The Ubiquitin E3 Ligase PUB17 Positively Regulates Immunity by Targeting a Negative Regulator, KH17, for Degradation

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ABSTRACT
Ubiquitination is a post-translational modification that regulates many processes in plants. Several ubiquitin E3 ligases act as either positive or negative regulators of immunity by promoting the degradation of different substrates. StPUB17 is an E3 ligase that has previously been shown to positively regulate immunity to bacteria, fungi and oomycetes, including the late blight pathogen Phytophthora infestans. Silencing of StPUB17 promotes pathogen colonization and attenuates Cf4/avr4 cell death. Using yeast-2-hybrid and co-immunoprecipitation we identified the putative K-homology (KH) RNA-binding protein (RBP), StKH17, as a candidate substrate for degradation by StPUB17. StKH17 acts as a negative regulator of immunity that promotes P. infestans infection and suppresses specific immune pathways. A KH RBP domain mutant of StKH17 (StKH17GDDG) is no longer able to negatively regulate immunity, indicating that RNA binding is likely required for StKH17 function. As StPUB17 is a known target of the ubiquitin E3 ligase, StPOB1, we reveal an additional step in an E3 ligase regulatory cascade that controls plant defense.

Key words: oomycete, plant disease, late blight, E3 ligase, KH RNA-binding protein

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INTRODUCTION
Plants are constantly subjected to attack by microbes in the environment. However, they have evolved a sensitive two-tier surveillance system that is able to recognize and thwart most attempted incursions. The first layer of defense comprises recognition of conserved microbe-associated molecular patterns (MAMPs) by cell surface pattern recognition receptors (PRRs). This up-regulation of immune responses is termed pattern-triggered immunity (PTI) and prevents infection by most microbes (Jones and Dangl, 2006). Host-adapted pathogens are able to suppress PTI through the activity of secreted effector proteins that can manipulate immunity; this is called effector-triggered susceptibility (ETS). The second layer of plant defenses involves the detection of these effectors, or their activities, by plant resistance (R) genes. This recognition results in a massively amplified defense response termed effector-triggered immunity (ETI), which can halt pathogen colonization (Jones and Dangl, 2006).

The plant immune responses can include the synthesis of antimicrobial compounds and defense hormones, cell wall reinforcement, generation of reactive oxygen intermediates (ROIs), and a form of programmed cell death (PCD) called the hypersensitive response (HR) (Dixon et al., 1994). While regulation of immunity requires huge alterations to the transcriptome (Li et al., 2016), changes in post-translational modifications (PTMs) are emerging as an important means of controlling and coordinating defense responses. One such PTM is ubiquitination, which involves the covalent attachment of ubiquitin (Ub) to a lysine residue in the protein of interest. There are three enzymes needed for ubiquitination. An E1 activating enzyme is required to recruit Ub; an E2 conjugating enzyme, which determines Ub transfer and type of Ub linkage; and an E3 ligase, which is responsible for selecting substrates for...
Plant Communications

ubiquitination (Sadanandom et al., 2012). Ubiquitination is a reversible process and there is a family of deubiquitinating enzymes (DUBs) that remove Ub (Isozo and Nagel, 2014). The precise form of ubiquitination (i.e., monoubiquitination or polyubiquitination) and the type of linkages in the Ub chain formation can specify different fates for the substrate; for example, by causing changes in localization or activity (Chen and Sun, 2009). However, the major mode of action is the addition of a polyubiquitin chain to target the substrate for degradation by the 26S proteasome. Ubiquitination has been shown to regulate many different processes in plants, from growth and development, including flowering, to responses to both abiotic and biotic stresses (Sharma et al., 2016). One interesting observation is that there has been a considerable proliferation in the number and type of E3 ligases in various plants compared to animals (Vierstra, 2003), indicating the relative importance of ubiquitination as a regulatory mechanism. There are several families of E3 ligases in plants with their classification based on their protein domains: these are homology to E6-Ap C terminus (HECT) domains, plant ubiquitin-like (UBL) domains, and really interesting new gene (RING) domains, with the latter group divided into those that work as monomers and those that work as part of a cullin-based E3 ligase complex (Chen and Hellmann, 2013).

Many E3 ligases act as negative regulators of plant defense. For example, PUB12 and PUB13 work together to ubiquitinate the flg22 receptor FLS2, resulting in its degradation (Lu et al., 2011). PUB13 is also able to down-regulate SA-dependent responses to both abiotic and biotic stresses (Sharma et al., 2016). One interesting observation is that there has been a considerable proliferation in the number and type of E3 ligases in plants compared to animals (Vierstra, 2003), indicating the relative importance of ubiquitination as a regulatory mechanism. There are several families of E3 ligases in plants with their classification based on their protein domains: these are homology to E6-Ap C terminus (HECT) domains, plant ubiquitin-like (UBL) domains, and really interesting new gene (RING) domains, with the latter group divided into those that work as monomers and those that work as part of a cullin-based E3 ligase complex (Chen and Hellmann, 2013).

