Direct ubiquitination of pattern recognition receptor FLS2 attenuates plant innate immunity

Dongping Lu[1,3], **Wenwei Lin**[2,3,4], **Xiquan Gao**[1,3], **Shujing Wu**[2,3], **Cheng Cheng**[1,3], **Julian Avila**[1], **Antje Heese**[5], **Timothy P. Devarenne**[1], **Ping He**[1,3,†], and **Libo Shan**[2,3,†]

[1]Department of Biochemistry and Biophysics, Texas A&M University, College Station, TX 77843, USA

[2]Department of Plant Pathology and Microbiology, Texas A&M University, College Station, TX 77843, USA

[3]Institute for Plant Genomics and Biotechnology, Texas A&M University, College Station, TX 77843, USA

[4]Key Laboratory of Biopesticide and Chemical Biology, Ministry of Education, Fujian Agriculture and Forestry University, Fuzhou, 350002, China

[5]Department of Biochemistry, University of Missouri-Columbia, Columbia, MO 65211, USA

Abstract

Innate immune responses are triggered by the activation of pattern-recognition receptors (PRRs). The *Arabidopsis* PRR FLS2 senses bacterial flagellin and initiates immune signaling by association with BAK1. The molecular mechanisms underlying the attenuation of FLS2 activation are largely unknown. We report that flagellin induces recruitment of two closely related U-box E3 ubiquitin ligases PUB12 and PUB13 to FLS2 receptor complex in *Arabidopsis*. BAK1 phosphorylates PUB12/13 and is required for FLS2-PUB12/13 association. PUB12/13 polyubiquitinate FLS2 and promote flagellin-induced FLS2 degradation, and the *pub12* and *pub13* mutants displayed elevated immune responses to flagellin treatment. Our study has revealed a unique regulatory circuit of direct ubiquitination and turnover of FLS2 by BAK1-mediated phosphorylation and recruitment of specific E3 ligases for attenuation of immune signaling.

Plants and animals rely on innate immunity to prevent infections by detection of pathogen- or microbe-associated molecular patterns (PAMPs/MAMPs) through pattern-recognition receptors (PRRs) (1–4). *Arabidopsis* FLAGELLIN-SENSING 2 (FLS2), a plasma membrane localized leucine-rich repeat receptor-like kinase (LRR-RLK), is the receptor for bacterial flagellin (5). Upon flagellin perception, FLS2 associates instantaneously with another LRR-RLK termed BAK1, which appears to function as a signaling partner of the growth hormone brassinolide receptor BRI1 and multiple PRRs (6–10). BIK1, a receptor-like cytoplasmic kinase in the FLS2/BAK1 complex, is rapidly phosphorylated upon flagellin perception (11–12). Ligand-induced FLS2 endocytosis has also been suggested to be coupled with the activation of flagellin signaling (13). Similar to PRR activation, down-

†Correspondence: Libo Shan, lshan@tamu.edu, Tel: 979-845-8818. Ping He, pinghe@tamu.edu, Tel: 979-458-1368, Institute for Plant Genomics and Biotechnology, Texas A&M University, College Station, TX 77843 Fax: 979-862-4790.

regulation of PRR signaling is crucial for preventing excessive or prolonged activation of immune responses which would be detrimental to the hosts. Far less is understood as to how the innate immune responses are attenuated following the PRR activation.

