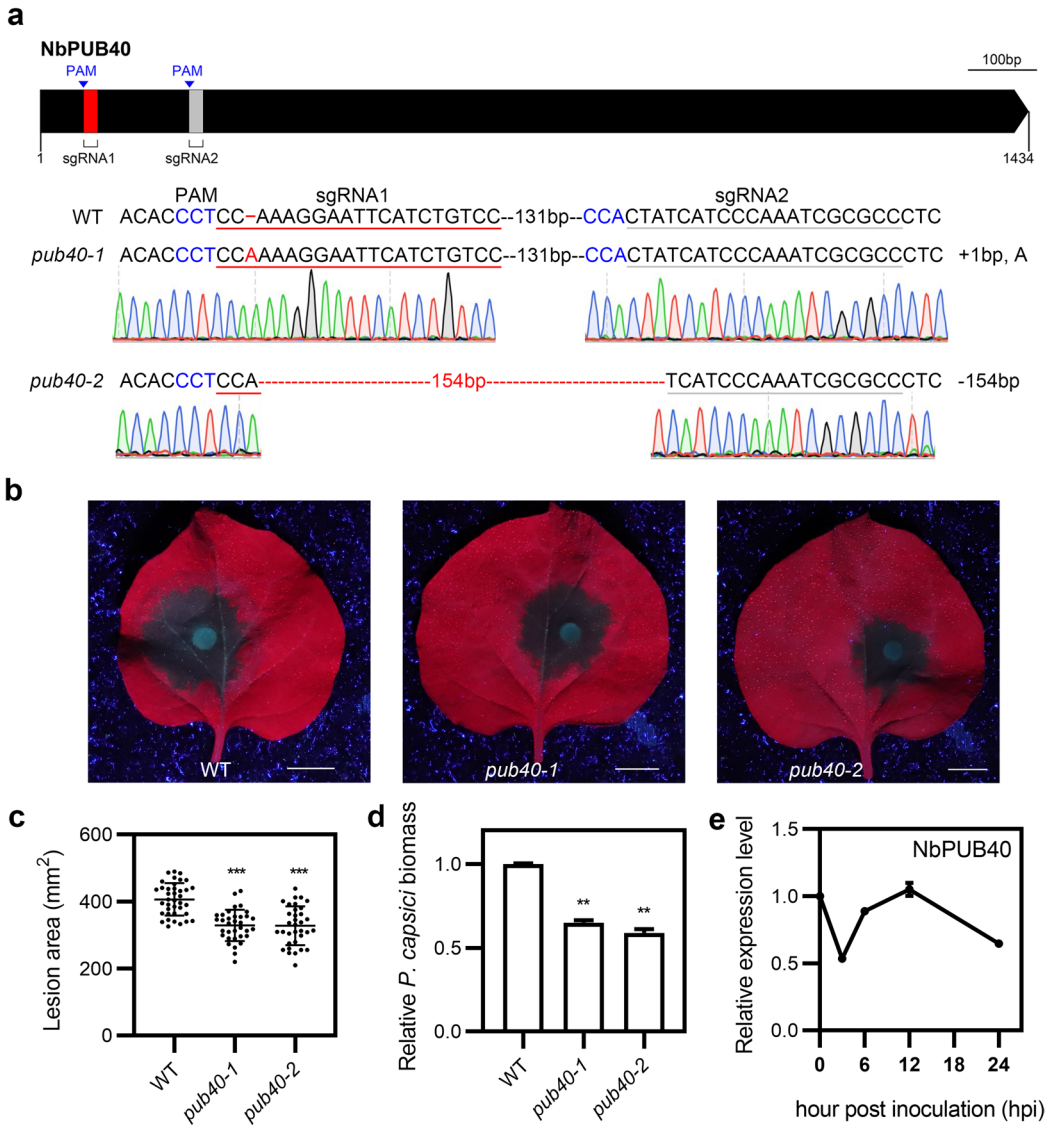

Fig. 2 NbPUB40 negatively regulates plant resistance to *P. capsici*. **a** Genotypes of two *N. benthamiana pub40* mutants (*pub40-1* and *pub40-2*) generated by CRISPR/Cas9-mediated genome editing. sgRNA sequences are highlighted with underlines. Protospacer adjacent motifs (PAMs) are represented in blue font. Insertion and deletion are in red font and dashed lines. **b, c** Both *pub40* mutants showed increased resistance to *P. capsici* infection. Leaves infected by *P. capsici* were photographed two days post-inoculation (dpi) under UV light. Average lesion areas were measured by ImageJ. Data included results from three independent experiments, with at least ten leaves in each experiment. Error bars indicate ± SD (Dunnett's post hoc test, *** $P < 0.001$). **d** Relative biomass was measured by quantitative PCR using *NbACT* as an internal reference gene. Error bars indicate ± SD (Dunnett's post hoc test, ** $P < 0.01$). **e** The expression pattern of NbPUB40 was analyzed after infection with *P. capsici* at various time points by quantitative PCR. The *NbACT* gene was used as the reference gene. Error bars indicate ± SD

between four-week *pub40* mutants and non-transgenic *N. benthamiana* (Additional file 2: Figure S4). In line with TRV:*P12/P13*-treated plants, *pub40* mutants showed significantly reduced disease symptoms (Fig. 2b, c) and pathogen biomass accumulation after *P. capsici* inoculation (Fig. 2d). We used RT-qPCR to further detect the expression pattern of *NbPUB40* after infection with *P. capsici*. As expected, the expression of *NbPUB40* was significantly inhibited in *N. benthamiana* after 3 h of infection (Fig. 2e). Collectively, NbPUb40 is a novel repressor of *P. capsici* resistance in *N. benthamian*.

NbPUB40 is subject to ubiquitination and proteasomal degradation *in planta*

An active E3 ubiquitin ligase, such as potato CMPG1 (Bos et al. 2010), usually can ubiquitinate itself for degradation by the 26S proteasome. When *N. benthamiana* leaves expressing HA-tagged NbPUB40 were treated with the protein synthesis inhibitor cyclohexmide (CHX), NbPUB40 protein abundance was notably reduced in a treatment time-dependent manner (Fig. 3a). NbPUB40 degradation could be suppressed by the proteasome inhibitor PS341 (bortezomib) and MG132, but not by the autophagy inhibitor E64d and DMSO (dimethyl sulfoxide) control (Fig. 3b, c and Additional file 2: Figure S5), indicating that NbPUB40 is subject to proteasomal degradation.

