

A regulatory module controlling homeostasis of a plant immune kinase

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Plant pattern recognition receptors (PRRs) perceive microbial and endogenous molecular patterns to activate immune signalling. The cytoplasmic kinase BIK1 acts downstream of multiple PRRs as a rate-limiting component, whose phosphorylation and accumulation are central to immune signal propagation. Previous work identified the calcium-dependent protein kinase CPK28 and heterotrimeric G proteins as negative and positive regulators of BIK1 accumulation, respectively. However, mechanisms underlying this regulation remain unknown. Here we show that the plant U-box proteins PUB25 and PUB26 are homologous E3 ligases that mark BIK1 for degradation to negatively regulate immunity. We demonstrate that the heterotrimeric G proteins inhibit PUB25/26 activity to stabilize BIK1, whereas CPK28 specifically phosphorylates conserved residues in PUB25/26 to enhance their E3 ligase activity and promote BIK1 degradation. Interestingly, PUB25/26 specifically target non-activated BIK1, suggesting that activated BIK1 is maintained for immune signalling. Our findings reveal a multi-protein regulatory module that enables robust yet tightly regulated immune responses.

Introduction

Plant pattern recognition receptors (PRRs) are plasma membrane-localized receptor kinases (RKs) or receptor-like proteins (RLPs) that detect a variety of endogenous and microbial molecular patterns generated during pathogen attacks (Couto and Zipfel, 2016; Tang et al., 2017). Many PRRs such as FLS2, EFR, PEPRs, and LYK5 function in complex with co-receptor kinases such as BAK1 and CERK1 (Liebrand et al., 2014; Ma et al., 2016; Zipfel and Oldroyd, 2017). These PRRs and co-receptors directly interact with and activate downstream immune signaling through a class of related receptor-like cytoplasmic kinases (RLCKs) that includes BIK1 and related PBS1-Like (PBLs) proteins (Lu et al., 2010; Zhang et al., 2010). BIK1 and PBLs are of central importance to the plant immune system as they regulate key signaling events, including transient bursts of calcium and reactive oxygen species (ROS) (Kadota et al., 2014; Li et al., 2014; Monaghan et al., 2015; Ranf et al., 2014), increased synthesis of salicylic acid (Kong et al., 2016), and activation of mitogen-activated protein kinases (Yamada et al., 2016). Multiple pathogen virulence effectors target BIK1 and PBLs to inhibit plant immunity (Feng et al., 2012; Liu et al., 2011; Zhang et al., 2010), further highlighting the importance of this family of kinases in plant immunity.

Recent work has demonstrated the importance of BIK1 regulation in immune homeostasis. Firstly, BIK1 phosphorylation, which is prerequisite for BIK1 activation, is subject to negative regulation by the protein phosphatase PP2C38 (Couto et al., 2016). Secondly, BIK1 stability is positively regulated by heterotrimeric G proteins composed of XLG2/XLG3 ($G\alpha$), AGB1 ($G\beta$) and AGG1/AGG2 ($G\gamma$), and negatively regulated by the calcium-dependent protein kinase CPK28 through the ubiquitin proteasome system (Liang et al., 2016; Monaghan et al., 2014). However, the identity of the E3 ubiquitin ligase(s) that target BIK1 and the detailed regulatory mechanisms that control its **stability** remain elusive.

Here we report the identification of two phylogenetically-related plant U-box domain-containing proteins, PUB25 and PUB26, as E3 ligases that target BIK1 for degradation and negatively regulate BIK1-mediated immunity. Both CPK28 and the heterotrimeric G proteins exert their regulations on BIK1 stability through PUB25/26.

Whereas CPK28 phosphorylates PUB25/26 to enhance E3 ligase activity and BIK1 degradation, the heterotrimeric G proteins directly inhibit PUB25/26 E3 ligase activity to stabilize BIK1. We additionally found that PUB25/26 specifically ubiquitinate non-activated BIK1, which is expected to indirectly limit the size of activated BIK1 pool. We propose a model in which PUB25/26, the G proteins, and CPK28 form a signaling module, which not only regulates the total amount, but also the proportion of activated BIK1 protein before and after immune activation, thereby controlling immune homeostasis.

Results

PUB25/26 Are Ubiquitin E3 Ligases That Promote BIK1 Degradation

In an effort to identify proteins interacting with BIK1, we transiently expressed BIK1-FLAG in *Arabidopsis* protoplasts, performed anti-FLAG immunoprecipitation (IP) and analyzed the immune complex composition by LC-MS/MS. One candidate protein identified in BIK1 IPs in two independent experiments, but absent in control IPs lacking a FLAG-tagged protein, was the U-box domain-containing protein PUB25 (Table S1). Split-luciferase complementation assays in *Nicotiana benthamiana* confirmed that PUB25 specifically interacted with BIK1, but not BAK1 (Figure 1A). This association was further confirmed using co-immunoprecipitation (co-IP) in *Arabidopsis* protoplasts (Figure S1A). A close homolog of PUB25, PUB26, similarly interacted with BIK1 (Figures 1B and S1B). Treatment of *N. benthamiana* plants or *Arabidopsis* protoplasts with flg22 greatly reduced the interaction, suggesting ligand-induced dissociation between the protein pairs. Flg22 treatment induces BIK1 phosphorylation and slower migration in SDS-PAGE (Fig. S1A; Lu et al., 2010; Zhang et al., 2010). This phosphorylated BIK1 is referred to as hyper-phosphorylated, whereas the fast migrating BIK1 before flg22 treatment is referred to as under-phosphorylated in the following text. Interestingly, the flg22-induced dissociation was not detected when a kinase-dead BIK1^{K105E}-HA mutant protein was used (Figures S1A and S1B). Unlike wild-type BIK1, BIK1^{K105E} does not undergo hyper-phosphorylation when plants or protoplasts are

treated with flg22 (Zhang et al., 2010), suggesting that the kinase activity or hyper-phosphorylation of BIK1 is required for flg22-induced dissociation. Only the under-phosphorylated BIK1 (Zhang et al., 2010) co-precipitated with PUB25/26, whereas the hyper-phosphorylated form did not (Figures S1A and S1B). Additional co-IP with FLAG-tagged BIK1 and HA-tagged PUB25 (Figure S1C) showed that both hyper- and under-phosphorylated BIK1 were equally pulled-down during immunoprecipitation, indicating that the lack of hyper-phosphorylated BIK1 in Figures S1A and S1B was not because of degradation of the hyper-phosphorylated BIK1 during immunoprecipitation. These data suggest that PUB25/26 **primarily** interact with the under-phosphorylated form of BIK1.

FLS2 is known to associate with additional RLCKs for signaling including PBL1 and BSK1 (Shi et al., 2013; Zhang et al., 2010). Co-IP assays in protoplasts showed that PUB25/26 can also interact with PBL1 and BSK1, and that the interactions were diminished upon flg22 treatment (Fig S1D-E), indicating that PUB25/26 may additionally target multiple RLCKs associated with FLS2.

In vitro ubiquitination assays (Zhao et al., 2012) were performed to determine whether BIK1 is a substrate of PUB25/26. Incubation of recombinant GST-tagged BIK1 with recombinant PUB25-FLAG-HIS, HA-ubiquitin (HA-UBQ), a His-tagged *Arabidopsis* E2 ubiquitin-conjugating enzyme UBC8, and a His-tagged wheat E1 ubiquitin-activating enzyme, led to **production of laddering bands only in the presence of E1, E2 and PUB25 (Figure 1C), indicating poly-ubiquitination of GST-BIK1**. In contrast, a GST-tagged RKS1, an RLCK not involved in FLS2 signaling (Wang et al., 2015), was not ubiquitinated. PUB25-FLAG-HIS was auto-ubiquitinated in both reactions. These data indicate that PUB25 is an E3 ubiquitin ligase and can ubiquitinate BIK1 *in vitro*.

To determine whether PUB25/26 can promote BIK1 degradation *in planta*, we co-expressed PUB25/26-FLAG with BIK1-HA in *Arabidopsis* protoplasts and examined BIK1-HA protein levels in the presence of cycloheximide (CHX), which inhibits *de novo* protein synthesis. Over-expression of PUB25/26-FLAG significantly reduced BIK1-HA abundance (Figure S1F), suggesting that PUB25/26 are capable of promoting BIK1

degradation. The abundance of kinase-dead BIK1^{K105E}-HA was similarly reduced when co-expressed with PUB25/26 (Figure S1F). Treatment of these protoplasts with the proteasome inhibitor MG132 restored BIK1-HA to a level comparable to protoplasts expressing BIK1-HA alone, which is consistent with the hypothesis that PUB25/26 promote BIK1 degradation through the ubiquitin proteasome system.

To further test whether PUB25/26 regulate BIK1 stability in plants, we transformed *Arabidopsis* Col-0 plants with *35S::PUB25-FLAG* or *35S::PUB26-FLAG* to generate multiple independent over-expression (OE) lines, all of which accumulated *PUB25/26* transcripts to high levels (Figure S1G) and developed a distinctive swirling rosette phenotype (Figure 1D), reminiscent of hyperactive brassinosteroid (BR) signaling caused by over-expression of *BRI1* or the BR-biosynthetic gene *DWF4* (Choe et al., 2001; Wang et al., 2001). As BIK1 is known negatively regulate BR signaling (Lin et al., 2013), the phenotype is consistent with a regulation of BIK1 stability. To facilitate detection of the BIK1 protein, we generated antibodies that specifically detected BIK1 in Col-0, but not *bik1* plants (Figure S1H). An examination of the *PUB25-OE1* line showed that its BIK1 protein level (Figure 1E), but not *BIK1* transcripts (Figure S1I), was greatly diminished compared to Col-0 plants, indicating *PUB25* overexpression reduced BIK1 level in a post-transcriptional manner. To further determine the role of PUB25/26 in BIK1 accumulation in plants, we isolated null *pub25* and *pub26* T-DNA insertional mutants and constructed a *pub25 pub26* double mutant (Figures S1J and S1K). All mutants were morphologically similar to Col-0 plants (Figure 1F). While the *BIK1* transcript level was not affected in the *pub25 pub26* double mutant (Figure S1L), the BIK1 protein was accumulated to higher levels in *pub25*, *pub26*, and *pub25 pub26* mutants compared to Col-0 (Figure 1G). In contrast, all plants accumulated similar amounts of FLS2, indicating that PUB25/26 specifically control BIK1 stability, at least in the context of the immune complex. It is interesting to note that the *pub25 pub26* double mutant did not show more BIK1 accumulation than did *pub25* and *pub26* single mutants, suggesting that both PUB25 and PUB26 are required to regulate BIK1 stability.

PUB25/26 Negatively Regulate BIK1-Mediated Immunity

We next tested whether PUB25/26 play a role in pattern-triggered immune response. The flg22-induced ROS burst was significantly elevated in *pub25* and *pub26* single mutants (Figures S2A and S2B), as well as the *pub25 pub26* double mutant compared to Col-0 (Figure 2A). We further inoculated these plants with the fungal pathogen *Botrytis cinerea* and the non-virulent bacterial strain *Pseudomonas syringae* pv. *tomato* (*Pto*) DC3000 *hrcC* to test whether PUB25/26 play a role in regulating disease resistance. We used the *Pto* DC3000 *hrcC* strain instead of the wild-type strain because the latter secretes a variety of effectors to suppress pattern-triggered immunity, which can obscure the analysis of this signaling pathway (Feng and Zhou, 2012; Macho and Zipfel, 2015). Compared to Col-0, *pub25*, *pub26*, and *pub25 pub26* mutants showed smaller lesions when inoculated with *B. cinerea* (Figure 2B) and reduced bacterial populations when inoculated with *Pto* DC3000 *hrcC* (Figure 2C). Because the *pub25 pub26* double mutant largely resembles *pub25* and *pub26* single mutants, these analyses suggest that both PUB25 and PUB26 function in *Arabidopsis* immunity. Comparatively, *PUB25-OE* lines were severely diminished in flg22-triggered ROS burst (Figure 2D) and exhibited increased susceptibility to both *B. cinerea* (Figure 2E) and *Pto* DC3000 *hrcC* (Figure 2F). These results demonstrate a negative role of PUB25/26 in flg22-induced immune responses and disease resistance to both fungal and bacterial pathogens. To determine whether the diminished BIK1 accumulation accounts for the impaired immunity in *PUB25-OE* plants, we introduced a *BIK1-HA* transgene under the control of its native promoter (*NP::BIK1-HA*; Zhang et al., 2010) into the *PUB25-OE1* line by crossing, which is known to increase BIK1 level in the recipient line. As previously reported (Liang et al., 2016; Monaghan et al., 2014), expression of the *NP::BIK1-HA* transgene increased flg22-triggered ROS burst (Figure 2G). While the *PUB25-OE1* line was severely impaired in flg22-triggered ROS burst, introduction of the *NP::BIK1-HA* transgene restored ROS production to wild-type levels (Figure 2G), indicating that the impaired immune response in the *PUB25-OE* line was indeed caused by the diminished BIK1 accumulation. Consistent with this result, the *NP::BIK1-HA* transgene also reduced *B. cinerea* susceptibility

in the *PUB25-OE* line (Figure 2H). Taken together, these findings demonstrated that *PUB25/26* negatively regulate flg22-triggered immune response and disease resistance, likely through controlling BIK1 stability.

***PUB25/26* Specifically Poly-Ubiquitinate Non-Activated BIK1**

The differential interactions between *PUB25/26* with hyper- or under-phosphorylated BIK1 (Figures S1A and S1B) prompted us to test whether these PUBs selectively target under-phosphorylated BIK1 for degradation. Indeed, whereas over-expression of *PUB25* or *PUB26* greatly destabilized BIK1 in CHX-treated protoplasts, it had a mild effect when the protoplasts were additionally treated with flg22 (Figures 3A and 3B). In contrast, the BIK1^{K105E} mutant protein, which does not undergo flg22-induced hyper-phosphorylation, was not stabilized upon flg22 treatment (Figure 3B). BIK1 is known to be phosphorylated at multiple sites following flg22 perception, including Ser236 and Thr237 located in the activation loop that are highly conserved in BIK1 and PBLs and necessary for kinase activation (Lu et al., 2010; Zhang et al., 2010). We generated phospho-dead BIK1^{S236A/T237A} (BIK1^{2A}) and phospho-mimetic BIK1^{S236D/T237D} (BIK1^{2D}) variants to determine whether the phosphorylation of these sites affects *PUB25/26*-BIK1 association and subsequent degradation. We measured the stability of these BIK1 variants co-expressed with *PUB25* in the presence of CHX. Similar to wild-type BIK1, phospho-dead BIK1^{2A} was destabilized by *PUB25* over-expression in protoplasts, whereas phospho-mimetic BIK1^{2D} was largely stable (Figure 3C), supporting that *PUB25* is unable to destabilize BIK1 when S236/T237 are phosphorylated. Co-IP assays showed that BIK1, BIK1^{2A}, and BIK1^{2D} similarly associated with *PUB25* in protoplasts (Figures S3A and S3B), indicating that phosphorylation at these sites did not affect BIK1 association with *PUB25/26*, and suggesting that other phosphosites may be responsible for flg22-induced dissociation. To further rule-out the potential impact of BIK1 degradation on co-IP experiments, we repeated the experiment in the presence of proteasome inhibitor MG132 and found that *PUB26* interacted equally with the BIK1 variants (Figure S3C), indicating that the insensitivity of BIK1^{2D} to *PUB25/26*-mediated degradation

was not simply due to lack of association. We therefore tested whether the phosphorylation of BIK1^{S236/T237} impacts PUB25-mediated poly-ubiquitination by co-expressing wild-type or mutant forms of BIK1-HA with PUB25-FLAG and FLAG-UBQ in protoplasts. The protoplasts were treated with the MG132 to allow accumulation of ubiquitinated proteins, and ubiquitinated BIK1-HA was isolated with anti-FLAG beads. Anti-HA immunoblot detected strong laddering bands indicative of poly-ubiquitination of BIK1-HA and BIK1^{2A}-HA proteins, whereas BIK1^{2D}-HA was much less poly-ubiquitinated (Figure 3D). Together, these results suggest that PUB25/26 associate with BIK1 irrespective of the S236/T237 phosphorylation status, but selectively mark under-phosphorylated BIK1 for ubiquitination and degradation.