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While E3 ligases such as PUB17 act as positive regulators of defense, the substrates targeted by these positive regulators are as yet unknown. PUB17 is required for selected HRs and resistance to a range of pathogens from different kingdoms, including bacteria, fungi, and the oomycete P. infestans (Yang et al., 2006; He et al., 2015). PUB20/CMPG1 is required for an overlapping but distinct set of PCD-promoting pathways, as well as being the target of P. infestans effector Avr3a (Gonzalez-Lamothe et al., 2006; Bos et al., 2010; Gilroy et al., 2011). StIRF1 and NbATL60 are MAMP-responsive RING-type E3 ligases, which positively regulate PTI and defense to P. infestans (Zhong et al., 2018). Finally, the potato ubox and kinase (StUBK) E3 ligase, a target of P. infestans effector PISF3, positively regulates immunity to P. infestans and flg22 signaling but has no known involvement in PCD (He et al., 2019).

Another area that is emerging as central to control of plant immunity involves RNA-binding proteins (RBPs). These typically form riboprotein complexes with RNA and thereby regulate the translation, stability, and transport of defense-associated RNAs, as well as aspects of gene silencing (Staiger et al., 2013; Hentze et al., 2018). Glycine-rich RNA-binding protein 7 (AtGRP7) is a RNA recognition motif (RRM)-type RBP that regulates the stability of its own transcript as well as those of PRRs FLS2 and EFR. AtGRP7 is targeted by the Pseudomonas syringae (Pst) effector HopU1, which ADP ribosylates the RRM, preventing it binding RNA, resulting in increased Pst colonization (Fu et al., 2007; Nicaise et al., 2013). Modifier of nsn1 (MOS2) is an RBP that is responsible for the correct splicing of the transcript of suppressor of npr1-1, constitutive1 (SN1), a TIR-NB-LRR gene (Copeland et al., 2013). PSR1-Interacting Protein 1 (PINP1) is an RBP with an RNA helicase domain. It acts to promote immunity by allowing the accumulation of small RNAs and microRNAs. PINP1 is targeted by the Phytophthora sojae effector Phytophthora Suppressor of RNA Silencing 1 (PSR1), which disrupts the formation of dicer-containing RNA processing complexes in the nucleus (Qiao et al., 2015). The K homology (KH) RBP AtESR1 regulates JA signaling and resistance to fungal pathogen Fusarium oxysporum by an unknown mechanism (Thatcher et al., 2015). Finally, the effector Pi04089 from P. infestans interacts with and stabilizes the KH RBP StKB1, which promotes pathogen colonization of the host (Wang et al., 2015).

The oomycete P. infestans is an economically important pathogen of potato, which is the fourth main staple food crop in the world after maize, rice, and wheat (Fry et al., 2015; Yildiz, 2018). Therefore, it is imperative to understand how the plant immune system responds to and is manipulated by P. infestans in order to find novel strategies to fight this pathogen. Previous studies have shown that StPUB17 positively regulates defense to a variety of pathogens, including P. infestans (Yang et al., 2006; He et al., 2015). As StPUB17 is itself degraded by the CUL3-based E3 ligase and negative regulator of immunity POB1 (Orosa et al., 2017), this places StPUB17 substrates in an E3 ligase cascade that controls defense to P. infestans. To this end, yeast-2-hybrid (Y2H) was used to identify the KH RBP StKH17 as a candidate substrate of StPUB17. StKH17 is
indeed turned over in the presence of StPUB17 in a proteasome-dependent manner. StKH17 acts as a negative regulator of immunity to *P. infestans* and an intact RNA-binding domain (BD) is required for this activity.

**RESULTS**

**The E3 Ligase StPUB17 Interacts with a Putative RNA Binding Protein StKH17**

In order to identify putative substrates of the E3 ligase StPUB17, a Y2H screen was conducted against a potato library generated from leaf material infected by *P. infestans* (Bos et al., 2010). Although the screen was carried out to a depth of $2.94 \times 10^6$ transformants, only six positive clones were recovered. Five of these encoded a putative RBP with a KH domain and a signal transducer and activator of RNA (STAR) domain (Supplemental Figure 1) corresponding to potato transcript PGSC0003DMT400071249 (hereafter referred to as StKH17). In order to confirm this interaction, a GAL4 DNA-BD fusion of StPUB17 was co-transformed into yeast with a GAL4 activation domain (AD) fusion of StKH17. Yeasts containing these constructs were positive in the three reporter gene assays, including the more stringent uracil assay, suggesting that the interaction between the two proteins was strong (Figure 1A). Additional controls, BD-Pi04089 and AD-StKRBP1, respectively comprising a *P. infestans* effector and a KH-type RBP previously shown to interact with each other in yeast and in planta (Wang et al., 2015), were also co-transformed into yeast yielding the expected reporter gene activation (Figure 1A). However, co-expression in yeast of BD-Pi04089 with AD-StKH17 failed to activate reporters, as did BD-StPUB17 with AD-StKRBP1, showing that the interaction between PUB17 and KH17 is specific. All yeast grew on the control media containing histidine (Figure 1A). To confirm whether the interaction also occurs in planta, co-immunoprecipitation was performed using Agrobacterium-mediated transient expression of protein fusions in *Nicotiana benthamiana*, a widely used model host for late blight disease (Whisson et al., 2016). Following incubation of samples with GFP-trap beads, GFP-StKH17 was observed to specifically co-immunoprecipitate cMYC-StPUB17, whereas a GFP-Pi04089 control did not (Figure 1B).