The U-box domain, especially its two highly conserved amino acid residues (Cys and Val), is crucial for the activity of E3 ubiquitin ligases (Gonzalez-Lamothe et al. 2006; Yang et al. 2006). Multiple sequence alignment showed that the U-box domain of NbPUB40 also contains these two conserved residues (Cys28 and Val41, Fig. 3d). In an *in-planta* ubiquitination assay, FLAG-tagged NbPUB40, NbPUB40-C28A, and NbPUB40-V41I mutants were co-expressed with HA-UBQ (ubiquitin) in *N. benthamiana* leaves. Ubiquitination signals of extracted total proteins immunoprecipitated with anti-FLAG beads were determined by immunoblots using anti-HA antibody. Compared to wild-type (WT) NbPUB40, C28A or V41I mutation led to a remarkably reduced ubiquitination signal (Fig. 3e). These findings suggested that NbPUB40 might be an active E3 ubiquitin ligase with U-box Cys28 and Val41 being critical for its activity.

NbPUB40 interacts with multiple MAPKs

Next, we identified candidate protein substrates of NbPUB40 by immunoprecipitation-mass spectrometry (IP-MS). Interacting proteins of an unrelated PUB (NbD002382 targeted by TRV:*P22*) were also identified in parallel to characterize potential false positive targets. Multiple MAPKs were found in the 586 candidate NbPUB40 substrate list, including a *Nicotiana* protein kinase 1 (NPK1)-like (NbNPL1: NbD027101) MAPKKK, two MAPKKs NbMEK2 (NbD050000) and NbSIPKK (NbD049175), and two MAPKs NbSIPK (NbD046763) and NbWIPK (NbD016498) (Additional file 1: Table S2).

We used split-LUC (luciferase) and co-immunoprecipitation (Co-IP) assays to confirm in vivo interactions between NbPUB40 and the abovementioned MAPK signaling proteins except for NbNPL1, which is lethal to *Agrobacterium tumefaciens* used for transient expression in *N. benthamiana*. Co-expression of NbPUB40 with NbSIPKK, NbSIPK, or NbWIPK generated high-intensity luminescence in split-LUC assays (Fig. 4a). For the Co-IP assay, NbPUB40 co-immunoprecipitated with NbSIPKK, NbMEK2, or NbWIPK (Fig. 4b). Since co-expression with NbSIPK dramatically reduced NbPUB40 protein expression in *N. benthamiana* (Additional file 2: Figure S6a), we successfully purified the other proteins than NPL1 from *Escherichia coli* and performed GST pull-down assays, where NbPUB40 was able to pull down NbSIPKK NbMEK2, NbSIPK, and NbWIPK in vitro (Fig. 4c). Yeast two-hybrid (Y2H) assays showed NbPUB40 interaction with NbNPL1, NbSIPKK, and NbSIPK (Additional file 2: Figure S6b). Based on these results, NbPUB40 is a key immune component associated with MAPK protein signaling (Fig. 4d).

The interaction network of NbPUB40-targeted MAPKs

MEK2-SIPK/WIPK is an essential MAPK immunity cascade in *N. benthamiana* (Sharma et al. 2003; Asai et al. 2008). As a negative regulator of MAPKKKε/MEK2/SIPK-WIPK, SIPKK suppresses SIPK activity (Gomi et al. 2005; Melech-Bonfil and Sessa. 2010). To test whether NbNPL1 interacts with NbSIPKK or NbMEK2, we constructed their interaction network via Co-IP, split-LUC, and Y2H assays. NbSIPKK but not NbMEK2 strongly interacted with NbNPL1 in Y2H (Additional file 2: Figure S7). Both NbMEK2 and NbSIPKK interacted with NbSIPK and NbWIPK in split-LUC and Co-IP assays (Fig. 5a–d). Y2H assays confirmed that NbMEK2 interacts with NbSIPK and NbWIPK, NSIPKK interacts with NbWIPK (Fig. 5e), but did not detect the interaction between NbSIPKK and NbSIPK (Fig. 5f). The interaction relationships are summarized in Fig. 5g.

NbPUB40 destabilizes the MEK2-SIPK/WIPK cascade

We then investigated whether NbPUB40 induced the degradation of these MAPKs. Protein abundances of HA-tagged NbMEK2, NbSIPK, and NbWIPK but not NbSIPKK, decreased notably when co-expressed with NbPUB40-GFP (Fig. 6a). Consistently, the *pub40* mutant showed increased protein abundances of NbMEK2, NbSIPK, and NbWIPK, but lower NbSIPKK accumulation (Fig. 6b). In in vivo ubiquitination assays,