CPK28 Acts Together with PUB25/26 to Regulate BIK1 Stability and Immune Responses

The ability of PUB25/26 to negatively regulate BIK1 and immunity is reminiscent of CPK28 (Monaghan et al., 2014), raising a possibility that they act in the same pathway.

Split-luciferase and co-IP assays showed that CPK28 associated with PUB25 and PUB26 regardless of flg22 treatment (Figures 4A, 4B and S4A), suggesting that CPK28 and PUB25/26 act together to control BIK1 stability. A *pub25 pub26 cpk28* triple mutant was generated to further test this possibility. Immunoblot analyses showed that endogenous BIK1 accumulated to a high level in *cpk28* compared to Col-0 (Figure 4C), which is consistent with the previous report (Monaghan et al., 2014). BIK1 accumulation was also increased in *pub25 pub26* plants, however slightly less than that in *cpk28*, and the *pub25 pub26 cpk28* triple mutant showed similar BIK1 levels compared to *cpk28* (Figure 4C). Consistent with this, *cpk28* and *pub25 pub26 cpk28* were similarly enhanced in flg22-induced ROS production (Figure 4D). The lack of an additive effect in the triple mutant supports the possibility that they act in the same pathway to promote BIK1 degradation. As E3 ligases, we reasoned that PUB25/26 may function downstream of CPK28 to regulate BIK1 stability. We therefore tested whether *PUB25* over-expression could reduce the flg22-triggered ROS burst in the

cpk28 mutant. Indeed, introgression of the *35S::PUB25-FLAG* transgene (*PUB25-OE1*) into *cpk28* by crossing significantly reduced flg22-induced ROS production in these plants (Figure 4E). These data support that PUB25/26 act downstream of CPK28 to buffer flg22-triggered immune signaling. Given that *cpk28* accumulated an even higher level of BIK1 and showed much greater flg22-induced ROS burst than did *pub25 pub26* double mutant (Figures 4C and 4D), it is possible that additional PUBs also control BIK1 stability downstream of CPK28. In *Arabidopsis*, three additional PUBs, PUB22, PUB23, and PUB24, are related to PUB25/26 in sequence and have been shown to negatively regulate pattern-triggered immunity (Trujillo et al., 2008). Co-expressing PUB22/23 strongly destabilized BIK1 in protoplasts (Figure S4B and S4C), suggesting that these E3 ligases also play a role in BIK1 **degradation**.

CPK28 can phosphorylate BIK1 *in vitro*, although the phosphorylation sites remain to be determined (Monaghan et al., 2014). In the absence of flg22 perception, CPK28 negatively regulates BIK1 stability. We first sought to determine whether BIK1 S236/T237 contribute to overall phosphorylation by CPK28 *in vitro*. Incubation with CPK28 resulted in phosphorylation of recombinant BIK1^{K105E} protein (Figure S4D). Introduction of S236A/T237A mutations into BIK1^{K105E} (BIK1^{K105E/2A}) did not impact overall phosphorylation by CPK28, indicating that S236/T237 are not major phosphosites for CPK28. Co-IP assays showed that the BIK1-CPK28 interaction was not affected by BIK1^{2A} and BIK1^{2D} mutations (Figure S4E), further suggesting that S236/T237 do not impact the regulation of BIK1 by CPK28. Furthermore, the BIK1-PUB25 interaction occurred similarly in *cpk28* mutant and CPK28-OE protoplasts (Figure S4F). Overall, these findings are consistent with previous findings that S236/T237 are phosphorylated by BAK1 upon flg22 perception (Lu et al., 2010; Feng et al., 2012), and that CPK28 does not affect BIK1-PUB25 interaction.

CPK28 Phosphorylates PUB25/26 to Enhance E3 Ligase Activity and BIK1 Degradation

Throughout our work, we found that PUB25/26-FLAG existed in slow and fast migrating

forms, and that treatment with flg22 often resulted in an increased amount of the slow migrating form (Figures S1A and S1B). Incubation of the protein samples with a protein phosphatase eliminated the slow migrating forms (Figures S5A and S5B), indicating that PUB25/26 are phosphorylated following flg22 perception. We affinity-purified PUB26-FLAG from flg22-treated protoplasts and conducted LC-MS/MS analysis to identify phospho-peptides. In total, eight phospho-sites in PUB26-FLAG were identified, including one in the U-box domain (Figure 5A), which is the E3 ligase catalytic domain (Cyr et al., 2002). Strikingly, seven phospho-sites are conserved in PUB25 (Figure 5A). Site-directed mutagenesis was used to substitute each of these residues to alanine. The T94A mutation, but not T407A and S63A mutations, specifically abolished the flg22-induced band shift in PUB26 (Figure S5C). Mutation of the corresponding site in PUB25, T95A, similarly eliminated flg22-induced phosphorylation (Figure S5H). These results indicated that T95/94 are the main flg22-induced phospho-sites in PUB25/26, respectively. It is interesting to note that this threonine is in a typical CPK phosphorylation motif ϕ XXXXTXB, where ϕ is a hydrophobic residue, X is any residue, T is threonine, B is a basic residue (Huang et al., 2001). This motif is also conserved in the related E3 ligases PUB22/23/24.

To test whether CPK28 and BIK1 are able to phosphorylate PUB25^{T95}/26^{T94}, we raised anti-phosphopeptide antibodies that specifically recognize an identical phospho-peptide spanning PUB25^{T95}/26^{T94} (Figure 5A; anti-pT94/95 antibodies). Immunoblot analyses detected strong phosphorylation of GST-PUB25/26 recombinant proteins at this site when incubated with GST-CPK28, but not HIS-BIK1 (Figures 5B and S5D). Importantly, the PUB25^{T95A}/26^{T94A} variants were not phosphorylated. Addition of the Ca²⁺ chelator EGTA to the reaction completely eliminated PUB26^{T94} phosphorylation (Figure S5E), a result consistent with Ca²⁺ dependency of CPK28 (Matschi et al., 2012; Bender et al., 2017). These data indicate that CPK28, but not BIK1, phosphorylates PUB25^{T95}/26^{T94}.

Anti-pT94/95 antibodies detected a strong phosphorylation on PUB25/26-FLAG affinity-purified from flg22-treated Col-0 protoplasts (Figures 5C and S5F), indicating a flg22-induced phosphorylation of PUB25^{T95}/26^{T94} in the plant cell. This phosphorylation was

reduced to ~40% for PUB25 and ~50% for PUB26 in *cpk28* protoplasts, whereas transfection of *cpk28* protoplasts restored the flg22-induced phosphorylation (Figures 5C and S5F), demonstrating that CPK28 is responsible for at least half phosphorylation *in vivo*. The flg22-induced PUB25^{T95} phosphorylation suggests an induction of CPK28 activity. Indeed, the CPK28-FLAG protein reproducibly displayed a slight flg22-induced mobility shift in SDS-PAGE which was eliminated upon phosphatase treatment (Figure S5G). To further test whether flg22 enhances CPK28 kinase activity, we transiently expressed CPK28-FLAG in *N. benthamiana* plants. CPK28-FLAG isolated from the flg22-treated leaves phosphorylated the recombinant GST-PUB26 protein more strongly *in vitro* compared to H₂O-treatment (Figure 5D), demonstrating that flg22 treatment indeed enhances CPK28 kinase activity in plants.

We next asked whether PUB25^{T95}/26^{T94} phosphorylation affects their function. Co-IP assays showed that PUB25^{T95A}/26^{T94A}, PUB25^{T95D}/26^{T94D}, and wild-type PUB25/26 associated similarly with BIK1 (Figures S5H and S5I), suggesting that phosphorylation at this site does not affect PUB25/26-BIK1 association. *In vitro* ubiquitination assays showed that auto-ubiquitination of PUB25^{T95A} and PUB25^{T95D} was notably decreased and enhanced, respectively (Figure 5E), suggesting that phosphorylation of PUB25^{T95} (and presumably PUB26^{T94}) is required for optimal E3 ligase activity. We further co-expressed PUB25-FLAG variants with BIK1-HA and FLAG-UBQ in protoplasts followed by MG132 treatment. Anti-HA immunoblot analyses showed that PUB25^{T95A} was much less capable of inducing BIK1 laddering compared to wild-type PUB25, whereas PUB25^{T95E} enhanced BIK1 laddering (Figure 5F). These results clearly demonstrated that the T95 phosphorylation enhances PUB25 E3 ligase activity and promotes BIK1 poly-ubiquitination. To determine whether PUB25^{T95} phosphorylation promotes BIK1 degradation, a BIK1-LUC translational fusion reporter construct was generated, so that luciferase activity could be used as an indication of BIK1 protein level. Transient expression of this reporter construct along with PUB25-FLAG or PUB25^{T95D}-FLAG in *N. benthamiana* plants strongly reduced luciferase activity, a result indicative of BIK1 degradation caused by PUB25 (Figure 5G). Transient expression of PUB25^{T95A} also resulted in a reduction of luciferase activity compared to the control, but it

was less pronounced compared to plants expressing the wild-type PUB25 (Figure 5G), indicating that the phosphorylation is required for optimum function of PUB25. Together, these results demonstrated that CPK28-mediated phosphorylation on PUB25^{T95}/26^{T94} promotes BIK1 degradation.

Since PUB25/26 target under-phosphorylated BIK1, we tested the impacts of the *cpk28* mutation on the removal of non-activated BIK1 and accumulation of activated BIK1 following flg22 treatment. In Col-0 protoplasts, the levels of under-phosphorylated BIK1 rapidly decreased upon flg22 treatment and were reduced to ~13% within 20 min, whereas the hyper-phosphorylated BIK1 remained relatively steady between 10-40 min following flg22 treatment (Figures S5J and S5K). In contrast, the levels of under-phosphorylated BIK1 decreased more slowly in *cpk28* protoplasts. Accordingly, the hyper-phosphorylated BIK1 accumulated to higher levels than that in Col-0 protoplasts. The results suggested that CPK28 actively promotes depletion of under-phosphorylated BIK1, which in turn restricts the amount of hyper-phosphorylated BIK1 following flg22 perception.

Heterotrimeric G Proteins Directly Inhibit PUB25/26 E3 Ligase Activity

Heterotrimeric G proteins composed of XLG2/XLG3 (G α), AGB1 (G β), and AGG1/AGG2 (G γ) positively regulate BIK1 stability by attenuating proteasome-dependent degradation of BIK1 (Liang et al., 2016). Split-luciferase and co-IP assays showed that PUB25/26 strongly associate with XLG2 and AGB1 irrespective of flg22 elicitation (Figures 6A-B, S6A-D), indicating that PUB25/26 and heterotrimeric G proteins may act in the same pathway to regulate BIK1 stability. To test this, we generated *pub25 xlg2 xlg3* and *pub25 pub26 agb1* triple mutants. Consistent with previous findings (Liang et al., 2016), *agb1* and *xlg2 xlg3* plants accumulated less BIK1 compared to Col-0 plants (Figures 6C and 6D). The triple mutants were largely restored in BIK1 accumulation, indicating that PUB25/26 act downstream of the heterotrimeric G proteins.

PUB25-FLAG, FLAG-UBQ, and BIK1-HA were co-expressed in *agb1* and *xlg2 xlg3*

protoplasts to test whether the G proteins regulate PUB25-mediated ubiquitination of BIK1. Anti-HA immunoblot detected enhanced laddering signal in BIK1-HA isolated from *agb1* and *xlg2 xlg3* mutants compared to Col-0 protoplasts (Figure 6E). Conversely, overexpression of HA-tagged heterotrimeric G proteins along with PUB26-HA and BIK1-FLAG in Col-0 protoplasts strongly diminished BIK1 laddering signal (Figure 6F). Together these results indicate that the heterotrimeric G proteins negatively regulate PUB25/26-mediated ubiquitination of BIK1.

Co-IP experiment showed that the PUB26 and BIK1 interacted normally in the *agb1* mutant protoplasts (Figure S6E), suggesting that the G proteins regulate BIK1 ubiquitination independent of PUB26-BIK1 interaction. We next performed *in vitro* ubiquitination assays to test whether the G proteins directly inhibit PUB25 E3 ligase activity. Because the full-length XLG2 is membrane-associated and difficult to express in *E. coli*, we purified a HIS-tagged C-terminal half of XLG2 (XLG2CT) recombinant protein containing the G α domain. We also purified GST-tagged full-length AGB1 and AGG2 recombinant proteins. Addition of the G proteins, but not BSA, to the reaction greatly reduced PUB25 auto-ubiquitination *in vitro* (Figure 6G), demonstrating that the heterotrimeric G proteins can directly inhibit PUB25 E3 ligase activity. Together, these results demonstrate that the heterotrimeric G proteins directly inhibit E3 ligase activity of PUB25/26, resulting in reduced poly-ubiquitination and increased stability of BIK1.

Discussion

Overall, our results demonstrate that PUB25/26 negatively regulate plant immunity by ubiquitinating and promoting degradation of BIK1 (Figure S7). We also show that PUB25/26 are subject to positive and negative regulation by CPK28 and heterotrimeric G proteins, respectively. Thus PUB25/26, CPK28, and the heterotrimeric G proteins form a signaling module to control BIK1 accumulation and immune homeostasis.

Phosphorylation is known to regulate E3 ligases (Vierstra, 2003). We found that

PUB25/26 possess basal E3 ligase activity and catalyzes poly-ubiquitination on BIK1. This activity is enhanced upon phosphorylation on T95/94, a site adjacent to the U-box domain. It is conceivable that this residue may regulate E3 ligase activity through a phosphorylation-induced conformational change in the U-box domain (Buetow et al., 2016; Caulfield et al., 2014; Dou et al., 2012). Consistently, a phospho-dead mutation of this site attenuated PUB25-mediated BIK1 degradation in *N. benthamiana*.

Several lines of evidence support that CPK28 acts together with PUB25/26 to regulate BIK1 stability and immunity. CPK28 interacts with and directly phosphorylates PUB25^{T95}/26^{T94}. The *cpk28 pub25 pub26* triple mutant and the *cpk28* single mutant are near identical in terms of BIK1 protein accumulation and flg22-induced ROS production, indicating that CPK28 and PUB25/26 act in the same pathway. The related E3 ligases PUB22/PUB23/PUB24 may also play a role in destabilizing BIK1 downstream of CPK28, as we show that they contain the conserved CPK phosphorylation site and that PUB22/23 can destabilize BIK1. Indeed, PUB22/PUB23/PUB24 are known to negatively regulate immunity (Trujillo et al., 2008). These findings explain greater amounts of BIK1 level and stronger immune responses in *cpk28* plants than that in *pub25 pub26* plants.

The *cpk28* mutant constitutively accumulates higher levels of BIK1 protein (Monaghan et al., 2014), suggesting that CPK28 regulates BIK1 stability in the absence of pattern recognition. A recent report showed that a recombinant, phosphorylated form of CPK28 is already fully activated *in vitro* at low Ca²⁺ concentrations (Bender et al., 2017). Nonetheless, our results suggest that the flg22-induced phosphorylation of PUB25^{T95}/26^{T94} is partially mediated by CPK28. Although previous in-gel kinase assays failed to detect an altered CPK28 auto-phosphorylation after flg22 treatment (Monaghan et al., 2015), the CPK28 mobility shift assay in this study suggests a slight flg22-induced phosphorylation of CPK28 (Figure S5G). Furthermore, CPK28 displays stronger kinase activity upon flg22 treatment (Figures 5D). Understanding the mechanism of CPK28 activation is an interesting area of future research.