To identify components in MAMP signaling, we performed a yeast two-hybrid screen using the BAK1 kinase domain as bait. One of the interactors isolated encodes the C-terminus of *Arabidopsis* PUB13 (At3g46510) (Table S1, Figs. S1 & S2A). PUB13 is a typical Plant U-box (PUB) E3 ubiquitin ligase with a U-box N-terminal Domain (UND), a U-box domain and a C-terminal ARMADILLO (ARM) repeat domain (Fig. S1) (14–15). To test whether full-length PUB13 and BAK1 interact *in vivo*, we performed a co-immunoprecipitation (Co-IP) assay following protoplast transient transfection. FLAG epitope-tagged PUB13 co-immunoprecipitated HA epitope-tagged BAK1 independent of flg22, a 22 amino acid peptide of flagellin (Fig. 1A). The interaction between FLS2 and PUB13 was barely detectable in the absence of flg22 treatment, however, PUB13 strongly associated with FLS2 upon flg22 stimulation (Fig. 1B). Deletion analysis indicated that the ARM domain of PUB13 was sufficient to associate with FLS2 (Fig. S2B). PUB12, the closest homolog of PUB13, but not PUB29, also associated with FLS2 upon flg22 stimulation (Fig. S2C). The association of FLS2 with PUB13 was further confirmed in transgenic plants expressing FLAG-tagged *PUB13* under the control of its native promoter (*pPUB13::PUB13-FLAG*) using an anti-FLS2 antibody (Fig. 1C).

Since flg22 induces the association of FLS2 with both BAK1 and PUB12/13, we tested whether flg22-induced FLS2-PUB12/13 association required BAK1. Significantly, flg22-mediated FLS2-PUB13 (Fig. 1D) or FLS2-PUB12 (Fig. S2D) association was not detectable in the *bak1-4* mutant plants, indicating that flg22-induced FLS2-PUB12/13 complex formation requires BAK1. However, the interaction of BAK1 and PUB12 does not require FLS2 (Fig. S2E), consistent with the observation that BAK1 constitutively interacts with PUB12 and PUB13 in the absence of ligand (Figs. 1A, S2A & S2E). Similar to the instantaneous association of FLS2 and BAK1, FLS2-PUB13 association was detected within 30 seconds upon flg22 stimulation (Fig. 1E). Together, the results suggest that BAK1 and PUB12/13 likely exist as a complex that is rapidly recruited to FLS2 receptor upon flg22 stimulation (Fig. S2F).

Interestingly, BAK1 directly phosphorylated PUB12 and PUB13 (Fig. 2A). The phosphorylation depended on the kinase activity of BAK1 since a BAK1 kinase mutant (BAK1Km) was unable to phosphorylate PUB12 or PUB13 (Fig. 2A). BIK1, an FLS2/BAK1-associated cytosolic kinase, did not phosphorylate PUB12 or PUB13 (Fig. S3A). However, BIK1 enhanced the ability of BAK1 to phosphorylate PUB13 (Fig. S3A). Consistent with our published data that BIK1 phosphorylates BAK1(11), it is likely that phosphorylation of BAK1 by BIK1 potentiates BAK1's activity to phosphorylate PUB12 and PUB13. To further examine whether flg22 could enhance BAK1-dependent phosphorylation of PUB13, we performed an immunocomplex kinase assay with flg22-treated protoplasts expressing full length BAK1. The immunoprecipitated BAK1, but not kinase-inactive BAK1Km, phosphorylated PUB13 (Fig. 2B). The phosphorylation of PUB13 was further enhanced upon flg22 treatment depending on its receptor FLS2 (Fig. 2B). These data suggest that PUB12/13 are substrates of BAK1, and that flg22 perception stimulates BAK1 phosphorylation of PUB12/13, which is likely further potentiated by BIK1 (Fig. S3C). To examine the biological significance of PUB12/13 phosphorylation, we tested whether phosphorylation is required for the flg22-induced FLS2-PUB12/13 association. Significantly, we found that the flg22-induced FLS2-PUB13 or FLS2-PUB12 association was blocked by the kinase inhibitor K252a (Figs. 2C & S3B). These results support the significance of phosphorylation events in FLS2-PUB12/13 association (Fig. S3C).