**StPUB17 and StKH17 Interact in the Nucleoplasm**

StPUB17 has been shown to localize to and act in the nucleus (He et al., 2015). Therefore, the localization of StKH17 was examined using confocal microscopy. GFP-StKH17 was found to accumulate strongly in the nucleoplasm but not in the nucleolus, and showed little or no cytoplasmic background (Supplemental Figure 2), whereas the RFP-StPUB17 wild-type (WT) and ubox-mutant fusion proteins exhibit the same localization as the previously published GFP fusions (He et al., 2015), namely nucleus and nucleolus with cytoplasmic background (Supplemental Figure 2). The dominant-negative ubox domain mutant StPUB17 Val314Ile, Val316Ile, was designed to abolish E3 ligase activity (Yang et al., 2006; He et al., 2015) and is hereafter referred to as StPUB17mut. Co-localization studies were performed using GFP-StKH17 with RFP-StPUB17 or RFP-StPUB17mut constructs. Curiously, upon co-localization with GFP-StKH17 both RFP-StPUB17 and RFP-StPUB17mut constructs no longer accumulate in the nucleolus, although they...
remain co-localized with GFP-StKH17 in the nucleoplasm (Figure 2). Fluorescence intensity plots drawn through the nucleus show a clear reduction in signal in the area corresponding to the nucleolus in the red channel when GFP-StKH17 is co-expressed with both RFP-StPUB17 and RFP-StPUB17mut. However, both RFP-StPUB17 and RFP-StPUB17mut show a peak in fluorescence intensity corresponding to the nucleolus when co-expressed with a free GFP control, showing that removal from the nucleolus is dependent on the presence of GFP-StKH17 (Figure 2). Analysis carried out using bimolecular fluorescence complementation (BiFC) confirms that YN-StKH17 and YC-StPUB17 interact in the nucleus to reconstitute YFP fluorescence, but are only observed in the presence of the 26S proteasome inhibitor MG132 (Supplemental Figure 3), suggesting that the complex may be turned over by the proteasome.

The StKH17-StPUB17 Complex Is Degraded in a Proteasome-Dependent Manner
As StPUB17 is an ubiquitin E3 ligase, protein stability was assessed to determine if StKH17 is a substrate targeted for degradation by the 26S proteasome. Agrobacterium transient expression was used to express GFP-StKH17 and RFP-StPUB17, either alone or together. When expressed together, the stability of StKH17 was reduced and this was at least partially prevented by addition of the proteasome inhibitor MG132 (Supplemental Figure 4). This indicates turnover by the proteasome, which suggests that StKH17 is a substrate of StPUB17. Interestingly, RFP-StPUB17 protein levels mirror those of GFP-StKH17; less stable upon co-expression with GFP-StKH17 while stability is restored by MG132 treatment (Supplemental Figure 4). Thus, it is plausible that the entire complex is degraded in a proteasome-dependent manner.
StKH17 is ubiquitinated in the presence of StPUB17. To further explore this, overexpression of NbKH17-like and NbKh17-like, which exist in a distinct cluster to StKH17 and NbKh17-like based on phylogenetic analysis (Supplemental Figure 8C). However, off-target silencing should not occur as no identical 21 nt stretches exist between NbKH17-like and NbKh17 VIGS constructs, or StKH17 RNAi construct (Supplemental Figure 8D–8F).

After challenge with *P. infestans*, a significant reduction in pathogen colonization and lower levels of sporulation were observed on *KH17* VIGS plants (Figure 4A and 4B; Supplemental Figure 7D). In agreement, potato RNAi lines also showed smaller disease lesion sizes compared to the control (Figure 4C and 4D). Reduced pathogen colonization when *KH17* is silenced suggests that StKH17 acts as a negative regulator of immunity to *P. infestans*. To further explore this, overexpression of StKH17 was carried out both in *N. benthamiana* and potato. Transient agroexpression of GFP-StKH17 and a free GFP control (Supplemental Figure 8D–8F). However, off-target silencing should not occur as no identical 21 nt stretches exist between NbKH17-like and NbKh17 VIGS constructs, or StKH17 RNAi construct (Supplemental Figure 8D–8F).