Our analyses of PUB25/26 further uncovered two mechanisms through which BIK1

stability is actively maintained both before and after pattern recognition (Figure S7). Firstly, the E3 ligase activity of PUB25/26 is subject to negative regulation through association with heterotrimeric G proteins, explaining our previous finding that they positively regulate BIK1 stability and pattern-triggered immunity (Liang et al., 2016). Secondly, PUB25/26 selectively target under-phosphorylated BIK1 for degradation. Flg22-induced S236/T237 phosphorylation in the activation loop protects BIK1 from the PUB25/26-mediated poly-ubiquitination and degradation. This phosphorylation may induce a conformational change in BIK1 that masks the poly-ubiquitination site, a possibility **that** awaits structural studies. Nonetheless, this mechanism contrasts the reported degradation of FLS2 by PUB12/13, which target the activated FLS2 for degradation (Lu et al., 2011), but is similar to the SINAT E3 ligase-mediated ubiquitination and degradation of dephosphorylated BES1 in BR signaling (Yang et al., 2017). Activation of the FLS2-BIK1 complex sets into motion immune signaling cascades that are required to protect plant cells from infection. To prevent excessive signaling and maintain homeostasis, we propose that any under-phosphorylated BIK1 is degraded by PUB25/26 to deplete the pool of signal-competent BIK1.

Regulated **degradation** of key signaling components allows plants to respond to environmental signals with speed and robustness, but with controlled duration and amplitude. **This** can be achieved by recruitment of E3 ligases and is often described as an ‘on-off’ switch (Ding et al., 2015a; Ding et al., 2015b; Dong et al., 2017; Lu et al., 2011; Ni et al., 2014; Spoel et al., 2009; Xu et al., 2015). Our findings show how PUB25/26-mediated **degradation** of BIK1 is dynamically controlled by multiple mechanisms, as illustrated in our working model (Figure S7). In the resting state, BIK1 exists primarily in an under-phosphorylated pool. The countering activities of CPK28 and heterotrimeric G proteins on PUB25/26 maintain BIK1 homeostasis, which ensures robust yet appropriate immune activation upon pathogen attack. Following activation by flg22, the FLS2 complex phosphorylates BIK1 to form an activated pool. Flg22 treatment additionally induces dissociation of the G proteins from the FLS2-BIK1 complex (Liang et al., 2016) and increased phosphorylation of PUB25/26 by CPK28. These are expected to rapidly deplete the under-phosphorylated pool and prevent

further accumulation of activated BIK1, thereby controlling the amplitude of immune responses. Thus, heterotrimeric G proteins, CPK28, and PUB25/26 form a regulatory module for BIK1 accumulation that is crucial for plant immune signaling homeostasis.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- CONTACT FOR REAGENT AND RESOURCE SHARING
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
 - Plant Materials and Growth Conditions
- METHOD DETAILS
 - Constructs and Transgenic Plants
 - Oxidative Burst Assay
 - Pathogen Inoculation and Disease Resistance Assays
 - Split-Luciferase Assay
 - Co-Immunoprecipitation Assay
 - *In Vitro* Ubiquitination Assay
 - Ubiquitination Assay in Protoplasts
 - BIK1 Protein Stability in Protoplasts
 - Protein Degradation Assay in *N. benthamiana* Plants
 - LC-MS/MS Analysis
 - Generation of Antibodies
 - *In Vitro* Phosphorylation Assays
 - Statistical Analysis

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and three tables can be found with this article online at [...](#)

AUTHOR CONTRIBUTIONS

J.W., J.M. and J.M.Z. designed the study. J.W., L.G., J.W., X.L., L.L., C.G., M.M., F.F., M.L., L.L., X.Z., and F.Y. performed the experiments. J.W., Q.X., S.C., J.M., and J.M.Z. analyzed the data. J.W., C.Z., J.M., and J.Z. wrote the paper.

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Figure Legends

Figure 1. PUB25/26 Interact with and Ubiquitinate BIK1.

(A and B) BIK1 interacts with PUB25/26 in *N. benthamiana*. Split-luciferase assays were conducted with the indicated constructs in the presence or absence of 1 μ M flg22 treatment. BAK1-HA-Nluc and Cluc-CPR5 were used as negative controls. Values represent mean relative luminescence units (RLU) \pm SDs (n = 8). Different letters indicate significant difference at p<0.05.

(C) PUB25 ubiquitinates BIK1. *In vitro* ubiquitination assays were carried out with the indicated recombinant proteins, and ubiquitination of GST-BIK1/RKS1 and **PUB25-FLAG-HIS** was detected by immunoblot using anti-GST and anti-HIS antibodies, respectively.

(D) Rosette morphology of independent *PUB25/26*-over-expression (OE) lines 5 w after germination grown in short-day conditions.

(E) Over-expression of *PUB25-FLAG* reduces BIK1 abundance in plants. Col-0 and the *PUB25-OE1* line were examined by immunoblot using anti-BIK1 antibodies. Anti-FLAG immunoblot confirmed the presence of PUB25-FLAG, and Ponceau S staining of Rubisco confirmed equal loading.

(F) Rosette morphology of *pub25*, *pub26* and *pub25 pub26* (*pub25/26*) double mutant plants alongside Col-0 at 5 w post-germination grown in short-day conditions.

(G) Mutations in *PUB25/26* increase BIK1 stability in plants. BIK1 levels in plants of the indicated genotypes were detected by immunoblot using anti-BIK1 antibodies. Accumulation of FLS2 and ACTIN was detected using anti-FLS2 and anti-ACTIN antibodies. Ponceau S staining of Rubisco is included as a loading control. Numbers indicate arbitrary units of BIK1 calculated from densitometry measurements normalized to total Rubisco protein.

The experiments were repeated at least twice (A, B) or three times (C, E, G) with similar results.

Figure 2. PUB25/26 Negatively Regulate Plant Immunity by Controlling BIK1 Levels.

(A-C) *pub25*, *pub26* and *pub25/26* mutants display enhanced ROS burst in response to flg22

(A), increased disease resistance to *B. cinerea* (B), and elevated resistance to *Pto* DC3000 *hrcC*.

(D-F) *PUB25-OE* lines are compromised in flg22-induced ROS burst (D), disease resistance to *B. cinerea* (E), and resistance to *Pto* DC3000 *hrcC* (F).

(G and H) Introduction of a transgenic copy of *PUB25* (*35S:PUB25-FLAG*) into *BIK1-OE* line through crossing suppresses restores flg22-induced ROS production (G) and disease resistance to *B. cinerea* (H). **Note that the accumulation of BIK1-HA is reduced in the presence of *PUB25-OE1* background (G).**

For ROS burst (A, D and G), leaves of the indicated genotypes were induced with 1 μ M flg22, and relative amounts of H₂O₂ (expressed as relative luminescence units) were measured immediately. Values are means \pm SDs (n = 8).

For disease resistance to *B. cinerea* (B, E and H), each leaf of the indicated genotype was inoculated with a droplet of ~2,500 spores of *B. cinerea*, and lesion size was measured at the indicated time. Values are means \pm SDs (n > 12). Photographs of representative infected leaves are shown under the histogram.

For resistance to *Pto* DC3000 *hrcC* (C and F), plants were spray-inoculated with a suspension of *Pto* DC3000 *hrcC*, and bacterial growth was assessed 3 d later. Values are means of colony forming units per leaf area (CFU/cm²) \pm SDs (n = 12).

Different letters indicate significant differences at p < 0.05. Each experiment was repeated three times with similar results. *pub25* and *pub26* refer to *pub25-1* and *pub26-1*, respectively.

Figure 3. PUB25/26 Specifically Mark Non-Activated BIK1 for Degradation.

(A and B) Flg22 treatment stabilizes BIK1-HA in *Arabidopsis* protoplasts over-expressing *PUB25-FLAG* (A) and *PUB26-FLAG* (B). Protoplasts expressing the indicated proteins were treated with 50 μ M cycloheximide (CHX) and 1 μ M flg22 for 2 h before total protein was examined with immunoblot. Ponceau S indicates equal loading. Numbers indicate arbitrary units of BIK1-HA calculated from densitometry measurements normalized to total Rubisco protein.

(C) *PUB25* destabilizes wild-type and phospho-dead forms BIK1, but not a phospho-mimetic

variant. Col-0 protoplasts co-expressing PUB25-FLAG with wild-type BIK1-HA, phospho-dead BIK1^{2A}-HA (BIK1^{S236A/T237A}), or phospho-mimetic BIK1^{2D}-HA (BIK1^{S236D/T237D}) were treated with 50 μ M cycloheximide (CHX) for 2 h before immunoblot analysis.

(D) BIK1^{2D} showed much less poly-ubiquitination promoted by PUB25 compared to that in BIK1 and BIK1^{2A} proteins in protoplasts. Col-0 protoplasts transfected with FLAG-UBQ, PUB25-FLAG, and BIK1-HA variants were incubated overnight in the presence of 5 μ M MG132. Ubiquitinated BIK1-HA was isolated with anti-FLAG beads, and BIK1 ubiquitination was detected with an anti-HA antibody.

Numbers indicate arbitrary units of BIK1 calculated from densitometry measurements normalized to total Rubisco protein (A-C).

Each experiment was repeated three times with similar results.

Figure 4. CPK28 Acts Together with PUB25/26 to Prevent Over-Accumulation of BIK1 and Hyper-Activation of Immunity.

(A and B) CPK28 interacts with PUB25 (A) and PUB26 (B) in plants. Split-luciferase assays were conducted with the indicated constructs in *N. benthamiana* with or without 100 nM flg22 treatment. Values are means \pm SDs (n = 12).

(C) *cpk28* and *pub25 pub26 cpk28* triple mutants show over-accumulation of BIK1. BIK1 protein was detected by immunoblot using anti-BIK1 antibodies. Ponceau S staining of Rubisco indicates loading control. Numbers indicate arbitrary units of BIK1 calculated from densitometry measurements normalized to total Rubisco protein.

(D) *pub25 pub26*, *pub25 pub26 cpk28*, and *cpk28* mutants display elevated ROS burst in response to flg22. Values are mean relative light units \pm SDs (n = 8).

(E) Over-expression of *PUB25* (*PUB25-OE1*) in *cpk28* restores wild-type levels of flg22-induced ROS burst. Values are mean relative light units \pm SDs (n = 12). Different letters indicate significant differences at $p < 0.05$.

Each experiment was repeated at least three times with similar results.

Figure 5. CPK28 Phosphorylates PUB25/26 to Enhance BIK1 Turn-Over and Dampen

Immunity.

(A) Schematic diagram of PUB26 phosphosites as listed in Table S2. The PUB26-FLAG protein was affinity-purified from Col-0 protoplasts pretreated with 1 μ M flg22 and subjected to phosphopeptide identification by LC-MS/MS. The identified phosphosites were aligned to PUB22, PUB23, PUB24 and PUB25 amino acid sequences and shown below the schematic diagram. The underlined sequence was used to generate anti-pT94/95 antibodies.

ϕ XXXXTXB denotes a predicted CPK phosphorylation motif, where ϕ represents a hydrophobic residue, X, B and T represent any residue, basic residue, and Threonine, respectively.

(B) CPK28, but not BIK1, phosphorylates PUB25^{T95}. Kinase assays were performed *in vitro* by incubating GST-PUB25 or GST-PUB25^{T95A} with HIS-BIK1, HIS-BIK1^{K105E} and GST-CPK28. Protein phosphorylation was detected by anti-pT94/95 immunoblots. The fast-migrating band across all lanes was caused by background signal.

(C) Flg22 induces PUB25^{T95} phosphorylation in a manner partially dependent on CPK28. Protoplasts of the indicated genotypes were transfected with the indicated constructs, treated with or without 1 μ M flg22 for 10 min before total protein was extracted. PUB25-FLAG protein was enriched by anti-FLAG affinity purification, and PUB25^{T95} phosphorylation was detected by immunoblot using anti-pT94/95 antibodies (upper panel). Total PUB25-FLAG and CPK28-HA in the input were detected by anti-FLAG and anti-HA immunoblot, respectively (lower panels). Values indicate relative amounts of phosphorylated protein normalized to total PUB25-FLAG. Ponceau S staining of Rubisco is included as a loading control. Numbers indicate arbitrary units of PUB25^{T95} phosphorylation calculated from densitometry measurements normalized to total PUB25-FLAG protein.

(D) Flg22 enhances CPK28 kinase activity in plants. *N. benthamiana* plants transiently expressing CPK28-FLAG were treated with flg22 or H₂O 20 min prior to protein isolation. CPK28-FLAG was then affinity-purified and incubated with the recombinant GST-PUB26 protein in the *in vitro* kinase buffer. PUB26 phosphorylation was detected by anti-pT94/95 immunoblots.

(E) T95 phosphorylation enhances PUB25 E3 ligase activity. *In vitro* ubiquitination assays

were performed with the indicated recombinant proteins, and PUB25 auto-ubiquitination was detected with immunoblot using an anti-HA antibody.

(F) T95 phosphorylation promotes BIK1 poly-ubiquitination. Col-0 protoplasts transfected with the indicated constructs were incubated overnight in the presence of 5 μ M MG132. Ubiquitinated BIK1-HA was affinity-purified with anti-FLAG beads and detected by anti-HA immunoblot.

(G) PUB25^{T95} phosphorylation positively regulates BIK1 degradation. A BIK1-LUC translational fusion construct was generated so that luciferase activity can be used as an indication of BIK1 protein level. The indicated constructs were transiently expressed in *N. benthamiana* plants, and luciferase activity was measured. Values are mean relative light units (RLU) \pm SDs (n = 8). Different letters indicate significant difference at p < 0.05.

Each experiment was repeated twice (B, D) or at least three times (C, E-G) with similar results.

Figure 6. Heterotrimeric G Proteins Stabilize BIK1 by Directly Inhibiting PUB25 E3 Ligase Activity.

(A and B) PUB25 interacts with AGB1 (A) and XLG2 (B) in plants. Split-luciferase assays were performed in *N. benthamiana* with the indicated constructs with or without 1 μ M flg22 treatment. Values are means of relative light units (RLU) \pm SDs (n = 8). Different letters indicate significant difference at p<0.05.

(C and D) *pub25* and *pub26* mutations restore BIK1 accumulation in *agb1* (C) and *xlg2 xlg3* mutants (D). BIK1 levels in plants of the indicated genotypes were determined by immunoblot using anti-BIK1 antibodies; Rubisco is included as a loading control. Numbers indicate arbitrary units of BIK1 calculated from densitometry measurements normalized to the Rubisco protein.

(E) Heterotrimeric G protein mutants display increased BIK1 poly-ubiquitination in protoplasts. The indicated constructs were expressed in protoplasts derived from Col-0, *agb1* or *xlg2 xlg3* mutants, incubated overnight in the presence of 5 μ M MG132. Ubiquitinated BIK1-HA was purified with anti-FLAG beads. BIK1 ubiquitination was determined with

anti-HA immunoblot. Ponceau-stained Rubisco is included as a loading control. .

(F) Overexpression of heterotrimeric G proteins inhibit BIK1 poly-ubiquitination in protoplasts. The indicated constructs were expressed in Col-0 protoplasts and incubated overnight in the presence of 5 μ M MG132. Total BIK1-FLAG was affinity purified with anti-FLAG beads. BIK1 ubiquitination was determined with anti-FLAG (F) immunoblot, respectively. Ponceau-stained Rubisco is included as a loading control.

(G) Recombinant heterotrimeric G proteins directly inhibit PUB25 auto-ubiquitination. *In vitro* ubiquitination assays were performed with the indicated recombinant proteins. BSA was added as a negative control. PUB25 auto-ubiquitination was determined by anti-HA immunoblot.