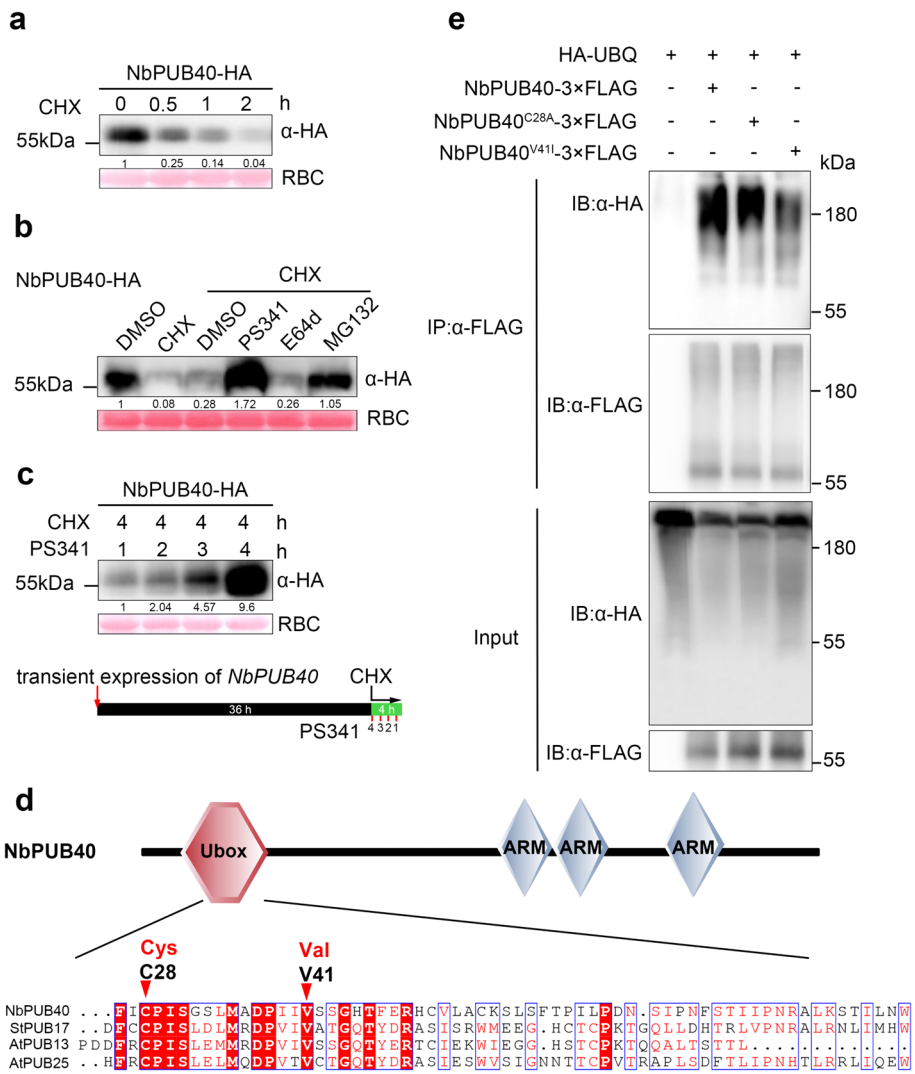

Fig. 3 NbPUB40 is subject to ubiquitination and proteasomal degradation *in planta*. **a** Inhibition of protein synthesis by CHX revealed *in planta* degradation of NbPUB40. After transient expression of NbPUB40 for 36 h, *N. benthamiana* leaves were treated with 50 μM CHX for 0, 0.5, 1, and 2 h before collecting samples for immunoblotting. **b** NbPUB40 degradation was suppressed by the 26S proteasome inhibitor PS341 and MG132. Leaves expressing NbPUB40-HA were treated with 0.5% DMSO (dimethyl sulfoxide), 50 μM CHX, CHX + DMSO, CHX + 50 μM PS341, CHX + 50 μM E64d, or CHX + 50 μM MG132 for 2 h before sample collection. The anti-HA antibody was used for immunoblotting. Protein band intensities were analyzed using ImageJ. **c** NbPUB40 degradation was suppressed by PS341 in a time-dependent manner. After transient expression of NbPUB40 for 36 h, leaves were treated with 50 μM CHX for 4 h before sampling and treated with 50 μM PS341 for 4, 3, 2, and 1 h before sampling. The anti-HA antibody was used for immunoblotting. Protein band intensities were analyzed using ImageJ. A simple illustration of the sampling timeline is shown. **d** Two conserved active sites (represented by red triangles) of the U-box domain in NbPUB40, potato StPUB17, and *Arabidopsis* AtPUB13 and AtPUB25. **e** Mutation of C28A or V41I significantly reduced NbPUB40 polyubiquitination

FLAG-tagged NbSIPKK, NbMEK2, NbSIPK, or NbWIPK were co-expressed with HA-UBQ in WT and *pub40-2* mutant plants. NbSIPK was later excluded from the assay since it severely affected the stable expression of UBQ in *N. benthamiana* for an unknown reason (Additional file 2: Figure S8). A strong polyubiquitination signal was detected in the NbSIPKK, NbMEK2, and NbWIPK immunoprecipitated product using anti-HA antibody (Fig. 6c–e). NbMEK2 and NbWIPK were less ubiquitinated in *pub40-2* than in WT, while the polyubiquitination levels of NbSIPKK showed no difference. These findings suggested that NbPUB40 targets the MEK2-SIPK/WIPK cascade.

The MEK2-SIPK/WIPK cascade contributes to resistance against *P. capsici*

The MEK2-SIPK/WIPK cascade is essential for resistance to Gram-negative bacteria, tobacco mosaic virus (TMV),

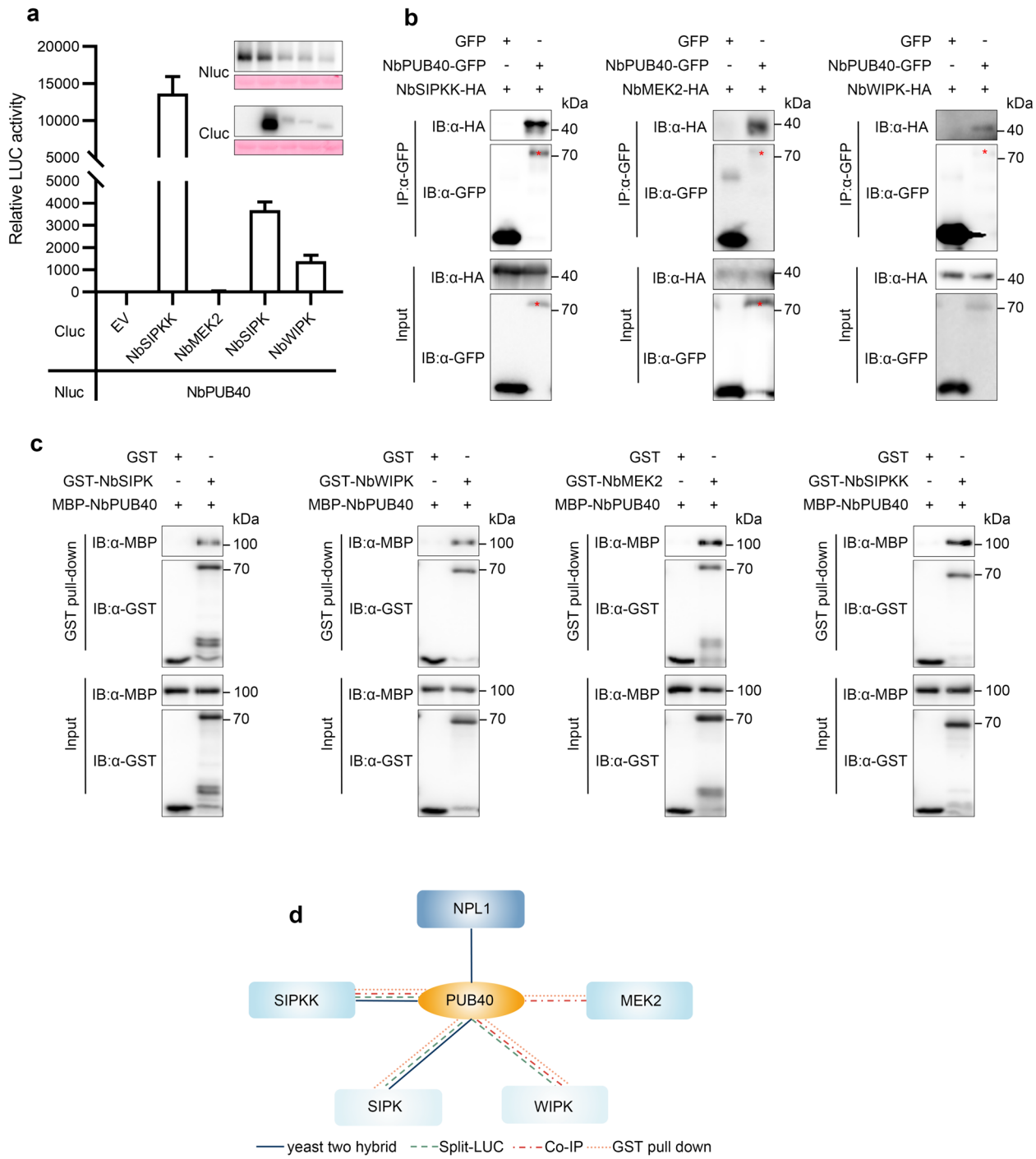

Fig. 4 NbPUB40 interacts with multiple MAPKs. **a** NbPUB40 was associated with NbSIPKK, NbSIPK, and NbWIPK in the split-LUC assay. The corresponding constructs were transiently expressed in *N. benthamiana,* and protein expression was confirmed by immunoblot assays. Protein interaction intensities were indicated by relative luminescence units (RLUs) (mean ± SD, n ≥ 6). **b** Co-IP assays confirmed NbPUB40 interaction with NbSIPKK, NbMEK2, and NbWIPK. GFP-tagged NbPUB40 transiently co-expressed with NbSIPKK-HA, NbMEK2-HA, or NbWIPK-HA. Extracted total plant proteins were immunoprecipitated with GFP-Trap agarose (Chromo Tek). Red asterisks indicate the band of NbPUB40-GFP. **c** NbPUB40 interact with NbSIPK, NbWIPK, NbMEK2, and NbSIPKK in vitro. Protein interactions were detected by GST pull-down assays with glutathione agarose beads. **d** A schematic illustration of interactions between PUB40 and MAPKs