An *in vitro* ubiquitination assay with GST-PUB12/13 fusion proteins in the presence of recombinant AtUBA1 (E1), AtUBC8 (E2), ATP and FLAG-tagged ubiquitin (Ub) demonstrates that PUB12 and PUB13 possess auto-ubiquitination activity as detected by either anti-FLAG or anti-GST antibody (Fig. S4A). Significantly, both PUB12 and PUB13 polyubiquitinated the cytosolic domain of FLS2 evidenced by detection of a ladder-like smear with high-molecular-weight proteins (Fig. 3A). Eliminating E1, E2, PUB13, Ub or ATP from the reaction blocked the ladder formation for FLS2 (Fig. S4B). Mutation of the conserved cysteine or tryptophan residue to alanine (C262A or W289A) within the U-box motif of PUB13 abolished its ubiquitin ligase activity on FLS2 (Fig. 3B). The specificity of FLS2 ubiquitination by PUB12 and PUB13 was further substantiated by the observation that several U-box E3 ubiquitin ligases, including PUB14 and PUB29, did not ubiquitinate FLS2 (Fig. S4C). Interestingly, PUB12 and PUB13 did not ubiquitinate the cytosolic domain of BAK1 or BIK1 (Fig. 3C). Thus, PUB12 and PUB13 specifically ubiquitinate the PRR FLS2, but not the signaling components BAK1 or BIK1 (Fig. S5C).

It has been shown that flg22 induces FLS2 translocation into intracellular vesicles, which is followed by degradation of FLS2 (13). FLS2 possesses a PEST-like motif in the C-terminus, required for FLS2 endocytosis. In addition, a potential phosphorylation site, Thr 867, is essential for FLS2 internalization and signaling (13). However, neither the T867V nor a PEST mutant P1076A abolished FLS2 ubiquitination by PUB13 or PUB12 (Fig. S5A), suggesting that FLS2 ubiquitination and internalization are likely uncoupled. It has been reported that the ubiquitination of FLS2 by a bacterial effector AvrPtoB is also independent of its PEST domain (16). An FLS2 kinase inactive mutant K898M also did not affect FLS2 ubiquitination by PUB13 (Fig. S5B), indicating that FLS2 kinase activity is not required for its ubiquitination. This is consistent with our data that PUB12/13 are phosphorylated by BAK1 and phosphorylation is required for flg22-induced FLS2-PUB12/13 association. Our data also suggest that FLS2 ubiquitination is distinct from mammalian receptor tyrosine kinase (RTK), in which a kinase-defective mutant blocked RTK ubiquitination (17).

To further demonstrate the biological function of PUB12 and PUB13 in plant innate immunity, we isolated their T-DNA insertional mutants. RT-PCR analysis indicated that *pub12-1* (SAIL_35_G10) displayed a slight reduction of transcript, while *pub12-2* (WiscDsLox497_01) showed a pronounced transcript reduction and was selected for further analyses (Fig. S6). The *pub13* mutant (SALK_093164) is a null mutant with undetectable transcript (Fig. S6). We examined the immune responses in wild-type (WT), *pub12-2* and *pub13* mutants. Flg22-induced FLS2/BAK1 association (Fig. S7A) and BIK1 phosphorylation (Fig. S7B) occurred similarly in *pub12-2* and *pub13* mutants and in WT plants, suggesting that PUB12 and PUB13 are not required for flg22 perception by FLS2. When treated with flg22, *pub12-2* and *pub13* mutants accumulated two- to threefold more H_2O_2 production than WT plants (Fig. 4A). Similarly, the *pub12-2* and *pub13* mutants showed more callose deposits than WT plants (Figs. 4B & S6C). The complementation lines of the *pub13* mutants transformed with a *pPUB13::PUB13-FLAG* construct displayed a similar level of H_2O_2 production (Fig. S8A) and callose deposition (Figs. S8B & S8C) as the WT plants upon flg22 stimulation. Compared to WT plants, the induction of immune responsive genes, *WRKY30*, *AP2* (*At3g23230*) and *FRK1*, was further enhanced in the *pub12-2* and *pub13* mutants upon flg22 treatment (Fig. 4C). Notably, the basal expression level of these genes was comparable in WT and *pub12-2* or *pub13* mutants, suggesting that *pub12-2* and *pub13* mutants are not constitutively expressing immunity-related genes. Together, these data indicate that flg22-mediated responses are potentiated in the *pub12* and *pub13* mutants, and that *PUB12* and *PUB13* play a negative role in FLS2 signaling.