**StKH17 Negatively Regulates Plant Immunity to *P. infestans***

As StPUB17 is a positive regulator of plant immunity to *P. infestans* (Ni et al., 2010; He et al., 2015), and StKH17 behaves as a substrate of StPUB17, the potential involvement of StKH17 in regulating defense to *P. infestans* was investigated. Virus-induced gene silencing (VIGS) was used to transiently silence *NbKh17* in *N. benthamiana* and stable RNAi transgenic lines were produced to silence *StKH17* in potato. Gene expression analysis using quantitative (q)RT–PCR showed a 70%–80% reduction in *NbKh17* levels in *N. benthamiana* plants expressing the independent VIGS constructs TRV-KH17 V1 and TRV-KH17 V2 (Supplemental Figure 7) and an 80%–90% reduction in *StKH17* transcript levels in potato RNAi lines #20, #33, and #34 (Supplemental Figure 8A), compared with the controls TRV–GFP and E potato-3, respectively. No obvious growth or morphological phenotypes were observed in any *KH17*-silenced plants, either transiently in *N. benthamiana* or in stably silenced potato lines (Supplemental Figures 7 and 8), suggesting that the gene does not contribute to development. We also found additional KH-type RBP-encoding genes, *StKH17-like* and *NbKh17-like*, which exist in a distinct cluster to *StKH17* and *NbKh17* based on phylogenetic analysis (Supplemental Figure 8C). However, off-target silencing should not occur as no identical 21 nt stretches exist between *NbKH17-like* and *NbKh17* VIGS constructs, or *StKH17* RNAi construct (Supplemental Figure 8D–8F).

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overexpressing StKH17 were also produced, although only two lines showed substantially increased expression (Supplemental Figure 8B). No obvious growth or morphological phenotypes were observed in the potato StKH17 overexpression lines. Following P. infestans infection, significantly increased colonization was also observed on the overexpression lines #23 and #29 compared to the E potato-3 control (Figure 4F). This supports the role of StKH17 as a negative regulator of immunity to P. infestans and suggests that the positive regulator of defense, StPUB17, acts by targeting a negative regulator, StKH17, for degradation by the 26S proteasome.

Overexpression of StKH17 Specifically Suppresses Cf4/Avr4-Induced Cell Death

A key function StPUB17 plays in immunity is the ability to promote certain cell death responses, such as that triggered by perception of the Cladosporium fulvum effector Avr4, by the tomato receptor Cf4 (Yang et al., 2006; He et al., 2015). To explore whether StKH17 is also involved in regulating this immune response, GFP-StKH17 was transiently co-expressed with Cf4 and Avr4 in N. benthamiana. The dominant-negative GFP-StPUB17mut, which is able to suppress this cell death (He et al., 2015), was used as a positive control and free GFP was used as a negative control. Both GFP-StKH17 and GFP-StPUB17mut were able to significantly suppress Cf4/Avr4-induced cell death to a similar extent (Figure 5A). As StPUB17 is known to have no involvement in cell death triggered by the P. infestans PAMP INF1, GFP-StKH17 was also tested to determine if it regulated this pathway. No significant effect on INF1-triggered cell death was observed following co-expression with either GFP-KH17 or GFP-StPUB17mut (Figure 5B), suggesting that, similar to StPUB17, StKH17 is not involved in this pathway. The fact that both proteins are involved in regulating the same Cf4-associated pathway further supports the hypothesis that StKH17 may be a substrate of StPUB17.

An Intact RNA Binding Domain Is Required for KH17 to Negatively Regulate Defense

KH RBPs typically function by binding RNA through the conserved GxxG motif in the binding cleft and this motif can be mutated to GDDG to prevent RNA binding but maintain protein stability (Hollingworth et al., 2012). In order to investigate the requirement for RNA binding to StKH17 function, the GxxG motif was mutated to GDDG using site-directed mutagenesis (SDM) to give StKH17GDDG. Firstly, Y2H analysis was used to

Figure 4. KH17 Silencing by VIGS or Stable RNAi Reduces P. infestans Infection while StKH17 Transient or Stable Overexpression Enhances P. infestans Colonization.

(A) Box plot showing lesion diameter is reduced in TRV-KH17-silenced N. benthamiana plants compared to the TRV-GFP control (one-way ANOVA p < 0.001, N = 145). The combined data are shown for six biological replicates comprising ~three leaves from ~four plants per replicate.

(B) Box plot showing the sporangia recovered per milliliter is reduced in TRV-KH17-silenced N. benthamiana plants compared to the TRV-GFP control (ANOVA p < 0.001, N = 216). The combined data are shown for six biological replicates comprising ~three leaves from ~four plants per replicate.

(C) Representative leaf images taken under UV light showing P. infestans lesions on E potato-3 control and transgenic potato RNAi and overexpression (OE) lines.

(D) Box plot showing lesion diameter is reduced in transgenic potato plants silencing StKH17 compared to the E potato-3 control (ANOVA p < 0.001, N = 30). The combined data are shown for three biological replicates comprising ~three leaves from ~five plants per replicate.

(E) Box plot showing the lesion diameter is increased in the halves of N. benthamiana leaves transiently overexpressing GFP-StKH17 compared to those overexpressing free GFP (ANOVA p = 0.006, N = 105). The combined data are shown for three biological replicates comprising ~three leaves from ~six plants per replicate.