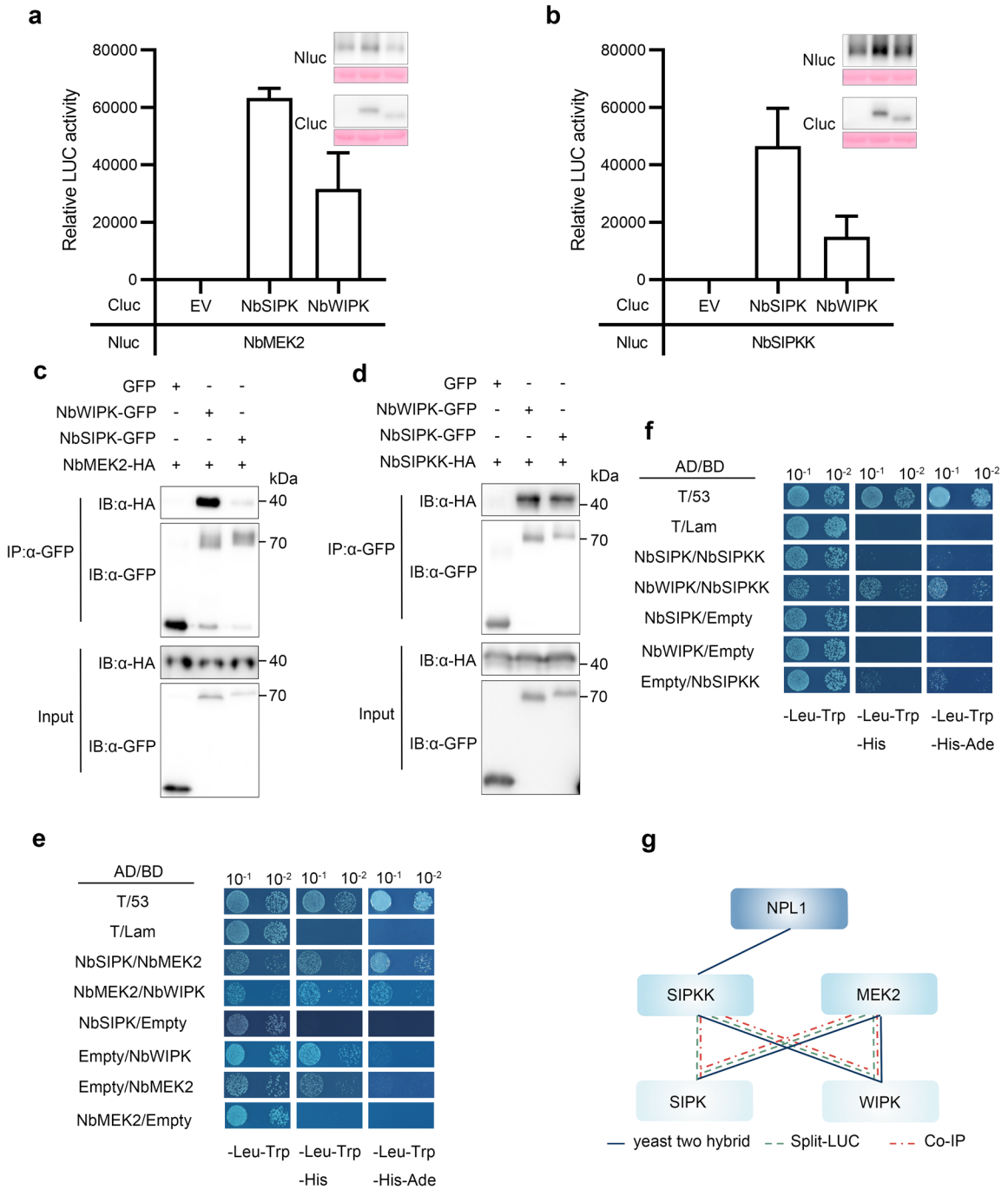

Fig. 5 The interaction network of NbPUB40-targeted MAPKs. **a, b** Split-LUC assay showed NbMEK2 and NbSIPKK interaction with NbSIPK and NbWIPK. Corresponding constructs were transiently expressed in *N. benthamiana*, and protein expression was confirmed by immunoblot assays. Protein interaction intensities were indicated by relative luminescence units (RLU) (mean ± SD, n ≥ 6). **c, d** NbMEK2 and NbSIPKK interacted with NbSIPK and NbWIPK in Co-IP assays. Anti-GFP and anti-HA antibodies were used for the western blots. **e, f** Interactions between NbMEK2/NbSIPKK and NbSIPK/ NbWIPK were confirmed by Y2H. T/53, positive control, T/Lam, negative control. AD, pGADT7; BD, pGBKT7. **g** The interaction network of NbPUB40-targeted MAPK signaling kinases in *N. benthamiana*

and the *Colletotrichum orbiculare* fungus (Jin et al. 2003; Tanaka et al. 2009; Melech-Bonfil and Sessa. 2010), but seems to have an independent defense mechanism against *Phytophthora* infection (Wang et al. 2018a). To confirm the role of this cascade in *Phytophthora* resistance, we constructed TRV vectors to silence relevant