Each experiment was repeated at least twice (A-D) or three times (E-G) with similar results.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-GST	TIANGEN	Cat#AB101-02
Mouse monoclonal anti-HA	CWBIO	Cat#CW0092M
Mouse monoclonal anti-HIS	Sigma	Cat#H1029
Rabbit polyclonal anti-ACTIN	EASYBIO	Cat#BE0027-100
Rabbit polyclonal anti-BIK1	This paper	N/A
Rabbit polyclonal anti-FLS2	Zhang et al. (2010)	N/A
Rabbit polyclonal anti-pT94/T95	This paper	N/A
Bacterial and Fungal Strains		
<i>Agrobacterium tumefaciens</i> GV3101	Holsters et al. (1980)	N/A
<i>Botrytis cinerea</i>	Institute of Plant Protection, Chinese Academy of Agricultural Sciences	N/A
<i>Escherichia coli</i> BL21	TransGen Biotech	Cat#CD901-03
<i>Escherichia coli</i> DH5 α	CWBIO	Cat#CW0808S
<i>Pseudomonas syringae</i> pv <i>tomato</i> (Pto) DC3000 <i>hrcC</i> -	Yuan and He, (1996)	N/A
Chemicals, Peptides, and Recombinant Proteins		
3 \times FLAG peptide	Sigma	Cat#F4799
Anti-Flag M2 Beads (affinity gel)	Sigma	Cat#A2220
Cycloheximide (CHX)	Sigma-Aldrich	Cat#R750107
D-Luciferin	BioVision	Cat#7903
Flg22 peptides	Sangon Biotech	Custom order
Glutathione Sepharose™4 (affinity gel)	GE Healthcare	Cat#17-0756-01
HA-Ubiquitin (HA-UBQ)	R&D Systems	Cat#U-110
Luminol	Fluka	Cat#09253
MG132	Sigma	Cat#C2211
Ni-NTA Agarose (affinity gel)	QIAGEN	Cat#30210
Peroxidase	Sigma	Cat#P6782
PPase	NEB	Cat#P0753L
Protease Inhibitor Cocktail Tablets	Roche	Cat#04693116001
Critical Commercial Assays		
Gateway LR Clonase II Enzyme Mix	Invitrogen	Cat#11791100
pENTR™/D-TOPO® Cloning Kit	Invitrogen	Cat#K240020SP
Experimental Models: Organisms/Strains		

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<i>Arabidopsis: 35S:PUB25-FLAG (PUB25-OE)</i>	This paper	N/A
<i>Arabidopsis: 35S:PUB25-FLAG-1 pBIK1::BIK1-HA</i>	This paper	N/A
<i>Arabidopsis: 35S:PUB26-FLAG (PUB26-OE)</i>	This paper	N/A
<i>Arabidopsis: agb1-2</i>	Ullah et al. (2003)	N/A
<i>Arabidopsis: cpk28 35S:PUB25-FLAG-1</i>	This paper	N/A
<i>Arabidopsis: cpk28-1 (GK-523B08)</i>	Monaghan et al. (2014)	N/A
<i>Arabidopsis: pBIK1::BIK1-HA</i>	Zhang et al. (2010)	N/A
<i>Arabidopsis: pub25-1 (SALK_147032)</i>	NASC	N/A
<i>Arabidopsis: pub25-1 pub26-1</i>	This paper	N/A
<i>Arabidopsis: pub25-1 pub26-1 agb1-2</i>	This paper	N/A
<i>Arabidopsis: pub25-1 pub26-1 cpk28-1</i>	This paper	N/A
<i>Arabidopsis: pub25-1 xlg2-1 xlg3-1</i>	This paper	N/A
<i>Arabidopsis: pub26-1 (GABI_308D07)</i>	NASC	N/A
<i>Arabidopsis: pub26-2 (GABI_270G01)</i>	NASC	N/A
<i>Arabidopsis: xlg2-1 xlg3-1</i>	Ding et al. (2008)	N/A
Oligonucleotides		
Primers see Table S3	Sangon Biotech	Custom order
Recombinant DNA		
pET28a	EMD Biosciences	69864-3
pGEX 6P-1	Amersham Biosciences	27-4597-01
pENTR	Invitrogen	K2400-20
pFAST-G01	Funakashi	IN3-VEC31
pCAMBIA1300	The Centre for Application of Molecular Biology to International Agriculture	N/A
pCAMBIA-35S:PUB25-FLAG	This paper	N/A
pCAMBIA-35S:PUB26-FLAG	This paper	N/A
pCAMBIA-35S-AGB1-HA-Nluc	Liang et al. (2016)	N/A
pCAMBIA-35S-BAK1-HA-Nluc	Liang et al. (2016)	N/A
pCAMBIA-35S-BIK1-Cluc	Liang et al. (2016)	N/A
pCAMBIA-35S-BIK1-HA-Nluc	Liang et al. (2016)	N/A
pCAMBIA-35S-CPK28-HA-Nluc	This paper	N/A
pCAMBIA-35S-CPR5-Cluc	Liang et al. (2016)	N/A
pCAMBIA-35S-PBL20-HA-Nluc	This paper	N/A
REAGENT or RESOURCE	SOURCE	IDENTIFIER

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Continued

pCAMBIA-35S-PUB25-Cluc	This paper	N/A
pCAMBIA-35S-PUB26-Cluc	This paper	N/A
pCAMBIA-35S-XLG2-HA-Nluc	Liang et al. (2016)	N/A
pCAMBIA-pBIK1:BIK1-HA	Zhang et al. (2010)	N/A
pET28a-HIS-BIK1	Liang et al. (2016)	N/A
pET28a-HIS-BIK1 ^{K105E}	Liang et al. (2016)	N/A
pET28a-HIS-E1 (GI: 136632)	Qi Xie, Institute of Genetics and Developmental Biology	N/A
pET28a-HIS-UBC8	Qi Xie, Institute of Genetics and Developmental Biology	N/A
pET28a-PUB25 ^{T95A} -HIS	This paper	N/A
pET28a-PUB25 ^{T95D} -HIS	This paper	N/A
pET28a-PUB25-HIS	This paper	N/A
pET28a-PUB25-FLAG-HIS	This paper	N/A
pET28a-XLG2CT-HIS	Liang et al. (2016)	N/A
pGEX 6P-1-GST-AGB1	Liang et al. (2016)	N/A
pGEX 6P-1-GST-AGG2	Liang et al. (2016)	N/A
pGEX 6P-1-GST-BIK1	Zhang et al. (2010)	N/A
pGEX 6P-1-GST-CPK28	This paper	N/A
pGEX 6P-1-GST-PUB25	This paper	N/A
pGEX 6P-1-GST-PUB25 ^{T95A}	This paper	N/A
pGEX 6P-1-GST-PUB26	This paper	N/A
pGEX 6P-1-GST-PUB26 ^{T95A}	This paper	N/A
pUC19-35S-FLAG-RBS	Li et al., (2005)	N/A
pUC19-35S-HA-RBS	Li et al., (2005)	N/A
pUC19-35S-AGB1-FLAG	Liang et al. (2016)	N/A
pUC19-35S-AGB1-HA	Liang et al. (2016)	N/A
pUC19-35S-AGG1-HA	Liang et al. (2016)	N/A
pUC19-35S-BIK1 ^{2A} -FLAG-RBS	This paper	N/A
pUC19-35S-BIK1 ^{2A} -HA	This paper	N/A
pUC19-35S-BIK1 ^{2D} -FLAG	This paper	N/A
pUC19-35S-BIK1 ^{2D} -HA	This paper	N/A
pUC19-35S-BIK1-FLAG	Zhang et al. (2010)	N/A
pUC19-35S-BIK1-HA	Zhang et al. (2010)	N/A
pUC19-35S-BIK1 ^{K105E} -HA	Zhang et al. (2010)	N/A
pUC19-35S-CPK28-HA	This paper	N/A
pUC19-35S-HA-UBQ	This paper	N/A

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<i>Continued</i>		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
pUC19-35S-FLAG-UBQ	This paper	N/A
pUC19-35S-PUB25-FLAG	This paper	N/A
pUC19-35S-PUB25-HA	This paper	N/A
pUC19-35S-PUB25 ^{T95A} -FLAG	This paper	N/A
pUC19-35S-PUB25 ^{T95D} -FLAG	This paper	N/A
pUC19-35S-PUB26-FLAG	This paper	N/A
pUC19-35S-PUB26-HA	This paper	N/A
pUC19-35S-PUB26 ^{T94A} -FLAG	This paper	N/A
pUC19-35S-PUB26 ^{T94D} -FLAG	This paper	N/A
pUC19-35S-XLG2-FLAG	Liang et al. (2016)	N/A
pUC19-35S-XLG2-HA	Liang et al. (2016)	N/A
pUC19-PUB26 ^{S63A} -FLAG	This paper	N/A
pUC19-PUB26 ^{T407A} -FLAG	This paper	N/A
pUC19-PUB26 ^{T95A} -FLAG	This paper	N/A
Software and Algorithms		
IBM SPSS Statistics 22	IBM	https://www.ibm.com/analytics/cn/zh/technology/spss/

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for reagents may be directed to and will be fulfilled by the corresponding author, Jian-Min Zhou (jmzhou@genetics.ac.cn).

EXPERIMENTAL MODELS AND SUBJECT DETAILS

Plant Materials and Growth Conditions

Arabidopsis and *N. benthamiana* plants were grown on soil at 23/21 °C day/night and 70% relative humidity with 10/14 h light/dark photoperiod. *Arabidopsis* materials used in this study are all derived from Col-0. Previously published lines include: *cpk28-1* (Matschi et al., 2012; Monaghan et al., 2014), *xlg2-1 xlg3-1* (Ding et al., 2008), *agbl-2* (Ullah et al., 2003), *NP::BIK1-HA* (Zhang et al., 2010). T-DNA insertion mutants *pub25-1* (SALK_147032), *pub26-1* (GABI_308D07) and *pub26-2* (GABI_270G01) were obtained from the European *Arabidopsis* Stock Centre (NASC),

and homozygous lines were obtained by genotyping using allele-specific primers. Additional *Arabidopsis* transgenic lines and higher order mutants generated in this study are described below. *35S::PUB25-FLAG* and *35S::PUB26-FLAG* were introduced into Col-0 plants to generate *PUB25/26* over-expression lines (*PUB25/26-OE*) using standard protocols. *PUB25-OE1/NP::BIK1-HA*, *PUB25-OE1/cpk28-1*, *pub25-1 pub26-1*, *pub25-1 xlg2-1 xlg3-1*, *pub25-1 pub26-1 agb1-2* and *pub25-1 pub26-1 cpk28-1* were generated in this study by crossing and confirmed by allele-specific genotyping, presence of transgenic antibiotic resistance, and/or immunoblotting.

METHOD DETAILS

Constructs and Transgenic Plants

Constructs for protoplast transfection experiments were generated by PCR-amplifying coding sequences from cDNA and inserting into the PUC19-35S-FLAG-RBS and PUC19-35S-HA-RBS vectors (Li et al., 2005).

To generate constructs for split-luciferase assays, coding sequences were PCR-amplified from cDNA and inserted into Cluc-pCAMBIA, pCAMBIA-Nluc or pCAMBIA-HA-Nluc (Chen et al., 2008; Liang et al., 2016).

To generate constructs for recombinant proteins, coding sequences were inserted into pGex6p.1 (Amersham Biosciences) or pET28a vector (EMD Biosciences) to create translational fusion to GST and HIS tags, respectively.

To generate *PUB25/26-OE* transgenic plants, *35S::PUB25/26-FLAG* fragments were excised from the PUC19-35S-PUB25/26-FLAG constructs and cloned into pCAMBIA1300 plasmid (The Centre for Application of Molecular Biology to International Agriculture, Canberra). The resulting constructs were introduced into Col-0 plants by *Agrobacterium*-mediated transformation using standard protocols.

To generate *PUB25-OE/NP::BIK1-HA* plants, the *PUB25-OE1* transgenic line was crossed with *NP::BIK1-HA* (Zhang et al., 2010), and sibling transgenic lines carrying both *PUB25-OE1* and *NP::BIK1-HA* transgenes or only the *NP::BIK1-HA* transgene were identified by immunoblot.

Point mutations were introduced into above constructs by site-directed mutagenesis. Primers used for construction and genotyping are listed in Table S3.

Oxidative Burst Assay

Leaves of 25-day-old-plants were sliced into approximately 1 mm² strips and floated overnight on sterile water in a 96-well plate. The following day the water was replaced with a solution of 20 mM luminol (Sigma) and 10 mg/mL horseradish peroxidase (Sigma) containing 1 μM flg22 (Zhang et al., 2007). Luminescence was captured by using GLOMAX 96 microplate luminometer (Promega). Alternatively, 4 mm-diameter leaf discs were collected from 4-5 week-old plants into 96-well plates as described previously (Monaghan et al., 2014), treated with 100 nM flg22 (EZ Biolabs), and luminescence recorded on a SpectraMax plate reader outfitted with the LUM module.

Pathogen Inoculation and Disease Resistance Assays

B. cinerea was cultured on potato dextrose agar (PDA) medium (200 g/L Potato, 20 g/L Glucose, 15 g/L Agar) and incubated at 24 °C with a 12 hr photoperiod. Conidia were collected by flooding the plates using distilled water, filtrated with nylon mesh and washed twice by centrifugation (1000 rpm, 5 min) before being resuspended in potato dextrose broth (PDB). A single droplet of 5 μL spore suspension (5×10^5 spores/mL) was placed on a leaf of 4-week-old soil-grown *Arabidopsis* plant. Inoculated plants were kept under a transparent dome to maintain high humidity until disease lesions were recorded 2 d later.

For bacterial resistance assays, 25-d old plants were sprayed with *Pto* DC3000 *hrcC*⁻ mutant bacteria (Yuan and He, 1996) at 5×10^8 cfu/mL, covered with a

transparent dome for 1 d, and bacterial population in the leaf was determined 3 d after inoculation as described previously (Zipfel et al., 2004).

Split-Luciferase Assay

The assay was performed as previously described (Chen et al., 2008) with modifications. Briefly, *A. tumefaciens* strains containing the desired constructs were infiltrated into fully expanded leaves of 5-to-6-week-old *N. benthamiana* plants. Leaf discs were taken after 2 d, incubated with 200 μ L water containing 1 mM luciferin in a 96-well plate for 10-15 min, and luminescence was captured with the GLOMAX 96 microplate luminometer or SpectraMax plate reader with LUM module. Equal expression of proteins was verified by immunoblot.

Co-Immunoprecipitation Assay

Protoplasts were isolated and transfected as previously described (Niu and Sheen, 2012), incubated overnight, and then treated with water or 1 μ M flg22 for 10 min. Total protein was extracted with buffer containing 50 mM HEPES [pH 7.5], 150 mM KCl, 1 mM EDTA, 0.2% Triton-X 100, 1 mM DTT, proteinase inhibitor cocktail (Roche). For anti-FLAG IP, total protein was incubated with 50 μ L agarose-conjugated anti-FLAG antibody (Sigma) for 4 h, washed 6 times with extraction buffer, and the bound protein was eluted by incubating with 60 μ L of 20 μ g/mL 3 \times FLAG peptide (Sigma) for 1 h. The eluted protein was separated by SDS-PAGE and detected with immunoblot.

In Vitro Ubiquitination Assay

His- and GST-tagged recombinant proteins were expressed in *E. coli* strain BL21 and affinity-purified with glutathione-sepharose (GE) and Ni-sepharose (Qiagen), respectively, following manufacturer's instructions. *In vitro* ubiquitination assays were performed as described (Zhao et al., 2012). In a basic reaction, 12.5 ng E1-HIS, 250 ng E2-HIS, 1 μ g PUB25-FLAG-HIS or PUB25-HIS, and 4 μ g HA-UBQ (Boston Biochem, cat. no. U-110) were incubated in 30 μ L of ubiquitination reaction buffer

(50 mM Tris-HCl pH 7.4, 10 mM MgCl₂, 5 mM ATP, 2 mM DTT) at 30 °C with oscillation in a thermomixer (Eppendorf) for 2 h. For BIK1/RKS1 poly-ubiquitination, 60 ng GST-BIK1 or GST-RKS1 was added to the reaction. For heterotrimeric G proteins inhibition on PUB25 E3 ligase, 500 ng/each of XLG2CT-HIS, GST-AGB1, and GST-AGG2 were added to the reaction. For negative control, 1.5 µg BSA was added in place of the G proteins. The reaction was terminated by adding 4× protein loading buffer and boiled at 100 °C for 5 min. The sample was separated by SDS-PAGE and detected by immunoblot using anti-HA, anti-GST and anti-HIS antibodies (see STAR METHODS). Poly-ubiquitination on GST-BIK1/RKS1 is indicated by ladders in the anti-GST immunoblot, whereas PUB25-HIS and PUB25-FLAG-HIS auto-ubiquitination is indicated by ladders in anti-HA and anti-HIS immunoblots, respectively.