(F) Box plot showing the lesion diameter is increased in transgenic potato plants overexpressing StKH17 compared to the E potato-3 control (ANOVA p < 0.001, N = 30). The combined data are shown for three biological replicates comprising ~two leaves from ~five plants per replicate.
That are negative regulators, several substrates have been identified to positively or negatively regulate immunity in plants. Of those, many ubiquitin E3 ligases have been demonstrated to either control the activity of immune-regulating GTPases through targeting the GTPase-activating proteins (GAPs) SPIN6 and Rab4A and guanine nucleotide exchange factor (GEF) SWAP70 for degradation (Antignani et al., 2015; Liu et al., 2015; He et al., 2018). To date, no examples of plant defense-associated RBPs have been identified to be the direct targets of E3 ligases. However, in mammalian systems, the RBP and translational repressor MEX3C contains both a RING-type E3 ligase domain in addition to a KH domain. MEX3C regulates immune responses to viral infection through ubiquitination of receptor RIG1 and the degradation of viral RNA (Kuniyoshi et al., 2014; Yang et al., 2017). The plant E3 ligase SPL11 has been shown to regulate flowering time through ubiquitination of substrate KB RBP SPL11-interacting Protein 1 (SPIN1) indicating that the E3 ligase/RBP combination is a conserved regulatory module (Vega-Sánchez et al., 2008).

E3 ligases that negatively regulate immunity have been shown to target positive immune regulators for degradation by the 26S proteasome; for example, POB1 targets SIPUB17 for 26S degradation (Orosa et al., 2017). Despite no substrates being identified, the hypothesis is that E3 ligases that positively regulate immunity would target negative regulators. Indeed, StKH17 is shown to be a negative regulator of defense to P. infestans as its overexpression expedites pathogen colonization and suppresses Cf4/Avr4-triggered cell death. The model (Figure 7) shows how an E3 ligase cascade regulates specific immune pathways. The E3 ligase POB1 was shown previously to suppress a range of immune responses, including Cf4-mediated cell death. It suppresses Cf4-mediated cell death by targeting the positive regulator PUB17 for degradation (Orosa et al., 2017; Figure 7). In turn, we show in this work that PUB17 targets StKH17 for degradation (Figure 7). Although Phytophthora does not trigger Cf4 cell death directly, several effectors from P. infestans (Avr3a, PexRD2, Pi22926) have been shown to suppress this pathway, suggesting that it is also triggered by an as yet unidentified Phytophthora MAMP (Gilroy et al., 2011; King et al., 2014; Ren et al., 2019).

Another example of an RBP that negatively regulates defense is StKBRP1 (Wang et al., 2015). StKBRP1 behaves as a susceptibility (S) factor that is co-opted and stabilized by the activity of the P. infestans effector Pi04089 in order to suppress immunity and promote conditions favorable for pathogen colonization. The fact that pathogen effectors have evolved the ability to interact with and manipulate RBPs such as StKBRP1, AtGRP7, and PINP1 (Nicaise et al., 2013; Qiao et al., 2015; Wang et al., 2015) suggests that these proteins are key nodes in the immune signaling regulatory network.

It is unknown why SIPUB17 and SIPUB17mut accumulate in the nucleolus. The nucleolus is mostly associated with ribosomal RNA synthesis and ribosome biogenesis, although there is new evidence for roles in growth and development, cell cycle, and stress responses (Kaliniina et al., 2018). However, it is worth noting that approximately 25% of P. infestans RxLR effectors have been shown to have nucleolar localization in the plant cell (Wang et al., 2019), perhaps suggesting that nucleolar

**DISCUSSION**

Many ubiquitin E3 ligases have been demonstrated to either positively or negatively regulate immunity in plants. Of those that are negative regulators, several substrates have been identified to be involved in the regulation of vesicle trafficking through the targeting of GTPase Rab4A, PI4K1/2, and exocyst subunit Exo70B2 for degradation (Stegmann et al., 2012; Antignani et al., 2015). E3 ligases are also known to regulate flowering time through ubiquitination of RBPs for degradation (Antignani et al., 2015; Liu et al., 2015; He et al., 2018). To date, no examples of plant defense-associated RBPs have been identified to be the direct targets of E3 ligases. However, in mammalian systems, the RBP and translational repressor MEX3C contains both a RING-type E3 ligase domain in addition to a KH domain. MEX3C regulates immune responses to viral infection through ubiquitination of receptor RIG1 and the degradation of viral RNA (Kuniyoshi et al., 2014; Yang et al., 2017). The plant E3 ligase SPL11 has been shown to regulate flowering time through ubiquitination of substrate KB RBP SPL11-interacting Protein 1 (SPIN1) indicating that the E3 ligase/RBP combination is a conserved regulatory module (Vega-Sánchez et al., 2008).

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Another example of an RBP that negatively regulates defense is StKBRP1 (Wang et al., 2015). StKBRP1 behaves as a susceptibility (S) factor that is co-opted and stabilized by the activity of the P. infestans effector Pi04089 in order to suppress immunity and promote conditions favorable for pathogen colonization. The fact that pathogen effectors have evolved the ability to interact with and manipulate RBPs such as StKBRP1, AtGRP7, and PINP1 (Nicaise et al., 2013; Qiao et al., 2015; Wang et al., 2015) suggests that these proteins are key nodes in the immune signaling regulatory network.