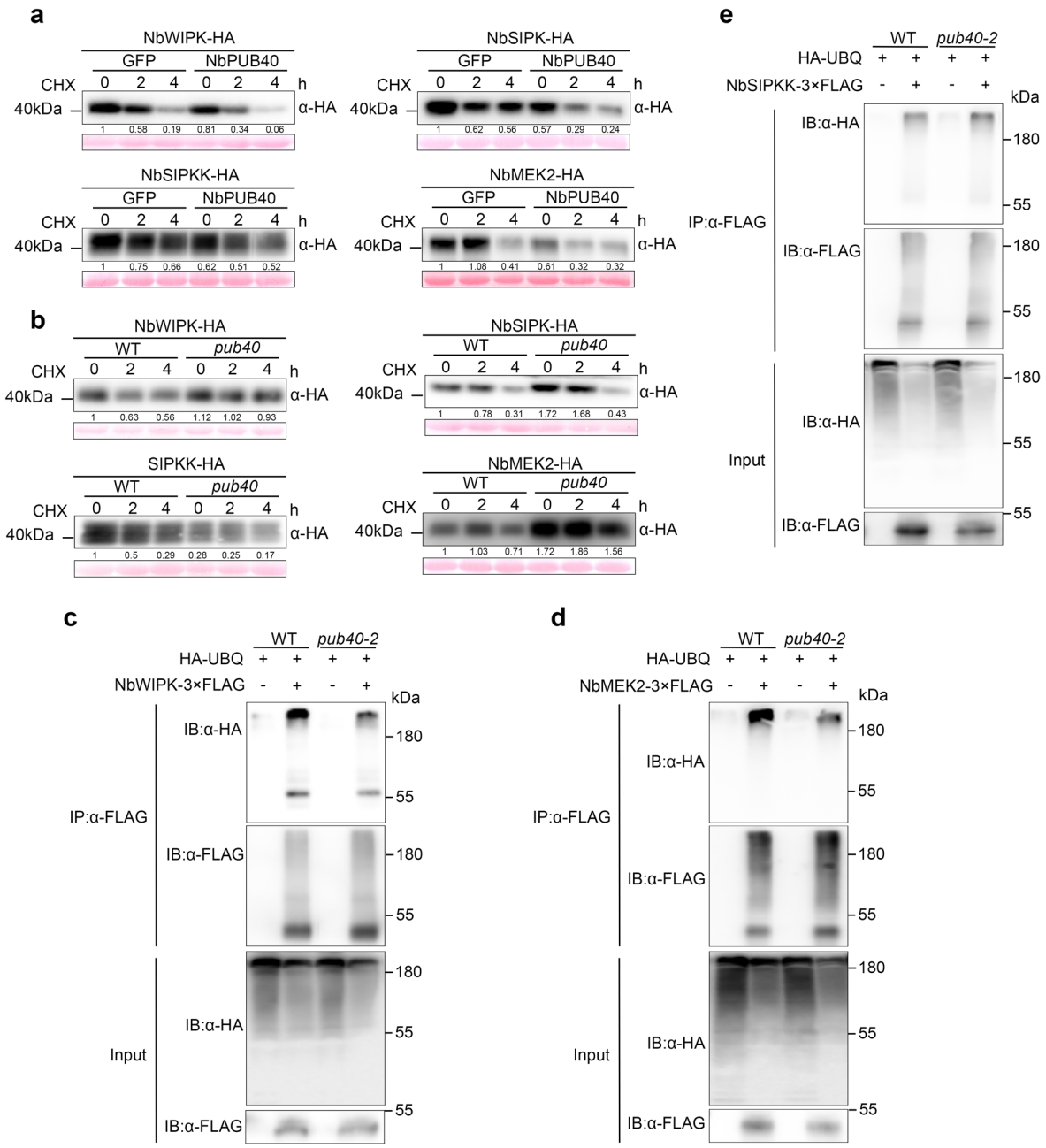

Fig. 6 NbPUB40 destabilizes the MEK2-SIPK/WIPK cascade. **a** NbPUB40 promoted NbMEK2, NbSIPK, and NbWIPK degradation *in planta*. HA-tagged NbMEK2, NbSIPK, or NbWIPK were co-expressed with NbPUB40-GFP or the GFP control for 36 h. *N. benthamiana* leaves were treated with 50 μM CHX for 0, 2, and 4 h before collecting samples for immunoblotting assay. Band intensities were analyzed using ImageJ. **b** Time-course degradation of NbMEK2, NbSIPK, and NbWIPK in WT and *pub40*. Designated MAPKs were transiently expressed in *N. benthamiana* and treated with 50 μM CHX for 0, 2, and 4 h before collecting samples for immunoblotting. **c, d, e** NbMEK2 and NbWIPK exhibited decreased polyubiquitination in *pub40*. FLAG-tagged NbMEK2, NbWIPK, or NbSIPKK were transiently expressed with HA-UBQ in WT and *pub40*

MAPKs before *P. capsici* inoculation. RT-qPCR analysis confirmed the effective silencing of these genes in TRV-treated plants (Additional file 2: Figure S9). Plants treated with TRV:*NbMEK2*, TRV:*NbSIPK*, TRV:*NbWIPK*, or TRV:*NbS/WIPK* were more susceptible to *P. capsici* infection than TRV:*GFP*-treated plants, as reflected by significantly larger lesion areas (Fig. 7a, b) and the higher levels of relative *P. capsici* biomass (Fig. 7c). These results indicated that MEK2-SIPK/WIPK positively regulates plant resistance to *P. capsici*.