Ubiquitination Assay in Protoplasts

Protoplasts transfected with HA-UBQ or FLAG-UBQ, PUB26-HA or PUB25-FLAG, BIK1-FLAG or BIK1-HA, and/or HA-tagged heterotrimeric G protein subunits were incubated overnight in the presence of 5 µM MG132 (Sigma). Total protein was extracted with buffer containing 50 mM HEPES [pH 7.5], 150 mM KCl, 1 mM EDTA, 0.3% Triton-X 100, 1 mM DTT, proteinase inhibitor cocktail (Roche) and incubated with agarose-conjugated anti-FLAG antibody (Sigma) in the presence of 5 µM MG132 for 4 h, washed 6 times with extraction buffer, and the bound protein was eluted with 3× FLAG peptide (Sigma) for 1 h. The immunoprecipitates were separated by SDS-PAGE. Poly-ubiquitination on BIK1-FLAG and BIK1-HA were indicated by laddering bands in anti-HA and anti-FLAG immunoblots, respectively.

BIK1 Protein Stability in Protoplasts

To determine BIK1 protein stability, protoplasts transfected with BIK1-HA and PUB25/26-FLAG were treated with 50 µM cycloheximide (CHX [Sigma]) in the presence or absence of 1 µM flg22 for 2 h. Total protein was extracted with extraction

buffer (50 mM HEPES [pH 7.5], 150 mM KCl, 1 mM EDTA, 0.3% Triton-X 100, 1 mM DTT, proteinase inhibitor cocktail [Roche]), separated in a SDS-PAGE gel and detected with anti-HA immunoblot.

Protein Degradation Assay in *N. benthamiana* Plants

A. tumefaciens strain GV3101 containing BIK1-LUC and *A. tumefaciens* strain containing WT or mutant variants of PUB25-FLAG were co-infiltrated into fully expanded leaves of *N. benthamiana* plants. Leaf discs were taken after 2 d and floated on 100 μ L 100 μ M CHX in a 96-well plate under dim light for 12-24 h. Then, 100 μ L of 2 mM luciferin was added into each well, and luminescence was recorded with a GLOMAX 96 microplate luminometer.

LC-MS/MS Analysis

To identify BIK1-interacting proteins, BIK1-FLAG was transiently expressed in WT protoplasts, and total protein was extracted with IP buffer I (50 mM HEPES [pH 7.5], 50 mM NaCl, 10 mM EDTA, 0.2% Triton X-100, 0.1 mg/mL Dextran [Sigma], proteinase inhibitor cocktail [Roche]). Protoplasts transfected with an empty vector pUC19-35S-FLAG-RBS (Li et al., 2005) were used as negative control. To identify PUB26 phosphosites, protoplasts expressing PUB26-FLAG were treated with 1 μ M flg22 for 10 min before protein extraction. The PUB26-FLAG protein was then enriched by immunoprecipitation using anti-FLAG antibody and eluted with 75 μ L 3 \times FLAG peptide (Sigma) for 40-60 min as previously described (Li et al., 2014; Liang et al., 2016; Liu et al., 2009). The eluted proteins were separated in 10% NuPAGE gel (Invitrogen), digested with trypsin, and subject to mass spectrometric analysis as previously described (Li et al., 2014).

Generation of Antibodies

Anti-BIK1 antibodies were produced by AbMart using the following peptide: C-TGTTKSSEKRFTQK. Anti-phospho-PUB26^{T94} antibodies were produced by AbMart using a phospho-peptide: Ac-CVERIPT(p)PKQ-NH₂. Antibodies that

recognize non-phosphorylated peptide were depleted by incubating antisera with non-phosphorylated peptide: Ac-CVERIPTPKQ-NH₂.

In Vitro Phosphorylation Assays

For phosphorylation assays, 200 ng GST-CPK28, HIS-BIK1 and HIS-BIK1^{K105E} proteins were incubated with 2 µg GST-PUB25 or GST-PUB26 in 20 µL reaction buffer (25 mM Tris-HCl [pH 7.5], 10 mM MgCl₂, 1 mM DTT, 100 mM ATP) for 30 min at 30 °C and GST-PUB25^{T95A} and GST-PUB26^{T94A} were used as negative control. The reaction was stopped by adding SDS loading buffer. Protein phosphorylation was detected by immunoblot using anti-pT95/pT94 antibodies which specifically react with phospho-PUB25^{T95} and phospho-PUB26^{T94}.

To determine flg22-induction of CPK28 kinase activity, CPK28-FLAG was transiently expressed in *N. benthamiana* plants using *Agrobacterium*-mediated transformation, and the leaves were treated with 1 µM flg22 or water for 20 min before protein was isolated with anti-FLAG beads. Equal amounts (approximately 100 ng) of CPK28-FLAG was incubated with 2 µg GST-PUB26 protein in an *in vitro* kinase buffer, and phosphorylation of PUB26T94 was detected with anti-pT94/95 immunoblot.

Statistical Analysis

Statistical significance among treatments were determined by one-way ANOVA, followed by Turkey's post-hoc test at p<0.05.