It is unknown why SIPUB17 and SIPUB17mut accumulate in the nucleolus. The nucleolus is mostly associated with ribosomal RNA synthesis and ribosome biogenesis, although there is new evidence for roles in growth and development, cell cycle, and stress responses (Kaliniina et al., 2018). However, it is worth noting that approximately 25% of P. infestans RxLR effectors have been shown to have nucleolar localization in the plant cell (Wang et al., 2019), perhaps suggesting that nucleolar
Overexpression of either GFP-StKH17 or dominant-negative GFP-StPUB17mut but not GFP-StKH17GDDG mutant is able to significantly reduce cell death triggered by the recognition of C. fulvum Avr4 by Cf4 compared to the overexpression of free GFP (Kruskal-Wallis one-way ANOVA on ranks \( p \leq 0.011, N = 45 \)). The combined data are shown for three biological replicates comprising \( >10 \) \( N. \) benthamiana plants per replicate.

(A) Box plot showing the lesion diameter is increased in the halves of leaves transiently overexpressing GFP-StKH17 compared to those overexpressing free GFP, whereas when the RNA-binding motif is mutated to GDDG there is no increase in colonisation observed for GFP-StKH17GDDG (ANOVA \( p < 0.001, N = 32 \)). The combined data are shown for three biological replicates comprising \( \sim\)three leaves from \( \sim4 \) \( N. \) benthamiana plants per replicate.

(B) Overexpression of either GFP-StKH17 or dominant-negative GFP-StPUB17mut but not GFP-StKH17GDDG mutant is able to significantly reduce cell death triggered by the recognition of C. fulvum Avr4 by Cf4 compared to the overexpression of free GFP (Kruskal-Wallis one-way ANOVA on ranks \( p \leq 0.011, N = 45 \)). The combined data are shown for three biological replicates comprising \( >10 \) \( N. \) benthamiana plants per replicate.

processes are targeted by this pathogen. Nuclear localization is clearly required for the immune activity of both POB1 and StPUB17 as fusion to nuclear export signals (NESs) abolished defense-related phenotypes (He et al., 2015; Orosa et al., 2017), but a need for StPUB17 nucleolar localization is unclear. As both the StPUB17 substrate (StKH17) and regulator (POB1) specifically accumulate in the nucleoplasm, this suggests that StPUB17 action occurs in the nucleoplasm rather than the nucleolus (Figure 2; Orosa et al., 2017).

StKH17 stability is clearly reduced in a proteasome-dependent manner in the presence of StPUB17 (Figure 3; Supplemental Figures 4 and 5). As PUB17 is also reduced in stability in a similar manner, it can be argued that the whole complex appears to be turned over by the proteasome. Indeed, there is evidence that E3 ligase stability can be regulated by ubiquitination in either a substrate-dependent or substrate-independent manner. E3 ligases can self-ubiquitinate or be ubiquitinated in \( \text{in vivo} \) by another ligase (De Bie and Ciechanover, 2011). Autoubiquitination of PUB17 has been demonstrated previously in \( \text{in vitro} \) ubiquitination assays (Yang et al., 2006; He et al., 2015). There was a much smaller reduction in protein stability observed when both StKH17 and StPUB17mut were co-expressed. This is interesting as it has been shown previously in \( \text{in vitro} \) ubiquitination assays that the mutation of PUB17 used in our assays results in a complete loss of E3 ligase activity (Yang et al., 2006). However, PUB E3 ligases are known to be activated by heterodimerizing or homodimerizing via their Ubox domains; auto-ubiquitination is thought to account for the general instability of E3 ligases \( \text{in planta} \) (Trujillo, 2018). The presence of an endogenous WT NbPUB17, either alone or dimerized with the mutant StPUB17mut form, could account for the partial reduction in KH17 when co-expressed with the mutant. It is also possible that the PUB17mut-KH17 complex may be partially targeted for degradation by another E3 ligase, such as POB1, which has previously been shown to regulate StPUB17 levels (Orosa et al., 2017). The fact that the StPUB17 mutant retains the same localization and substrate-binding affinity of the WT StPUB17 allows it to act as an efficient dominant-negative protein.

There are a variety of different protein domains in RBPs that are able to recognize and bind in a sequence-specific manner to RNA; these include the KH domain, RRM, or DEAD box helicase domain (Hentze et al., 2018). The KH domain is thought to recognize up to four nucleotides, with binding occurring through the GxxG motif. Many KH RBPs contain multiple KH domains to improve RNA recognition specificity (Hollingworth et al., 2012). However, STAR-type KH RBPs are able to form dimers to enhance recognition specificity (Feracci et al., 2016). Thus, it is possible that StKH17, which contains a STAR domain, may act as a dimer. Moreover, there is evidence that the binding of RNA itself is crucial for the activity of RBPs. AtGRP7 binds to defense-associated FLS2 and EFR transcripts in order to control their stability, ADP-ribosylation of the RRM by HopU1 abolishes this binding, resulting in heightened susceptibility to pathogens (Fu et al., 2007; Nicaise et al., 2013). In the same way, when the GxxG KH RNA-binding motif in StKH17 was mutated to GDDG to disrupt RNA binding, the resulting StKH17GDDG protein was no longer able to promote \( P. \) infestans colonization or suppress Cf4-triggered cell death.