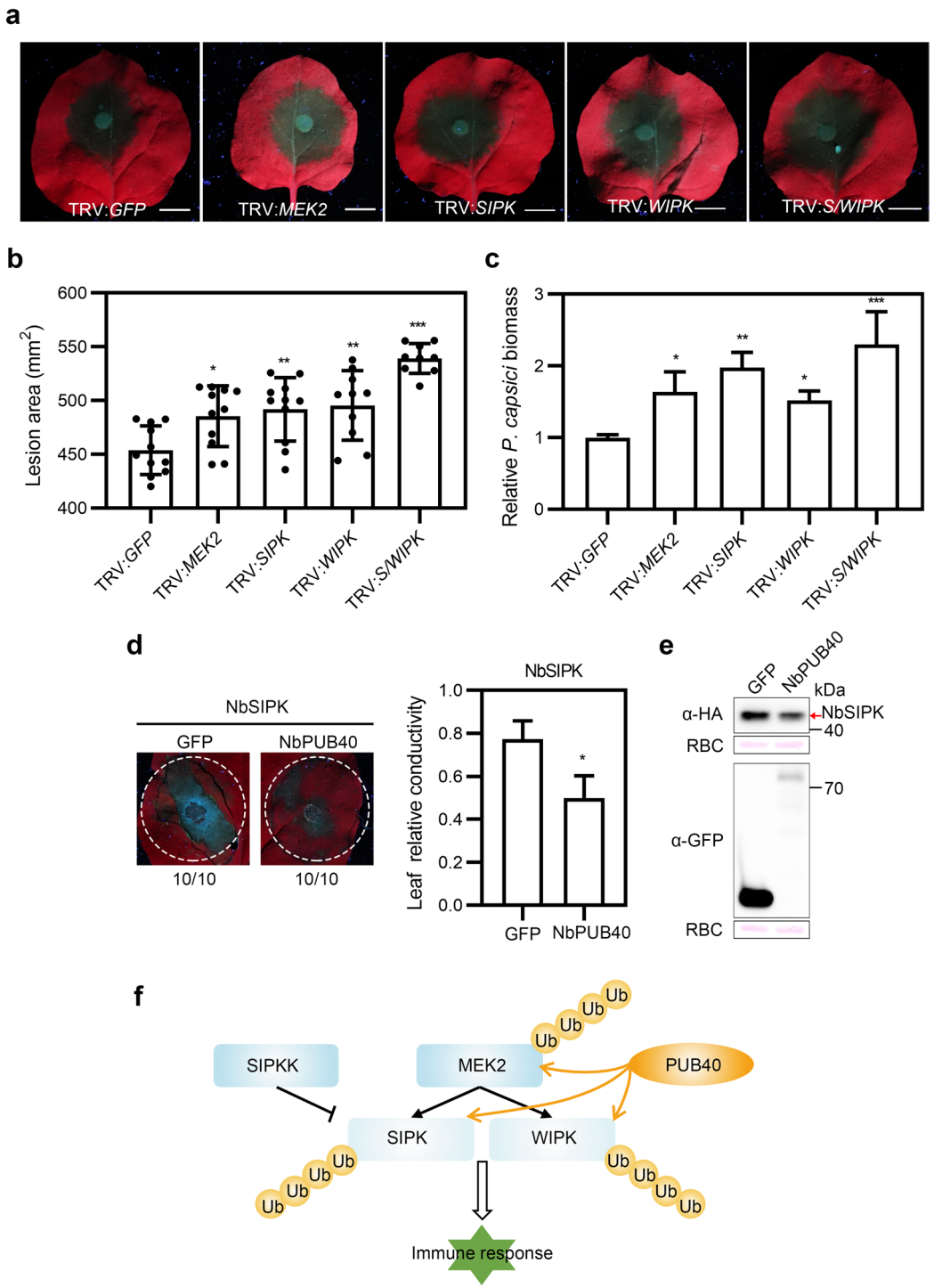

Fig. 7 The MEK2-SIPK/WIPK cascade is essential for plant resistance to *P. capsici*. **a** Representative leaf lesions caused by TRV:*MEK2*, TRV:*SIPK*, TRV:*WIPK*, or TRV:*S/WIPK* treatment followed by *P. capsici* infection. Photos were taken 2 dpi under UV light. **b** Lesion areas were measured by ImageJ. Independent experiments were repeated three times, and similar results were obtained. Error bars indicate ± SD (Dunnett's post hoc test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). **c** Relative *P. capsici* biomass was analyzed by qPCR using *NbACT* as an internal reference. Error bars indicate ± SD (Dunnett's post hoc test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). **d** Transient co-expression of *NbPUB40* inhibited NbSIPK-triggered cell death compared to the GFP control. Photos were taken under UV light. Relative conductivity was measured two days after infiltration. This assay was repeated using 10 leaves (Student's *t*-test, * $P < 0.05$). **e** Relevant protein expression was confirmed by immunoblotting assay. The anti-HA antibody was used to detect HA-tagged NbSIPK. The anti-GFP antibody was used to detect PUB40-GFP and the GFP control. Ponceau staining (RBC) showed equal protein loading. **f** A schematic diagram of NbPUB40-mediated suppression of plant immunity via polyubiquitinating and destabilizing the MEK2-SIPK/WIPK cascade

NtSIPK has been reported to induce cell death in tobacco (Yang et al. 2001; Zhang and Liu. 2001). We observed a similar phenotype when transiently expressed NbSIPK (Fig. 7d). NbSIPK-induced cell death and electrolyte leakage were significantly suppressed when co-expressed with NbPUB40 (Fig. 7d). Proper expression of all proteins was confirmed by immunoblotting (Fig. 7e). These results led us to explore the effect of PUB40 on MAPK activation, we detected MAPK activation after treating WT and *pub40* mutants with *P. capsici* spores for 6 h, and found that MAPK proteins were strongly activated in *pub40* mutants (Additional file 2: Figure S10). These results demonstrated that NbPUB40 attenuates *P. capsici* resistance by destabilizing the MEK2-SIPK/WIPK cascade in *N. benthamiana* (Fig. 7f).

Discussion

Arabidopsis PUB40 and its two homologs, PUB41 (AT5G62560) and PUB39 (AT3G47820), are involved in the degradation of BRASSINAZOLE RESISTANT1 (BZR1), which is a transcription factor regulating plant root growth and tolerance to phosphate (Pi) starvation (Kim et al. 2019). In the absence of brassinosteroids (BRs), BRASSINOSTEROID-INSENSITIVE2 (BIN2) phosphorylates and stabilizes PUB40 in roots to enhance its binding to BZR1, which leads to BZR1degradation (Kim et al. 2019). However, whether PUB40 participates in plant immunity is unknown. *P. capsici* can significantly reduce the yield of many crops due to its broad host range and potent destructiveness (Barchenger et al. 2018). We found that NbPUB40, an ortholog of PUB40 in *N. benthamiana* (Fig. 1b), acts as a negative regulator of plant *P. capsici* resistance.

A growing number of studies have shown the importance of other PUB proteins in plant immunity (Trujillo. 2018). *Arabidopsis* PUB13 and its homologs degrade immune receptors FLS2, PSKR1, and SDS2 (Lu et al. 2011; Fan et al. 2018; Hu et al. 2023). PUB25 and PUB26 ubiquitinate unphosphorylated BIK1, which regulates key signaling events downstream of PRRs (Wang et al. 2018b). PUB22 mediates the degradation of Exo70B2, a subunit of the exocyst complex involved in PTI. PUB22 also interacts with MPK3 but does not promote its degradation (Stegmann et al. 2012; Furlan et al. 2017). Soybean GmSAUL1 represses plant immunity likely by inhibiting the activation of MPK3, but it is unclear whether GmSAUL1 could degrade MPK3 (Li et al. 2023b). There have been no reports of U-box type E3 ubiquitin ligase interacting with MAPK protein to regulate plant immune response. In our study, NbPUB40 directly interacts with MEK2-SIPK/WIPK and SIPKK (Fig. 4c), MEK2-SIPK/WIPK contributes to plant resistance to *P. capsici* (Fig. 7a). These results indicated a new mechanism by which MAPK proteins are the targets of PUBs as well, demonstrating the significant impact of PUBs on plant immunity.

MAPK proteins are also the main targets of pathogen effectors, particularly in *Phytophthora* species. For instance, multiple effector proteins from the plant pathogenic oomycete *P. infestans* have been shown to target potato MAPK cascades. Pi22926, PexRD2, and Pi17316 interact with distinct MAPKKKs (King et al. 2014; Murphy et al. 2018; Ren et al. 2019), while PITG20300 and PITG20303 stabilize potato StMKK1 to negatively regulate plant immunity (Du et al. 2021). *Phytophthora sojae* effector Avh331 promotes the infection by manipulating the MAPK signaling of *A. thaliana* and *N. benthamiana*. (Cheng et al. 2012). Unfortunately, the effector of *P. capsici* that inhibits MAPK activity has not been reported so far; for this reason, it is important to investigate the negative regulators involved in the plant response to *P. capsici* infection. We observed that NbPUB40 negatively regulates the infection of *P. capsici*. As a potentially active E3 ubiquitin ligase (Fig. 3), NbPUB40 is essential for the precise regulation of the target MAPK cascade. NbPUB40 influences the ubiquitination of MEK2-SIPK/WIPK in plants (Fig. 6c, d), the mutation of *PUB40* enhances their stability, and the MAPK activation following *P. capsici* infection in *pub40* mutants was significantly stronger than in WT (Additional file 2: Figure S9). These findings clarify the mechanism of NbPUB40 promoting the infection of *P. capsici* by negatively regulating the protein levels of MEK2, SIPK, and WIPK.