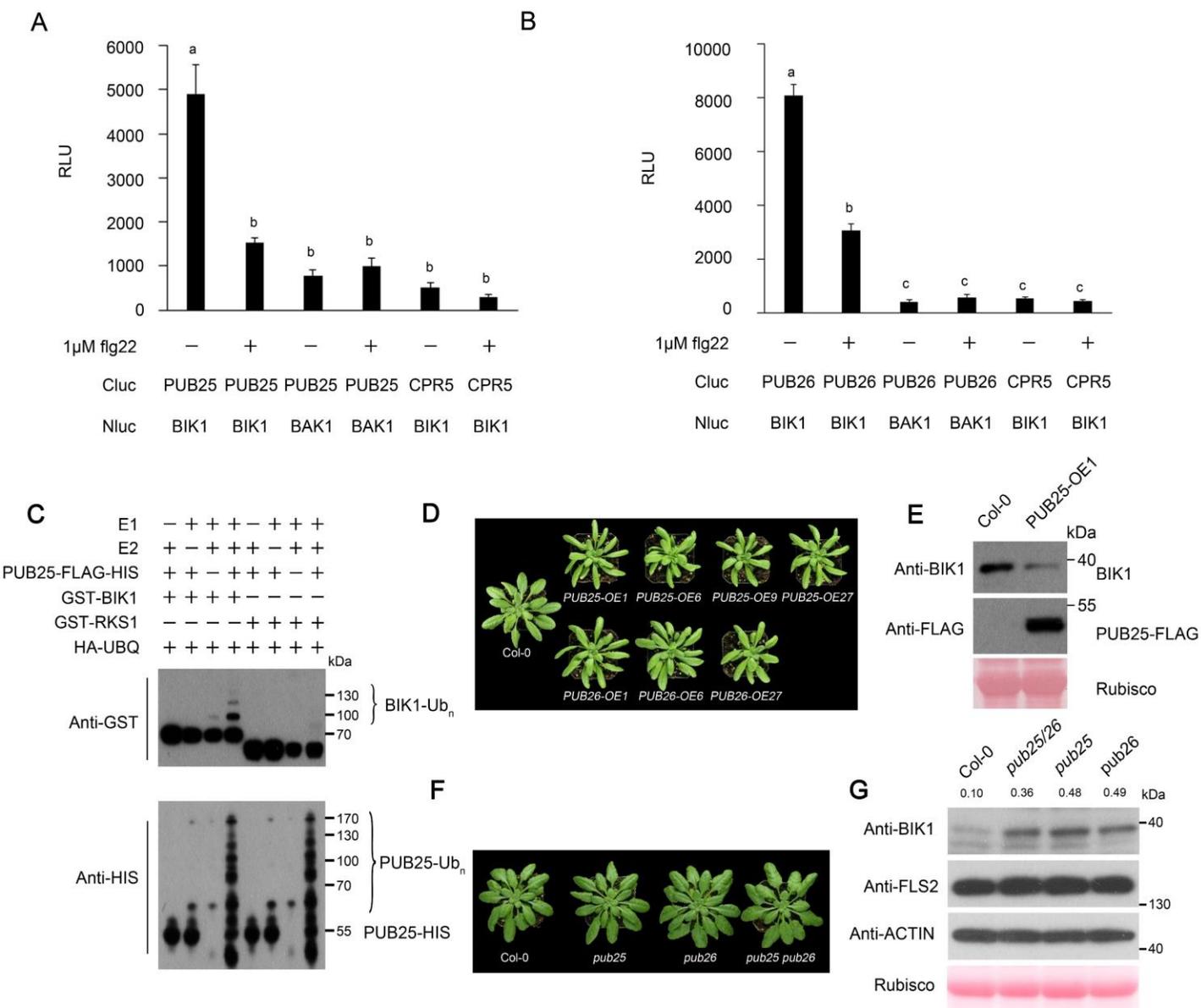


Fig. 1

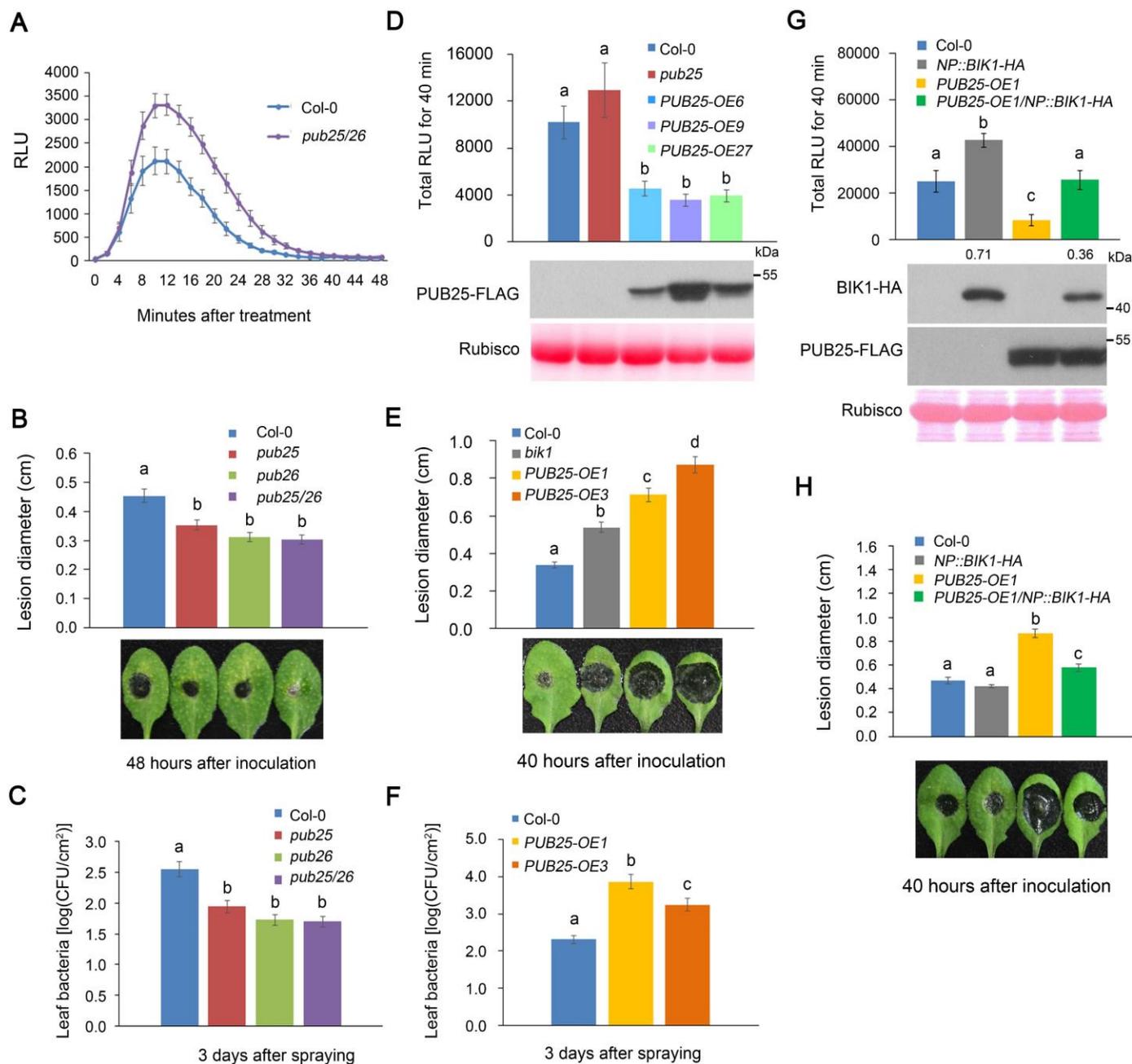


Fig. 2

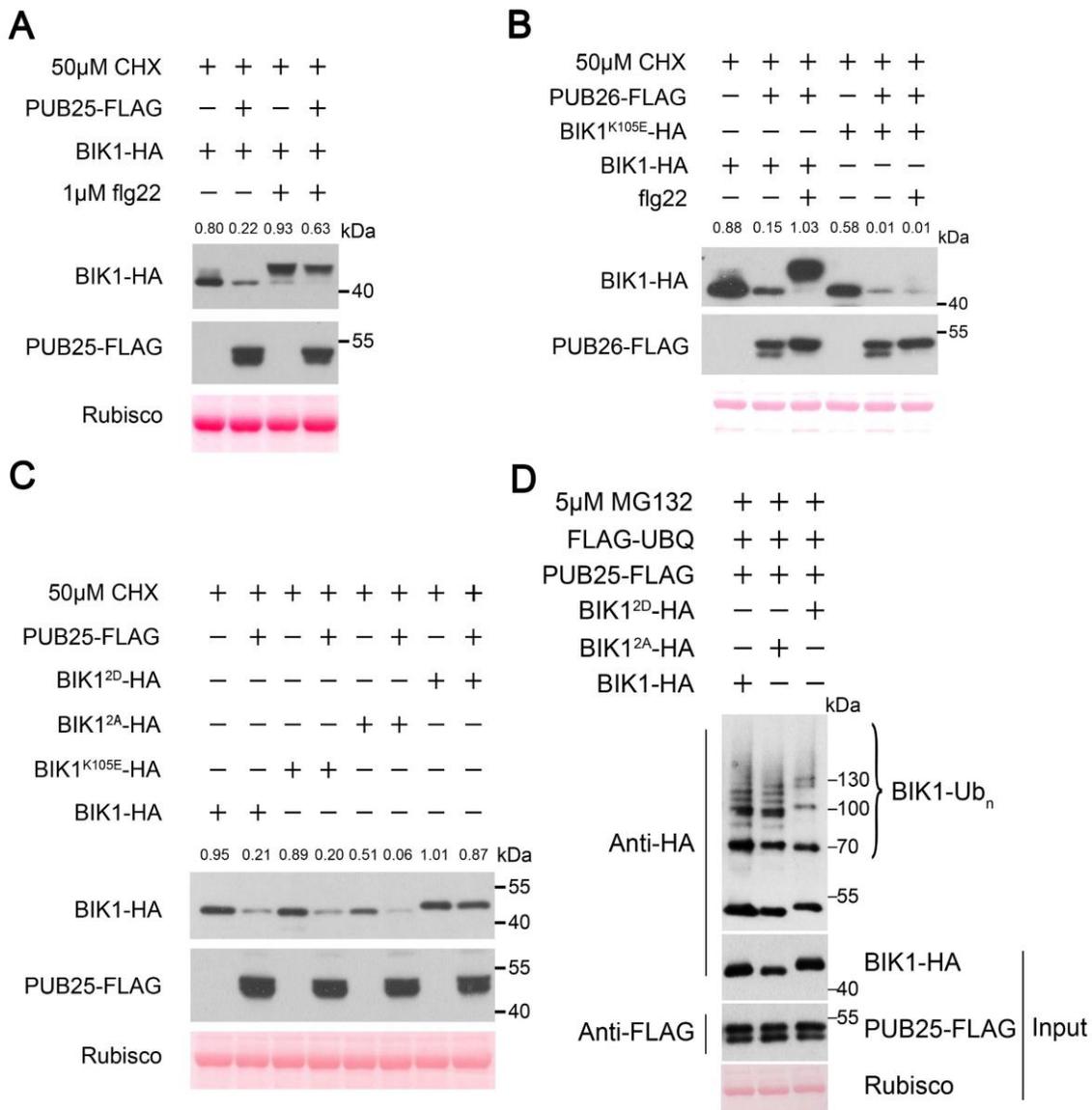


Fig. 3

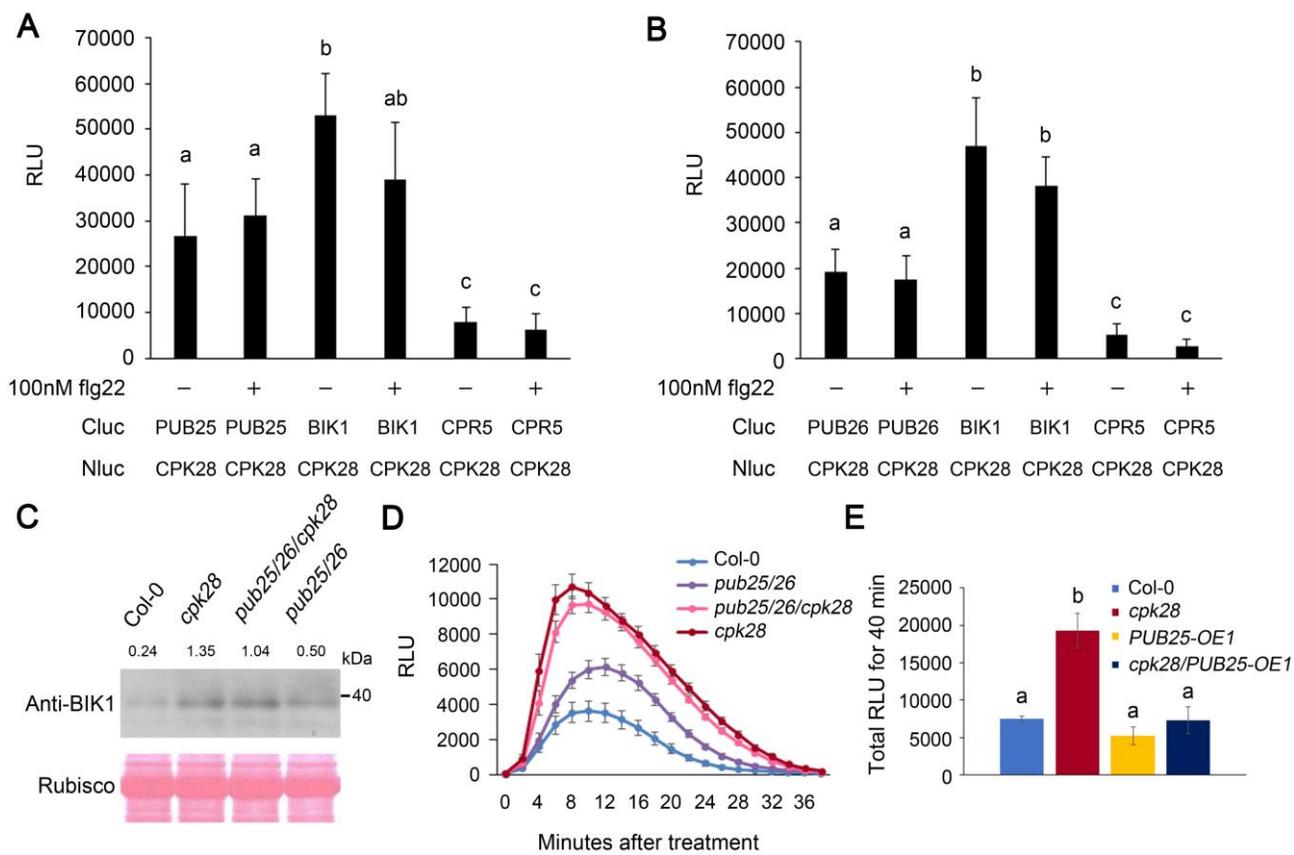


Fig. 4

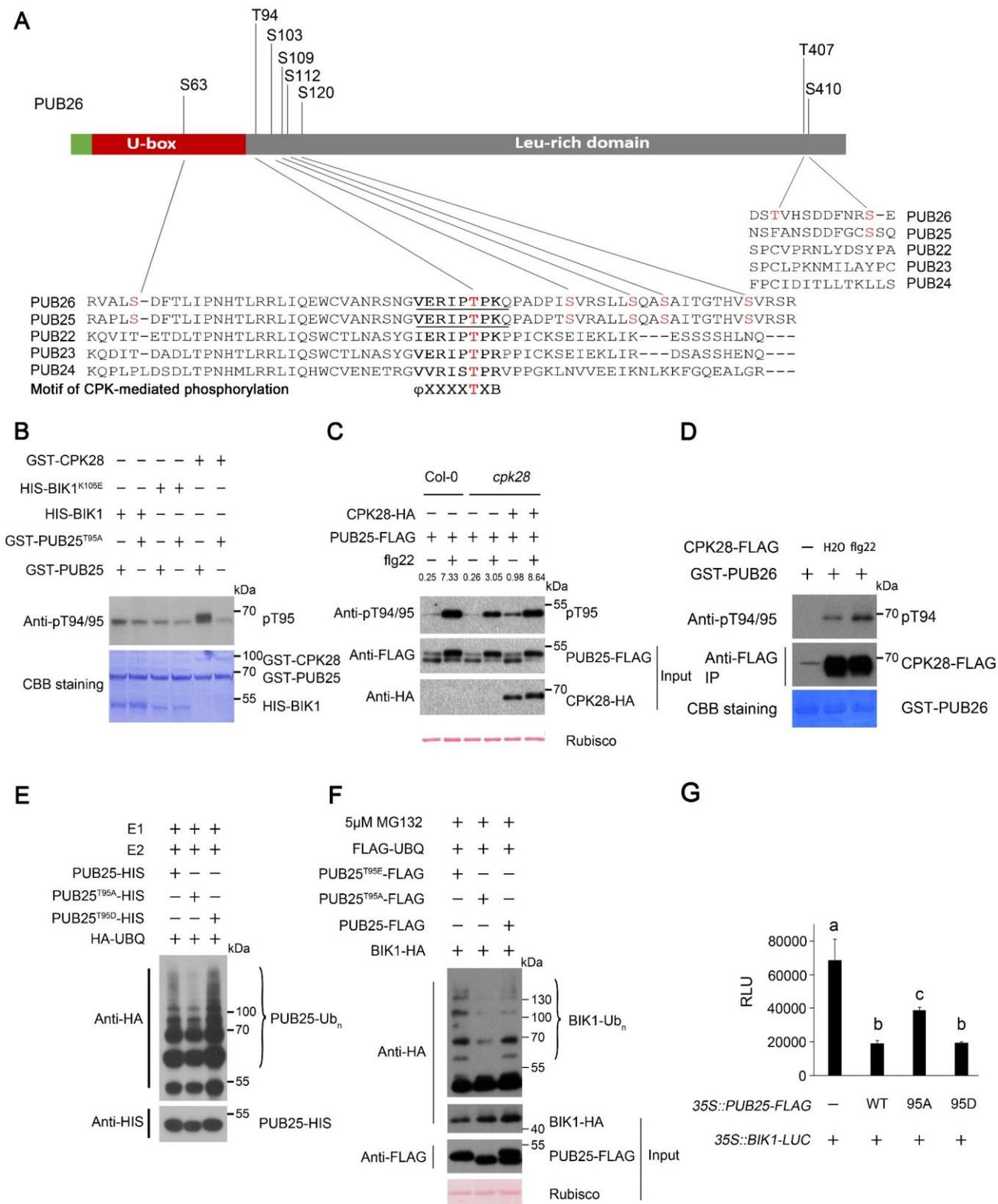


Fig. 5

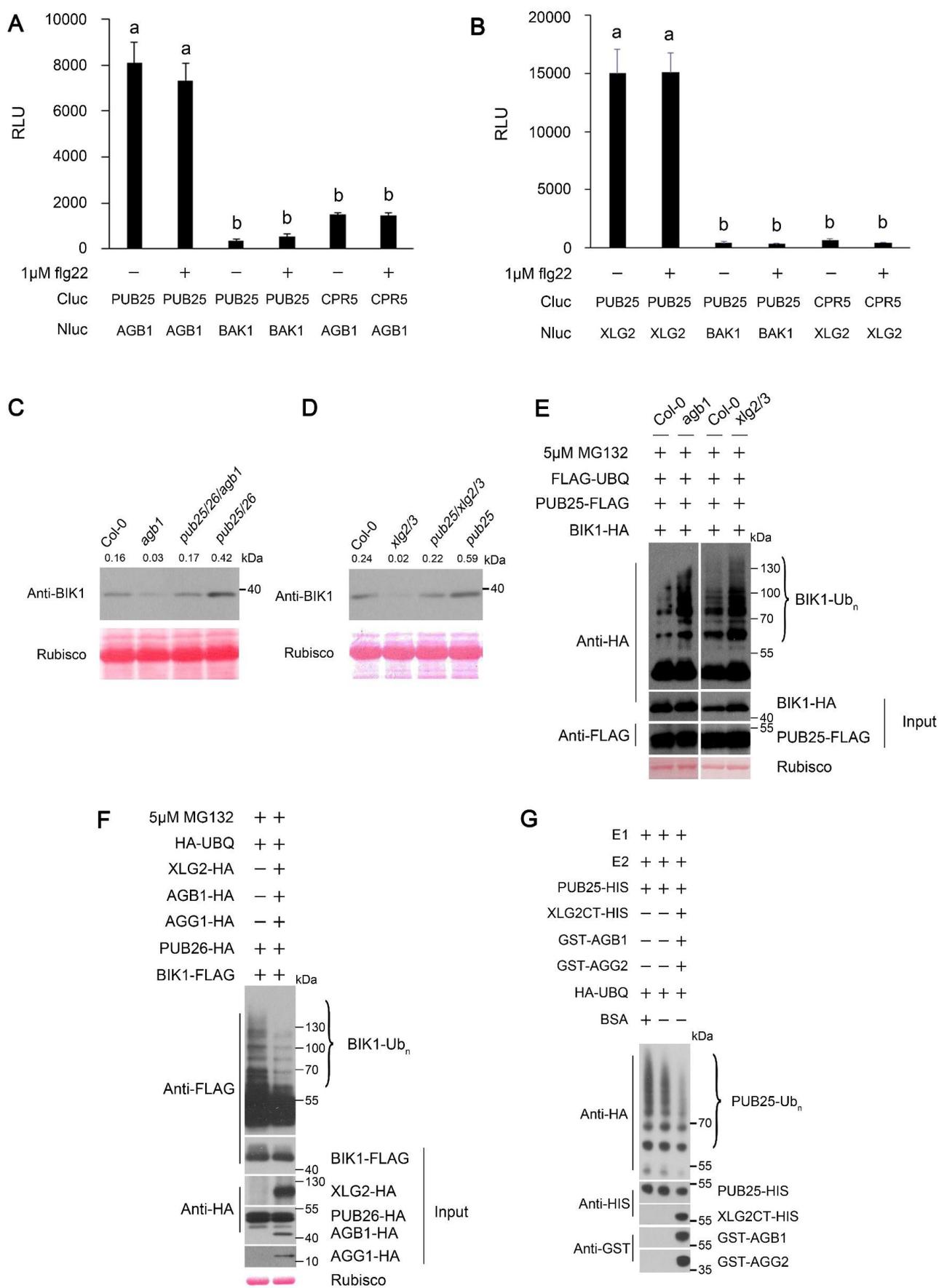


Fig. 6

Supplemental Information

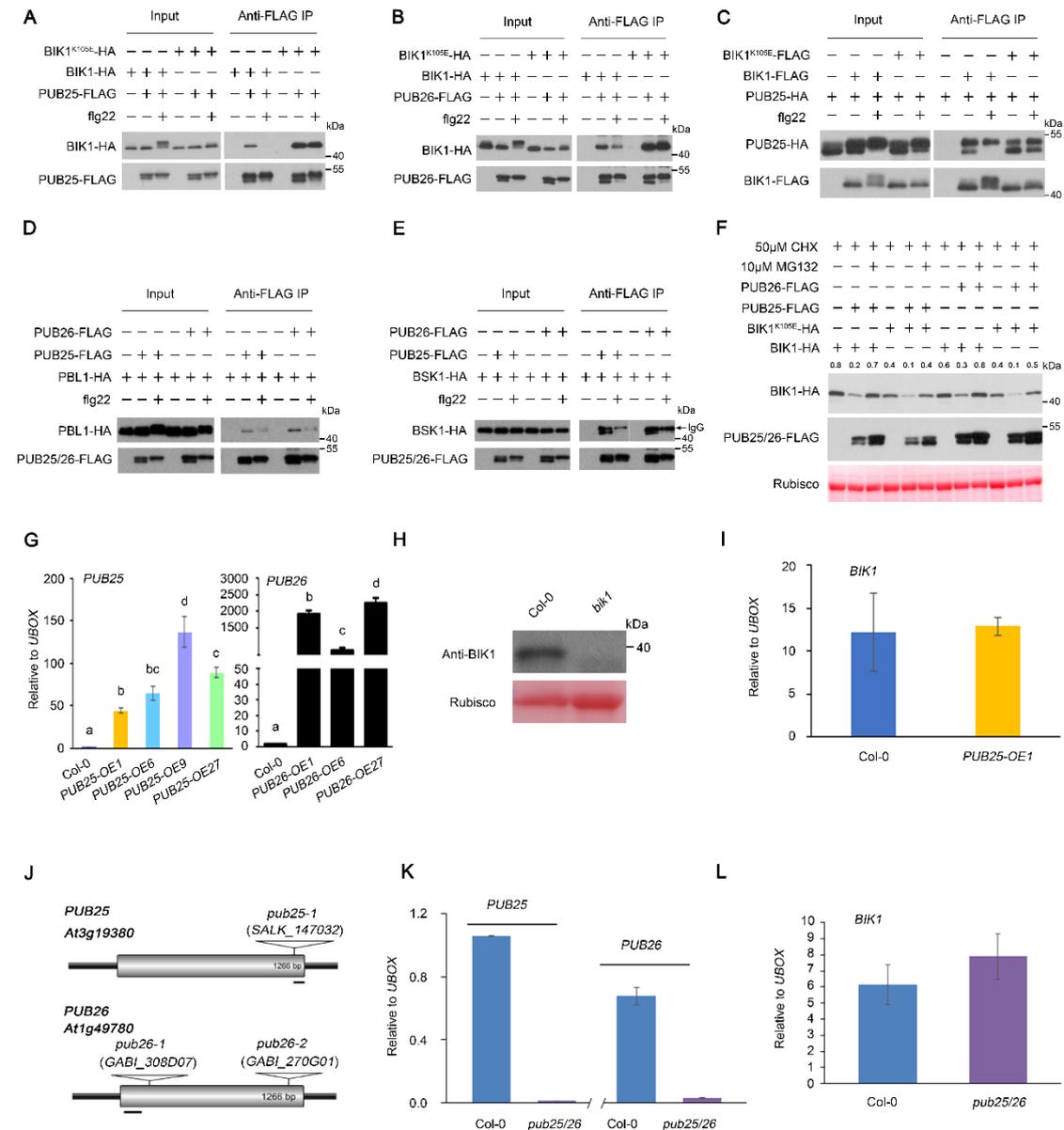


Figure S1, related to Figure 1. PUB25/26 Interact with and Promote Degradation of BIK1.

(A-C) BIK1 associates with PUB25 (A and C) and PUB26 (B) in protoplasts. Col-0 protoplasts were transfected with the indicated constructs, and Co-IP assays were performed and detected with anti-FLAG and anti-HA immunoblot. The BIK1^{K105E} mutant is defective in ATP-binding and does not possess kinase activity (Zhang et al., 2010). Note that the hyper-phosphorylated form of BIK1 does not associate with PUB25/26.

(D and E) PUB25/26 associate with PBL1 (D) and BSK1 (E) in protoplasts. Col-0 protoplasts

were transfected with the indicated constructs, and Co-IP assays were performed and detected with anti-FLAG and anti-HA immunoblot.