RBPs are involved in regulating plant defense through the post-transcriptional control of RNA processing, stability, and localization, reviewed in Staiger et al. (2013). This can take many different forms, from control of alternative splicing and nonsense-mediated decay to changes in stability or localization of mRNAs. RBPs such as the argonautes are also involved with small RNA and microRNA generation, targeting, and epigenetic regulation (Staiger et al., 2013). The next steps would be to identify the specific RNAs that are substrates for StKH17 in order to determine its mode of action in the negative regulation of specific immune pathways.

**MATERIALS AND METHODS**

**Plant Materials and Growth**

\( N. \) benthamiana was grown at \( 22^\circ \)C in 16-h days with nights at \( 18^\circ \)C. Light levels were maintained between 200 and 450 W/m\(^2\). Potato plantlets were grown \( \text{in vitro} \) in Murashige and Skoog (MS) medium (4% sucrose and...
PUB17 Targets Negative Regulator KH17

Figure 7. Model Showing the Role of StKH17 in Defense Regulation.

The E3 ligase POB1 is a negative regulator of plant defense and overexpression promotes P. infestans colonization while negatively regulating INF1 and PVX/Rx PCD. POB1 also negatively regulates CH4/Avr4 PCD by targeting the E3 ligase StPUB17 for 26S degradation (Orosa et al., 2017). StPUB17 is a positive regulator of immunity and restricts P. infestans colonization while promoting CH4/Avr4 PCD (He et al., 2015). Here the RNA-binding protein StKH17 is shown to be a negative regulator of plant defense and StKH17 overexpression promotes P. infestans colonization and negatively regulates CH4/Avr4 PCD. StPUB17 positively regulates immunity by targeting the negative regulator StKH17 for 26S proteasome degradation.

0.7% agar), 3-week-old plantlets were transferred to individual pots in a greenhouse at 20–26°C with humidity above 80%.

Cloning

StKH17 was amplified from potato cDNA and attB recombination sites were added using nested PCR; primer sequences are shown in Supplemental Table 1. Gateway entry clones were generated by recombining attB-effector PCR products with pDonr201 and clones were recombined into pB7WGF2 and transferred into recombining attB-effector PCR products with pDonr201 and clones were transformed into yeast to test pairwise interactions.

Y2H

A screen with StPUB17 was carried out using the Invitrogen ProQuest system and yeast strain MaV203. Briefly, DNA-BD “bait” fusions to StPUB17 were generated using Gateway recombination with an entry clone. This was transformed MaV203 cells and recovered using nutritional selection and tested for reporter gene auto-activation. Competent cells were generated for BD-PUB17 and were transformed with a potato DNA AD “prey” Y2H library. Interacting clones were selected based on the reporter gene activity (i.e., ability to grow on media lacking histidine or uracil and gain of β-galactosidase activity). Interacting clones were sequenced. WT and mutant bait and prey clones were then co-transformed into yeast to test pairwise interactions.

P. infestans Growth

P. infestans strain 88069 was grown for 2 weeks at 19°C on Rye agar plates before sporangia were harvested by flooding with sterile distilled water (SDW), scraping with a plastic spreader, and filtering through a 70 µm nylon cell strainer (Coming) to remove hyphae. The resulting suspension was centrifuged at 2750 rpm for 10 minutes and the pellet re-suspended in SDW to 50 000 sporangia per milliliter using a counting chamber.

Agrobacterium-Mediated Transient Infection Assays

Agrobacterium strains GV3101 or AGL1 with STKH17 and STPUB17 WT and mutant constructs were grown in yeast extract and beef (YEBS) media supplemented with the appropriate antibiotics at 28°C overnight. Cultures were centrifuged at 4000 rpm before resuspension in 10 mM 2-[(N-morpholino)ethanesulfonic acid: 10 mM MgCl2 with 200 µM acetylseringone and adjusted to an optical density 600 (OD600) of 0.05 for confocal analysis and 0.5 for western and cell death assays. An OD600 of 0.1 was used for Phytophthora virulence assays where test and control suspensions were infiltrated in two spots on either half of an N. benthamiana leaf (three leaves per plant; six plants per replicate) before being drop inoculated 24 h later with 10 µL of P. infestans inoculum at 50 000 sporangia per milliliter and one-way ANOVA was performed to determine statistically significant differences.