E3 ligase activity is often regulated by phosphorylation. MPK3 phosphorylates Thr62 and Thr88 residues of PUB22 to reduce its autoubiquitination, which leads to increased PUB22 accumulation and inhibition of immune signals (Furlan et al. 2017). The phosphorylation of KEG leads to its degradation (Liu and Stone. 2010). BRI1 phosphorylates the Ser-344 residue of PUB13, which is required for PUB13-mediated degradation and endocytosis of BRI1 (Zhou et al. 2018). In addition, the phosphorylation status of E3 ligase substrates is also linked to degradation. For instance, PUB4, PUB25, and PUB26 specifically ubiquitinate and degrade the non-phosphorylated BIK1 (Wang et al. 2018b; Yu et al. 2022). In our results, the co-expression of NbPUB40 and NbSIPK significantly reduced NbPUB40 protein levels (Additional file 2: Figure S6a), suggesting that NbPUB40 may be phosphorylated by NbSIPK. Whether NbPUB40 is phosphorylated and/or activated by its MAPK substrates and the phosphorylation status of NbPUB40-degradable MAPKs remains to be determined. In addition to NbPUB40, we identified two NbPUB40-like (NbD030710/NbD036202) proteins on the same screen. They likely play similar roles in destabilizing MAPKs and attenuating plant immunity.

Their acting mechanisms and possible coordination with NbPUB40 need to be explored in the future.

Conclusion

Plant MAPKs are key modules of immune signaling. Precise regulation is crucial to prevent their hyperactivation. However, only limited studies have investigated the regulation of MAPK protein abundance. In this study, we reported that a U-box E3 ligase NbPUB40 attenuates plant resistance to *P. capsici* by targeting and destabilizing the MEK2-SIPK/WIPK cascade via the 26S proteasome system.

Methods

Plant and microbial materials

Wild-type *N. benthamiana* and *pub40-1* and *pub40-2* mutant plants were grown in a glasshouse (25°C, 14-h day/10-h night, and 60% relative humidity). *Phytophthora capsici* was grown at 25°C on vegetable juice (V8) medium (100 mL filtered V8 juice and 0.2 g calcium carbonate per liter). *Escherichia coli* DH5α and *Agrobacterium tumefaciens* GV3101 strains were respectively cultured at 37°C and 28°C on Luria Bertani (LB) medium (5 g yeast extract powder, 10 g tryptone, and 10 g NaCl per liter).

Plasmid vector construction

Designated gene fragments were amplified from *N. benthamiana* cDNA and ligated into corresponding vectors using ClonExpress® II One Step Cloning Kit (C112). DNA fragments for gene silencing in *N. benthamiana* were cloned into the pTRV2 vector (Liu et al. 2002). For split-luciferase assays, *NbPUB40, NbNPL1, NbSIPKK, NbMEK2, NbWIPK,* and *NbSIPK* coding sequences were inserted into pCAMBIA1300-Cluc-3×FLAG and pCAMBIA1300-Nluc-HA vectors. For co-immunoprecipitation (Co-IP) assays, constructs were generated using pCAMBIA1300-GFP, pCAMBIA1300-3×FLAG, and pCAMBIA1300-HA vectors. For yeast two-hybrid assays (Y2H), all designated genes were constructed into pGADT7 and pGBKT7 vectors. PCR Primers used in this study are listed in Additional file 1: Table S1 and Table S3.

CRISPR/Cas9-mediated *N. benthamiana* genome editing

N. benthamiana pub40 mutants were generated as described previously (Wang et al. 2022). Briefly, two guide RNAs (sgRNA1: GGACAGATGAATTCCTTTGG, sgRNA2: GGCGCGATTTGGGATGATAG) targeting *NbPUB40* were designed using the online program CCTop (Stemmer et al. 2015). The pHEE401E construct harboring sgRNAs was transformed into *Agrobacterium* strain LBA4404. *Agrobacterium*-mediated leaf disc transformation of *N. benthamiana* was performed as previously described (Ellis et al. 1987). Gene-edited T_0 plants obtained through tissue culture were verified by PCR and sequencing. Seedlings expressing prematurely terminated NbPUB40 were transferred to soil for DNA isolation and genotyping. Homozygous T_2 plants were used for subsequent studies.

Virus-induced gene silencing in *N. benthamiana*

Agrobacterium strains harboring pTRV1 and pTRV2 constructs were mixed with infiltration buffer at a ratio of 1:1 and infiltrated into two-week-old leaves of *N. benthamiana*. After 18 days, the two largest true leaves were used for *P.capsici* infection assay. Silencing efficiencies were detected by RT-qPCR, with TRV:*GFP* used as a control. Primers used for RT-qPCR are listed in Additional file 1: Table S3.

P. capsici infection assay

Fresh mycelial plugs of *P. capsici* strain LT263 were collected using a cork-borer set (Sigma, Ø 0.5 cm) and inoculated on the back of detached *N. benthamiana* leaves. Inoculated leaves were placed in dark boxes with high humidity for 36 h. Photos were taken under the ultraviolet lamp. Lesions were measured by ImageJ. Relative *P. capsici* biomass in infected plant leaves was measured by qPCR (Yu et al. 2012).

RT-qPCR

Total RNA samples were extracted from *N. benthamiana* leaves pre-treated with TRV constructs. For silencing efficiency evaluation, top leaves were collected 20 days after TRV treatment. TriZol kit (Zoman) and reverse transcription kit (Zoman) were used for RNA extraction and first-strand cDNA synthesis, respectively. qPCR was performed using 2×HQ SYBR qPCR Mix (Zoman) on QuantStudio 1 (Thermo).

Identification of NbPUB40-interacting proteins

To identify potential NbPUB40 interacting proteins, FLAG-tagged NbPUB40 was transiently expressed in *N. benthamiana* leaves for 48 h. Total proteins were extracted using lysis buffer: 10 mM Tris–HCl, 150 mM NaCl, 0.5 mM EDTA, 0.5% v/v Triton X-100, 2% (w/v) polyvinylpolypyrrolidone, and 10% (v/v) glycerol. Centrifuged supernatant was incubated with anti-FLAG M2 agarose (Sigma) at 4℃ for 2 h. The beads were then washed with lysis buffer and TBS buffer. Finally, immunoprecipitated proteins were used for LC-MS/MS analysis. Five MAPK proteins with high Qscores were selected as NbPUB40-interacting candidate proteins, as shown in Additional file 1: Table S2.