(F) Over-expression of *PUB25/26* promotes proteasome-dependent degradation of wild-type BIK1 and BIK1^{K105E} mutant. Col-0 protoplasts were transfected with the indicated constructs, incubated overnight, and then treated with 50 μM cycloheximide (CHX) and/or 10 μM MG132 for 2 h prior to analysis by immunoblot. Ponceau-stained Rubisco indicates equal loading. Numbers indicate arbitrary units of BIK1-HA calculated from densitometry measurements normalized to total Rubisco protein.

(G) Over-accumulation of *PUB25/26* transcripts are much higher in *PUB25/26* over-expression lines compared to Col-0 plants. These assays were repeated three times with similar results and representative data are shown.

(H) Anti-BIK1 antibodies specifically detected BIK1 in Col-0, but not *bik1* plants.

(I) *BIK1* transcripts accumulate normally in *PUB25-OE1* plants as indicated by quantitative RT-PCR. The assay was repeated three times with similar results and one representative experiment is shown.

(J) Schematic diagrams of T-DNA insertional mutations in *PUB25/26*.

(K) The *pub25 pub26* (*pub25/26*) double mutant is abolished in wild-type *PUB25/26* transcript accumulation. *PUB25/26* expression in the *pub25/26* double mutant was determined by quantitative RT-PCR analysis of amplicons close to but not flanking the insertion sites indicated as lines in (D).

(L) *BIK1* transcripts accumulate normally in *pub25 pub26* plants as indicated by quantitative RT-PCR. These assays were repeated three times with similar results and one representative experiment is shown.

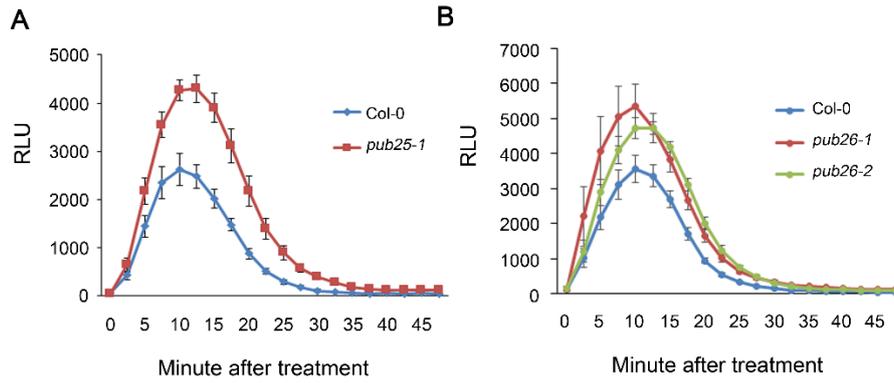


Figure S2, related to Figure 2. *pub25* and *pub26* Mutants Exhibit Elevated ROS Burst in Response to flg22 Treatment.

(A and B) ROS burst in *pub25-1* (A) and *pub26-1*, *pub26-2* (B) leaves treated with 1 μ M flg22. Values are means of relative light units \pm SDs (n = 8). Different letters indicate significant difference at $p < 0.05$. Experiments were repeated at least three times with similar results.

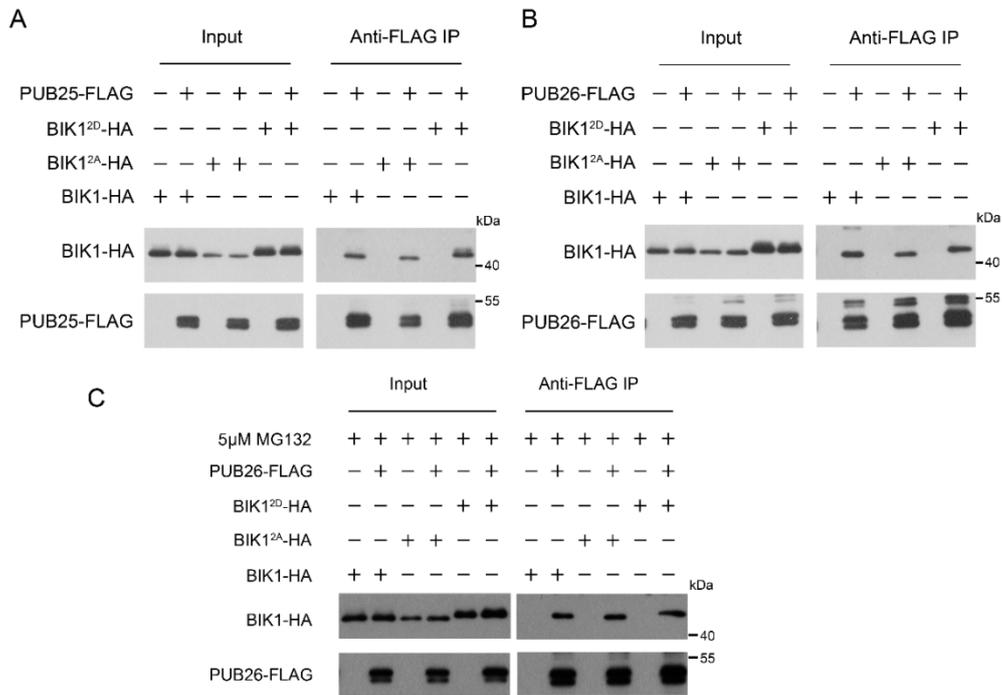


Figure S3, related to Figure 3. BIK1, BIK1^{2A} and BIK1^{2D} Interact Equally with PUB25/26.

(A) Interaction of BIK1 variants with PUB25.

(B) Interaction of BIK1 variants with PUB26.

(C) Interaction of BIK1 variants with PUB26 in the presence of MG132. Col-0 protoplasts were transfected with the indicated constructs, and FLAG-tagged proteins were affinity-purified. The immunoprecipitates were detected with anti-FLAG and anti-HA immunoblot. All assays were performed twice with similar results.

Note that experiments in A and B were performed in the absence of CHX. With constant *de novo* protein synthesis, over-expression of BIK1 probably obscured the destabilizing effects of PUB25/26, which resulted in similar amounts of BIK1 protein in different lanes.

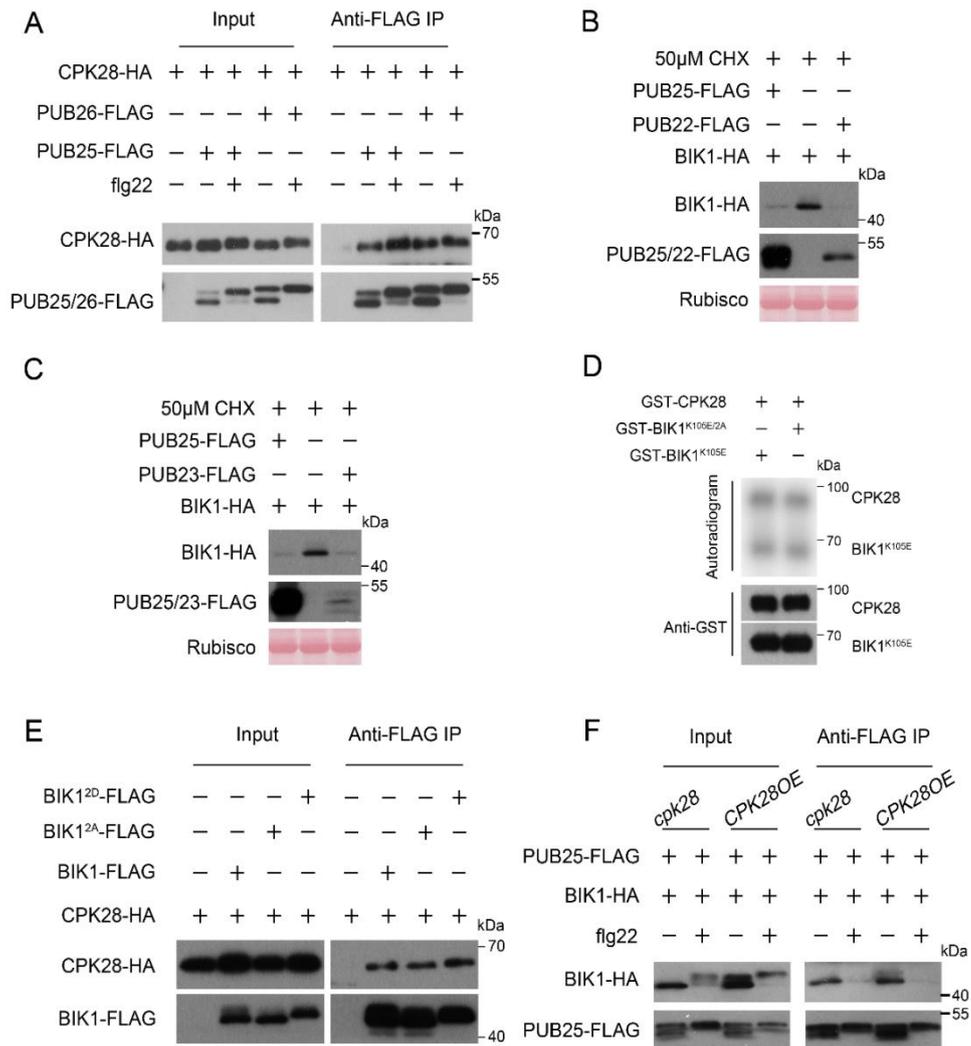


Figure S4, related to Figure 4.

(A) CPK28 Interacts with PUB25/26. Col-0 protoplasts were transfected with the indicated constructs, and co-IP assay was performed using agarose-conjugated anti-FLAG antibody. The presence of proteins was determined by immunoblot using anti-HA and anti-FLAG antibodies.

(B and C) PUB22 (B) and PUB23 (C) destabilize BIK1 in protoplasts. Col-0 protoplasts were transfected with the indicated constructs, incubated overnight, and then treated with 50 μM CHX for 2 h prior to immunoblot analysis. Ponceau-stained Rubisco indicates equal loading.

(D) CPK28 equally phosphorylates BIK1^{K105E} and BIK1^{K105E/2A} *in vitro*. GST-CPK28 was incubated with GST-BIK1^{K105E} or GST-BIK1^{K105E/2A} in the presence of ³²P-γ-ATP and

analyzed by autoradiography. Loading is indicated by Coomassie Brilliant Blue (CBB) staining of the gel.

(E) Ser236 and Thr237 do not impact CPK28-BIK1 interaction in protoplasts. Col-0 protoplasts were transfected with the indicated constructs, and co-IP was performed.

(F) The BIK1-PUB25 interaction occurs equally in protoplasts derived from *cpk28* and *CPK28OE* plants. Protoplasts were transfected with the indicated constructs, and co-IP was performed. Note that the effect of the *CPK28OE* transgene on the steady state level of BIK1-HA is not observed in the co-IP assay because CHX was not included in the assay. The slight difference of BIK1-HA levels in different samples likely reflect variations caused by transfection efficiency.

Each experiment was performed three times (A) or twice with similar results (B-F).

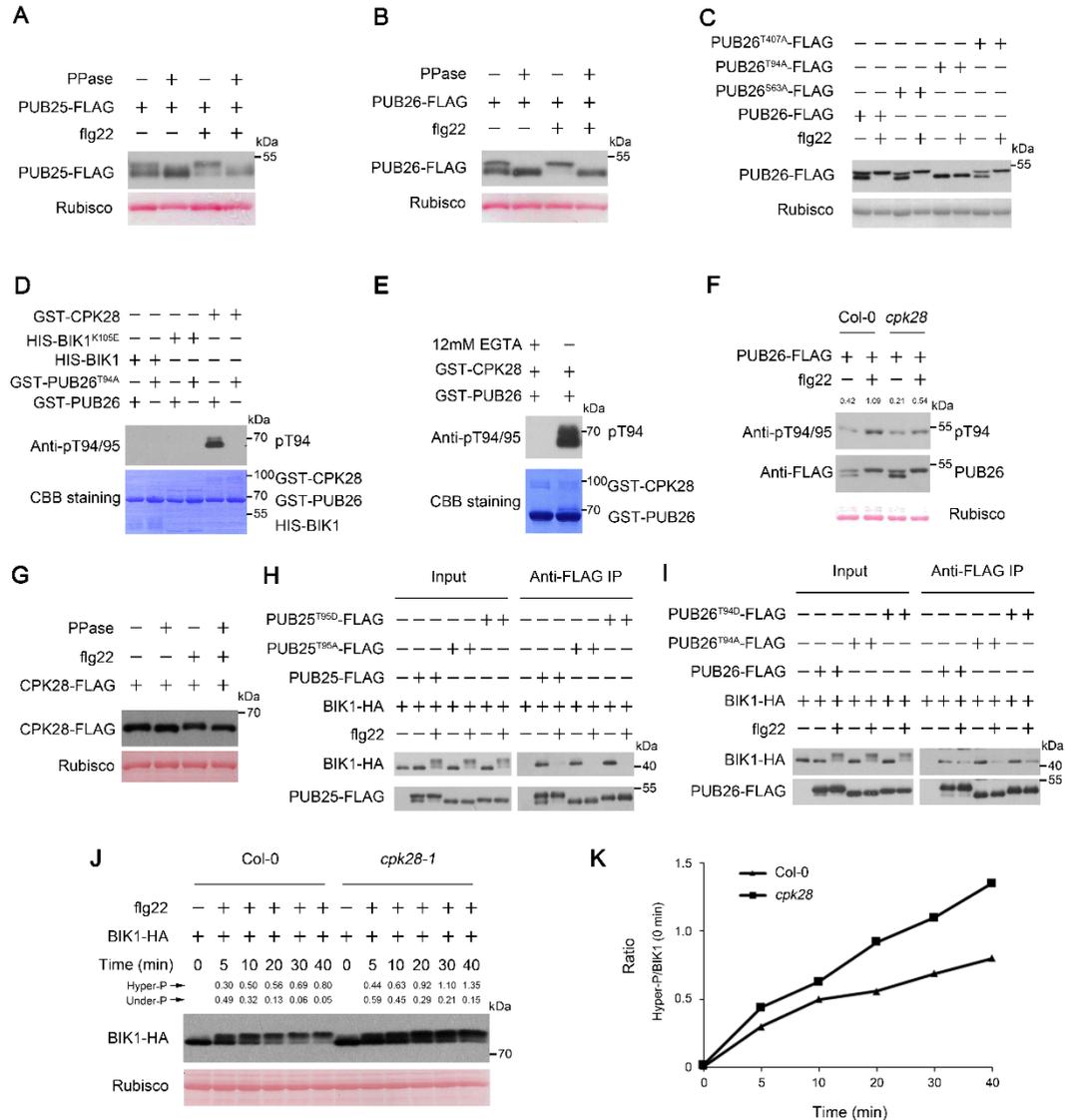


Figure S5, related to Figure 5. Flg22 Induces Phosphorylation on PUB25^{T95} and PUB26^{T94} That Is in Part Mediated by CPK28.

(A and B) Flg22 induces phosphorylation on PUB25 (A) and PUB26 (B). Col-0 protoplasts were transfected with the indicated constructs, treated with 1 μ M flg22 for 10 min, and the mobility of PUB25/26-FLAG protein was analyzed by anti-FLAG immunoblot. Protein phosphorylation was confirmed by treating the protein sample with protein phosphatase (PPase).

(C) Thr94 is the main flg22-induced phospho-site in PUB26. Col-0 protoplasts were transfected with wild-type and mutated forms of PUB26-FLAG, treated with 1 μ M flg22, and

the migration of PUB26-FLAG was examined by anti-FLAG immunoblot.

(D) CPK28, but not BIK1, phosphorylates PUB26^{T94} *in vitro*. Protein phosphorylation was detected by anti-pT94/95 immunoblots. Loading is indicated by Coomassie Brilliant Blue (CBB).

(E) EGTA eliminates PUB26^{T94} phosphorylation by CPK28 *in vitro*. The indicated proteins were incubated in kinase assays as described above, with and without 12 mM EGTA for 30 min before analysis by immunoblot. Loading is indicated by Coomassie Brilliant Blue (CBB).