VIGS

VIGS constructs were made by cloning two individual ~170 bp PCR fragments from NbK17 into TRV vectors (Ratcliffe et al., 2001). N. benthamiana is an allotetraploid resulting from the hybridization of two unknown progenitors. It typically contains two similar copies of each gene, one from each parent (Bombarley et al., 2012). Therefore, the VIGS constructs and qPCR primers were designed to knock down and amplify both NbK17 genes (NbhK17a and NbhK17b) respectively. Primer sequences are shown in Supplemental Table 1. A TRV construct expressing GFP was used as a control (He et al., 2015). Agrobacterium tumefaciens strains containing a mixture of RNA1 and each NbK17 VIGS construct at an OD600 of 0.5 were infiltrated into the two leaves of the four-leaf-stage N. benthamiana plant. Systemic leaves were detached, analyzed by qRT–PCR, and used for P. infestans infection 2–3 weeks later. P. infestans lesions were measured at 7 days post inoculation (dpi) and sporangia counts were performed at 10 dpi on samples where three leaves were pooled and sporangia recovered in 3 mL of SDW. Counts were carried out using a cell counter and results were analyzed using one-way ANOVA to determine statistically significant differences.

Quantitative RT–PCR

Total RNA was extracted from the leaves of potato transgenic lines and N. benthamiana VIGS plants using a Qiagen RNeasy plant mini kit according to the manufacturer’s instructions. The cDNA was synthesized using Invitrogen superscript II kit and qRT–PCR was carried out using SYBR green as described previously (McLellan et al., 2013). Primers for real-time PCR are shown in Supplemental Table 1 and gene expression levels were analyzed using the comparative Ct method as described by Livak and Schmittgen, (2001) and Cikos et al. (2007).
Plant Communications

with mRFP-SIPUB17mut. Cells expressing fluorescent protein fusions were observed using a Zeiss 710 confocal microscope no more than 4 days post infiltration using a low OD600 of 0.05. GFP was excited with a 408 nm laser and the emissions were detected between 500 nm and 530 nm. mRFP was excited with a 561 nm laser and emissions detected between 600 nm and 630 nm. On co-expression, fluorophores were imaged sequentially to minimize cross-talk. Images were processed with propriety confocal software.

Cell Death Assay
Agrobacterium strains (expressing INF1 or CI4/Avr4) were co-infiltrated into leaves of N. benthamiana WT plants with free GFP or GFP-KH17, GFP-KH17DD, or GFP-SIPUB17mut. The number of positive HRs (i.e., more than 50% of the inoculated region produces clear cell death) were counted as described previously (Gilroy et al., 2011) and expressed as the mean percentage of total inoculations per plant. The error bars represent ± SEs of combined data from at least three biological replicates. One-way ANOVA was performed to determine statistically significant differences.

Western Blotting
Proteins were transiently overexpressed for 2dpi in N. benthamiana and were tested by western blotting to assess protein presence and stability. Proteins were extracted using GTEN buffer (10% glycerol; 25 mM Tris pH 7.5; 1 mM EDTA; 150 mM NaCl; 1 mM PMSF; 10 mM DTT; 0.5% Nonidet p40; PI inhibitor tablet) then mixed with 2× SDS–PAGE sample buffer and loaded on 12% SDS–PAGE gels. Gels were blotted onto nitrocellulose membrane and Ponceau stained to show loading. Membranes were blocked in 4% milk in 1× PBST (137 mM NaCl; 12 mM phosphate; 2.7 mM KCl; pH 7.4; 0.2% Tween-20) before addition of the primary antibodies: a monoclonal GFAP antibody at 1:2000 dilution (sc9998; Santa Cruz), a monoclonal anti-AMY antibody raised in mouse at 1:500 (SC-40; Santa Cruz), a monoclonal anti-HRP antibody produced in rabbit at 1:4000 (5F8; Chromotek), or a polyclonal ubiquitin antibody produced in rabbit (UBO11; Agrisera). The membrane was washed with 1× PBST (0.2% Tween 20) five times before addition of the secondary antibody at 1:5000 dilution with anti-mouse Ig-HRP antibody (A9044; Sigma-Aldrich), anti-rat Ig-HRP (ab6836; Abcam), or anti-rabbit Ig-HRP antibody (A2875; Sigma-Aldrich), followed by more washing and ECL (Amersham) development according to the manufacturer’s instructions. Relative band intensity was quantified using the Gel Analysis method in ImageJ software.

Phylogenetic Analysis
Protein sequences were obtained for the following genes: NbKH17-like b (Niben101Scf00244g03017.1), At2g38610, AT3G08620, StKH17 (XM_006359919.2), NbKH17a (Niben101Scf08926g07008.1), NbKH17b (Niben101Scf09096g02028.1), NbKH17-like a (Niben101Scf02665g15001.1), NbKH17-like b (Niben101Scf0244g03017.1), A2g38610, AT3G08620, and out-grouper StKRBP1 (PGSC0003DMT400066837). CLUSTALW was used to construct an alignment for the full aa sequence. This alignment was imported into TOPOLi v2.5 and a bayesian phylogenetic tree (MrBayes) was constructed.

SUPPLEMENTAL INFORMATION
Supplemental Information is available at Plant Communications Online.

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AUTHOR CONTRIBUTIONS
H.M., P.R.J.B., and Z.T. conceived and designed the experiments. H.M., K.C., Q.H., X.W., and P.C.B. performed experiments and analyzed data. H.M. and P.R.J.B. wrote the manuscript with input from all authors. P.R.J.B. and Z.T. independently secured funding for the research.

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