Co-IP assays

HA and FLAG/GFP-tagged proteins of interest were transiently co-expressed in *N. benthamiana*. After protein extraction and co-immunoprecipitation, products were incubated with anti-FLAG M2 agarose (Sigma) or GFP-Trap®_A (Chromotek) at 4°C with slight shaking for 2 h. The beads were collected and washed five times with TBS buffer. Eluted proteins were separated in SDS-PAGE and detected with anti-HA, anti-FLAG, or anti-GFP antibodies. The buffer used for protein extraction has been previously described (Zhang et al. 2023).

Split-luciferase complementation assay

Agrobacterium culture harboring Nluc- and Cluc-tagged constructs were co-infiltrated in *N. benthamiana*. After 48 h, leaf discs were collected in a 96-microplate and incubated in ddH_2O, which was then replaced with 1 mM luciferin (Biovision). Relative luciferase activities were measured using Tecan Infinite F200.

Pull-down assay

Recombinant proteins were expressed in *E. coli* and purified using glutathione agarose beads for GST-tagged proteins or dextrin beads 6FF for MBP-tagged proteins. An amount of 3 μg GST and GST-tagged proteins were incubated with MBP-NbPUB40 with 30 μL glutathione agarose beads in 1 mL GST buffer (25 mM Tris–HCl, 100 mM NaCl, 1 mM DTT, pH 7.5) for 2 h. The glutathione agarose beads were washed 6–7 times with GST wash buffer (25 mM Tris–HCl, 100 mM NaCl, 1 mM DTT, 0.1% Triton, pH 7.5) and eluted with GST buffer containing 15 mM GSH. Eluted proteins were tested by anti-MBP and anti-GST immunoblotting.

Yeast two-hybrid assay

Designated *MAPK* genes were cloned into pGBKT7 and pGADT7 vectors. Constructs for protein interaction tests were co-transformed into yeast strain AH109. Transformed yeast cells were grown on 2D (Trp-/Leu-) deficient culture medium at 28℃ for 3 days, and then transferred to fresh 2D, 3D (Trp-/Leu-/His-), and 4D (Trp-/Leu-/His-/Ade-) media at 28℃ for 3 days for interaction examinations.

Electrolyte leakage assay

Plant cell death was quantified by measurement of electrolyte leakage (Hatsugai and Katagiri. 2018). *N. benthamiana* leaf disks were placed in ddH_2O at room temperature for 5 h before measured electrical conductivity measurements. Samples were boiled for 20 min and cooled to room temperature for the second round of solution conductivity measurements. Finally, relative electrolyte leakage was calculated by the ratio of sample conductivities before and after boiling. Sample conductivity was measured using a conductivity meter (Mettler Toledo. LE703).

DAB and trypan blue staining

DAB staining was used to determine the H_2O_2 accumulation. *N. benthamiana* leaves were placed in DAB staining solution (1 mg/mL) incubated on a shaker at 100 rpm for 8 h, and then boiled with 95% alcohol for decolorization until the green color faded completely. Trypan blue staining was used to detect cell death. *N. benthamiana* leaves were boiled in trypan blue staining solution (0.02 g trypan blue, 10 mL lactic acid, and 10 g phenol, dissolved in 10 mL ddH_2O) for 5 min, and then transferred into a chloral hydrate solution (2.5 g/mL chloral hydrate solution) for decolorization until the green color faded completely.

In planta ubiquitination assay

Detection of ubiquitination in *N. benthamiana* was performed as described previously (Wang et al. 2022). The ubiquitin protein (UBQ) of *N. benthamiana* and FLAG-tagged MAPK proteins were transiently co-expressed in *N. benthamiana* and immunoprecipitated using anti-FLAG M2 agarose (Sigma). Ubiquitin signals were detected using the anti-HA antibody.

Protein stability *in planta*

For protein stability determination in *N. benthamiana*, HA-tagged protein was transiently expressed and treated with 50 μM CHX in a time-dependent manner. To determine NbPUB40 impact on MAPK stability, NbPUB40 or the GFP control was co-expressed with designated MAPKs in *N. benthamian* leaves treated with 50 μM CHX. Total plant proteins were extracted from leaves with the RIPA buffer added for immunoblot analyses. HA immunoblots were quantified in ImageJ and normalized according to the intensity of Ponceau staining.

Abbreviations

ARM	Armadillo
BIK1	Botrytis-induced kinase 1
CHX	Cycloheximide
CRCK3	Calmodulin-binding receptor-like cytoplasmic kinase 3
CRL	Cullin-RING Ligases
ETI	Effector-triggered immunity
FLS2	Flagellin-sensing2
GFP	Green fuorescent protein
LB	Luria Bertani
LRR	Leucine-rich repeat
LYK5	LysM-containing receptor-like kinase 5
MAPKs	Mitogen-activated protein kinases
MAPKKs	MAPK kinases
MAPKKKs	MAPKK kinases
MEK2	Mitogen-activated protein kinase 2
PAMPs	Pathogen-associated molecular patterns
PTI	PAMP-triggered immunity
PRRs	Pattern recognition receptors

PSKR1	Phytosulfokine receptor 1
PUB	Plant U-box protein
RLCKs	Receptor-like cytoplasmic kinases
SIPK	SA-induced protein kinase
TRV	Tobacco rattle virus
WIPK	Wound-induced protein kinase

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42483-024-00249-6.

Additional file 1: Table S1. pTRV2 construct primer information of PUB genes. Table S2. LC–MS/MS identified five potential NbPUB40 interacting proteins. Table S3. Primers used in this study.

Additional file 2: Figure S1. TRV based silencing of *P22* in *N. benthamiana*. Figure S2. Screening of NbPUBs involved in *P. capsici* resistance via TRV-induced gene silencing. Figure S3. The sequence of *pub40* mutants showing premature termination. Figure S4. The growth phenotypes of *pub40* mutants. Figure S5. NbPUB40 degradation was suppressed by PS341 or MG132 in a time-dependent manner. Figure S6. NbPUB40 interacts with multiple MAPKs. Figure S7. NbNPL1 interacts with NbSIPKK in yeast two-hybrid assay. Figure S8. NbSIPK disturbs stable expression of UBQ. Figure S9. *MAPK* gene silencing efficiencies in corresponding TRV-treated plants. Figure S10. MAPK activity was detected in WT, *pub40-1*, and *pub40-2* lines at the indicated time points after infection with *P. capsici*.

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Authors' contributions
ZY conceived and designed the experiments. YZ, JW, LP, and NW performed experiments and analyzed data. YZ, ZY, and DD wrote the manuscript with editing by HP and GX.

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Availability of data and materials
Sequences mentioned this study can be found in the Oxford Research Archive using the following accession numbers (Kourelis et al. 2019): NbPUB40, NbD017068; NbNPL1, NbD02710; NbSIPKK, NbD04917; NbMEK2, NbD050000; NbSIPK, NbD04676; NbWIPK, NbD01649.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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