(F) CPK28 contributes to PUB26^{T94} phosphorylation in protoplasts. Protoplasts of the indicated genotypes were transfected with PUB26-FLAG, treated with or without 1 μ M flg22 for 10 min, and PUB26 phosphorylation was detected by anti-pT94/95 immunoblot. The experiment was repeated at least three times with similar results. Loading is indicated by Ponceau S staining of Rubisco. Numbers indicate arbitrary units of PUB26^{T94} phosphorylation calculated from densitometry measurements normalized to total PUB26-FLAG protein.

(G) Flg22 induces CPK28 phosphorylation. Col-0 protoplasts were transfected with the indicated constructs, treated with 1 μ M flg22 for 10 min, and the mobility of CPK28-FLAG protein was analyzed by anti-FLAG immunoblot. Protein phosphorylation was confirmed by treating the protein sample with protein phosphatase (PPase).

(H and I) PUB25^{T95} and PUB26^{T94} phospho-site mutants associate with BIK1 normally in protoplasts (co-IP). Col-0 protoplasts were transfected with the indicated constructs, treated with or without 1 μ M flg22 for 10 min, and co-IP using FLAG beads was performed.

(J) CPK28 is required for the rapid removal of under-phosphorylated BIK1 and the restriction of hyper-phosphorylated BIK1 accumulation following flg22 treatment. Protoplasts derived from Col-0 and *cpk28* mutant plants were transfected with BIK1-HA, treated with 0.5 μ M flg22 for the indicated time points in the presence of 50 μ M CHX, and the BIK1-HA protein was analyzed by anti-FLAG immunoblot. Upper and lower numbers indicate arbitrary units of hyper- and under-phosphorylated BIK1, respectively, calculated from densitometry measurements normalized to BIK1 protein at 0 min time point. Ponceau-stained Rubisco indicates equal loading.

(K) Plot of hyper-phosphorylated BIK1 observed in (J).

Each experiment was done twice with similar results.

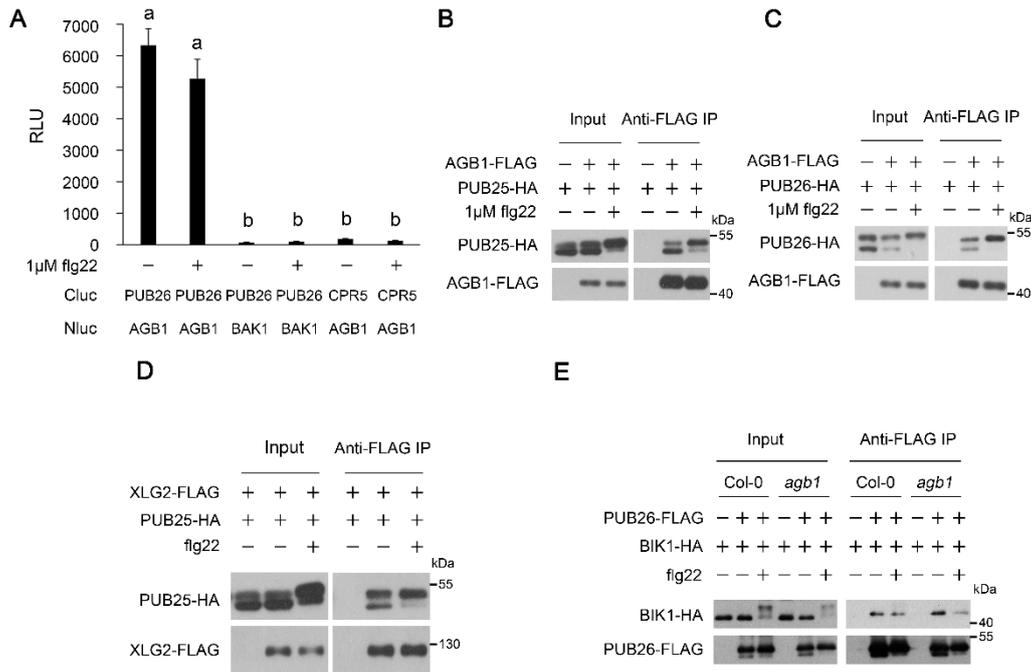


Figure S6, related to Figure 6. Heterotrimeric G Proteins Interact with PUB25/26 to Regulate BIK1 Stability.

(A) PUB26 associates with AGB1 in *N.b.* plants in split luciferase assays. AGB1-HA-Nluc and Cluc-PUB26 constructs were transiently expressed in *N.benthamiana* leaves, BAK1-HA-Nluc and Cluc-CPR5 were used as negative controls. Leaf discs were treated with or without 1 μ M flg22 for 10 min before luminescence was determined. Values are mean relative luminescence units \pm SDs (n = 8). Different letters indicate significant difference at $p < 0.05$.

(B-D) AGB1 (B and C) and XLG2 (D) constitutively associate with PUB25/26.

(E) The PUB26-BIK1 interaction occurred similarly in protoplasts derived from Col-0 and *agb1* mutant plants.

In B-E, Col-0 protoplasts were transfected with the indicated constructs, treated with 1 μ M flg22 for 10 min, and co-IP was performed. These experiments were performed twice with similar results.

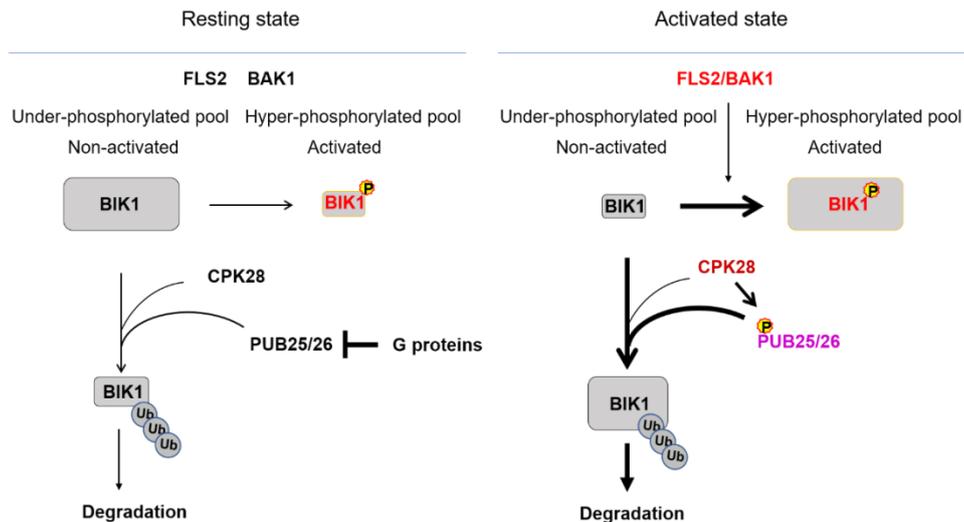


Figure S7. Model for The PUB-Mediated Control of BIK1 Homeostasis.

In *Arabidopsis* plants, BIK1 exists in a non-activated pool and an activated pool, the latter consists of BIK1 that is phosphorylated in the activation loop. The two pools are in equilibrium and are modulated by a number of components. In the resting state, the vast majority of BIK1 is in the non-activated pool. The basal E3 ligase activities of PUB25/26 specifically mark the non-activated BIK1 for proteasome-dependent degradation. CPK28 may also destabilize non-activated BIK1 through phosphorylation at unknown residues in BIK1 (Monaghan et al., 2014). The heterotrimeric G proteins directly inhibit PUB25/26 E3 ligase activity so that the degradation of BIK1 is kept to a minimum. Upon activation by flg22, the PRR (FLS2) complex phosphorylates BIK1 in the activation loop to rapidly increase activated BIK1 pool. The activation by flg22 also leads to increased phosphorylation of PUB25^{T95}/PUB26^{T94} by CPK28 and dissociation of the heterotrimeric G proteins from the FLS2-BIK1 complex (Liang et al., 2016). These lead to increased E3 ligase activities and rapid depletion of the non-activated BIK1 pool. As a result, the size of activated BIK1 pool stops increasing when the non-activated BIK1 pool is too small. The protection of the activated BIK1 pool from degradation may be beneficial for the phosphorylation of downstream targets. Protein phosphatases such as PP2C38 may convert the activated BIK1 pool to non-activated pool, which can then be degraded and the system returns to resting state.

Table S1, related to Figure 1 and S1. Identification of A PUB25 Peptide in FLAG-Tagged BIK1 Immunoprecipitation Product.

Bait	PUB25 peptide		
	Mascot score	Matched queries	Matched peptides
BIK1	40	2	1
Empty vector		0	0

Arabidopsis protoplasts were transfected with FLAG-tagged BIK1 or an empty vector (negative control). After immunoprecipitation with anti-FLAG beads, the products were subject to LC-MS/MS analysis.

Table S2, related to Figure 5 and S5. Identification of Phosphorylated Peptides in Protoplast-Expressed PUB26.

Position	Phospho-peptide sequence	H ₂ O	flg22
S63	VAL(pS)DFTLIPNHTLR	No	Yes
	SNGVERIP(pT)PK	Yes	Yes
T94	IPT(pP)K	Yes	Yes
	IP(pT)PKQPAD	No	Yes
	IP(pT)PKQPADPISVR	Yes	Yes
S103	QPADPI(pS)VR	No	Yes
S109	SLL(pS)QASAITGTHVSVR	Yes	Yes
S112	SLLSQA(pS)AITGTHVSVR	Yes	No
S120	SLLSQASAITGTHV(pS)VR	Yes	Yes
T407	DSWPDDS(pT)VHSDDFNR	No	Yes
S410	DSWPDDSTVH(pS)DDFNR	Yes	Yes

Arabidopsis protoplasts were transfected with FLAG-tagged PUB26 construct, treated with or without 1 μ M flg22 for 0 min before immunoprecipitation with anti-FLAG beads. The affinity-purified products were subject to LC-MS/MS analysis. The presence (Yes) and absence (No) of phospho-peptides in different treatments are shown on the right. Protein sequence coverage with or without flg22 treatment was 83% and 84%, respectively.

Table S3, related to Figure 1-6. Overview of the primers used for genotyping, cloning, and qRT-PCR.

Gene name	Primers
Primers for genotyping mutant lines	
PUB25-1_LP	ATGTGTGTTTGCTCGTCTTCC
PUB25-1_RP	TGGTTGATTGTTTCTCCACC
PUB26-1_LP	GTGACACCAATATCTCTACG
PUB26-1_RP	TCAACACCATCTCAATCAAC
PUB26-2_LP	TTGATTGAGATGGTGTGAC
PUB26-2_RP	TACACGGAACCTAAGCATACT
CPK28-1_F	GCGGCGGATTCTTTGACTAA
CPK28-1_R	AGTACACAACGGCTCATTATGAA
Primers for molecular cloning	
PUB25_KpnI-FLAG/HA-F	CGGGGTACCATGCCTAGGAATATAGAACCAT
PUB25-FLAG-R	agAAAAGGGGACCCTTGCTGCTCAACCAAAG
PUB25_SalI-HA-R	ACGCGTCGACAAAAGGGGACCCTTGCTGCTG
PUB26_KpnI-FLAG/HA-F	CGGGGTACCATGCCGGGAATTTAGAGCCGT
PUB26-FLAG-R	agAAACGGGCGCCACTTCGCTACGATTGAAATC
PUB26-SalI-R	ACGCGTCGACAAAACGGGCGCCACTTCGCTACGA
CPK28_KpnI-HA-F	CGGGGTACCATGGGTGTCTGTTTCTCCGCCA
CPK28_XhoI-HA-R	CCGCTCGAGTCGAAGATTCCTGTGACCTGCA
Cluc-PUB25_KpnI-F	CGGGGTACCATGCCTAGGAATATAGAACCAT
Cluc-PUB25-XhoI-R	CCGCTCGAGTCAAAAAGGGGACCCTTGCTGCTG
Cluc-PUB26_KpnI-F	CGGGGTACCATGCCGGGAATTTAGAGCCGT
Cluc-PUB26-SalI-R	ACGCGTCGACTCAAAACGGGCGCCACTTCGCTACGA
PBL20-Nluc-KpnI-F	GCCGGTACCATGAATGTCTGTTCTTGT
PBL20-Nluc-SalI-R	CAAGTCGACGCCTCTAATGCCAACAGAA
GST-PUB25-SalI-F	ACGCGTCGACATGCCTAGGAATATAGAACCAT
GST-PUB25-XhoI-R	CCGCTCGAGTCAAAAAGGGGACCCTTGCTGCTG
GST-PUB26-EcoRI-F	CCGGAATTCATGCCGGGAATTTAGAGCCGT
GST-PUB26-SalI-R	ACGCGTCGACTCAAAACGGGCGCCACTTCGCTACGA
GST-CPK28-BamHI-F	CGCGGATCCATGGGTGTCTGTTTCTCCGCCA
GST-CPK28-EcoRI-R	CCGGAATTCCTATCGAAGATTCCTGTGACCTG
PUB25-HIS-NcoI-F	CATGCCATGGATGCCTAGGAATATAGAACCAT
PUB25-HIS-SalI-R	ACGCGTCGACAAAAGGGGACCCTTGCTGCTG
UBQ-KpnI-HAtag-F	CGGGGTACCATGTACCCATACGATGTTTCTGACTATGCGGGCTATCCCT ATGACGTCCCAGACTATGCAGGATCCTATCCATATGACGTTCCAGATTA CGCTATGCAGATCTTTGTCAAGAC
UBQ-SalI-R	ACGCGTCGACTTAACCTCCACGGAGACGCAAC

Primers for site-directed mutagenesis

PUB25-T95A-F	AGTCGAACGTATCCCTgCTCCTAAACAACCAGCTG
PUB25-T95A-R	CAGCTGGTTGTTTAGGAGCAGGGATACGTTGACT
PUB25-T95D-F	AGTCGAACGTATCCCTgaTCCTAAACAACCAGCTG
PUB25-T95D-R	CAGCTGGTTGTTTAGGATCAGGGATACGTTGACT
PUB26-T94A-F	CGTCGAACGGATTCCtgCACCTAAACAACCGGCGG
PUB26-T94A-R	CCGCCGGTTGTTTAGGTGCAGGAATCCGTTGACG
PUB26-T94D-F	CGTCGAACGGATTCCgatCCTAAACAACCGGCGG
PUB26-T94D-R	CCGCCGGTTGTTTAGGATCAGGAATCCGTTGACG
BIK1-S236A,T237A-F	GTGATTTGAGTTATGTTgcTgCAAGGGTCATGGGTACTTATGG
BIK1-S236A,T237A-R	CCATAAGTACCCATGACCCTTGACGCAACATAACTCAAATCAC
BIK1-S236D,T237D-F	GTGATTTGAGTTATGTTgaTgacAGGGTCATGGGTACTTATGG
BIK1-S236D,T237D-R	CCATAAGTACCCATGACCCTGTCATCAACATAACTCAAATCAC
GST-PUB25-NoProtease-F	CTGGAAGTTCTGTTCCAGGTCGACATGCCTAGGAAT
GST-PUB25-NoProtease-R	ATTCCTAGGCATGTCGACCTGGAACAGAACTTCCAG
PUB25-HIS-removeadditionalATG-F	AACTTTAAGAAGGAGATATACCATGCCTAGGAATATAGAACCAT
PUB25-HIS-removeadditionalATG-R	ATGGTTCTATATTCCTAGGCATGGTATATCTCCTTCTTAAAGTT
PUB26-63A-F	CACACGTGTCGCTCTcgCCGACTTCACTCTCATCC
PUB26-63A-R	GGATGAGAGTGAAGTCGGCGAGAGCGACACGTGTG
PUB26-407A-F	TTGGCCTGACGATTCTgCCGTTCAATCCGACGATT
PUB26-407A-R	AATCGTCGGAATGAACGGCAGAATCGTCAGGCCAA

Primers for qRT-PCR

qPUB25_F	CTACAATTCCTTCGCTAACTCC
qPUB25_R	AAAAGGGACCACTTGGCTGCTGC
qPUB26_F	GCCGGGAATTTAGAGCCGTTG
qPUB26_R	GGTTAGGGATGAGAGTGAAGTC
qUBOX_F	TGCGCTGCCAGATAATACTACTATT
qUBOX_R	TGCTGCCAACATCAGGTT
qBIK1_F	TGGGCTCGACCGTACCTCACA
qBIK1_R	CGGGCGGACTTGGGTCAA