We performed pathogen infection assays with WT and *pub* mutants. Neither *pub12-2* nor *pub13* mutants showed significantly altered disease symptoms or bacterial multiplication

following *Pseudomonas syringae* pv. *tomato* DC3000 infection compared to WT plants (Fig. S9A). To reveal potentially functional redundancy, we generated a *pub12/13* double mutant. The *pub12/13* mutant did not display any obvious growth defects under normal growth conditions (data not shown), which is consistent with that *pub12-2* and *pub13* mutants did not constitutively activate immunity-related genes. Alternatively, additional components might play redundant functions with PUB12 and PUB13 in the control of detrimental effect of constitutively active defense systems. Importantly, the *pub12/13* mutant was more resistant to DC3000 infection than WT plants. Three days after infection, the bacterial population in the *pub12/13* mutant was about 10-fold lower than that in WT plants (Fig. 4D). The disease symptoms were also less severe in the *pub12/13* mutant compared to WT plants six days after infection (Fig. S9B). Similarly, the *pub12/13* mutants were also more resistant to *P. syringae maculicola* ES4326 infection as measured by bacterial growth and disease symptoms (Figs. 4D & S9B).

Ubiquitination could lead to protein degradation, or in some cases, modulation the activity or localization of a protein target. We examined FLS2 protein level in WT and the *pub12/13* mutant with an anti-FLS2 antibody. We repeatedly observed an apparent reduction of FLS2 protein level within 30 min after flg22 treatment in WT plants (Fig. 4E), which is consistent with the report of ligand-induced FLS2 endocytosis/degradation (13). This degradation was diminished by treatment with MG132, an inhibitor of proteasome-mediated degradation (Fig. 4E), suggesting the involvement of the 26S proteasome in FLS2 degradation. Importantly, FLS2 protein level was not significantly reduced in the *pub12/13* mutant treated with flg22 (Figs. 4E & S10A), suggesting the involvement of *PUB12* and *PUB13* in flg22-mediated FLS2 degradation. Similar to the *pub12/13* mutant, the reduced FLS2 level triggered by flg22 was not evident in the *bak1-4* mutant (Fig. S10B). This is consistent with the FLS2-PUB12/13 association being dependent on BAK1 (Figs. 1D & S2D). We further developed an *in vivo* ubiquitination assay with protoplast transient transfection of FLAG-tagged UBQ10. The treatment of flg22 enhanced FLS2 ubiquitination as detected by an anti-FLS2 antibody (Fig. S10C). The flg22-mediated FLS2 ubiquitination was reduced in the *pub12/13* mutant (Fig. S10C), which substantiates the role of PUB12/13 in the ubiquitination of endogenous FLS2 (Fig. S11).

Ubiquitination has been implicated in plant innate immunity (18–19). Two Avr9/Cf9 rapidly elicited *PUB* genes are required for programmed cell death and defense in tobacco, tomato and *Arabidopsis* (20–21). Three closely related *Arabidopsis* PUBs, PUB22/23/24, negatively regulate flagellin signaling (22). Rice SPL11, one of the closest homologs to PUB12/13, is a negative regulator of plant cell death (23). Similarly, a rice RING finger E3 ligase XB3 could be phosphorylated by PRR XA21 mediating bacterial resistance (24). However, the modes of action of these E3 ligases from upstream activators to downstream substrates are largely unknown. Identification of PUB12/13 as negative regulators of flagellin signaling by direct ubiquitination of FLS2 not only contributes to the general understanding of innate immune signaling, but also improves our ability for genetic manipulation of disease resistant crop plants without deleterious side effects.

Ubiquitination-mediated Toll-like receptor (TLR) degradation serves as one of the mechanisms to down-regulate TLR signaling (25). Triad 3A, an RING finger E3 ligase mediating ubiquitination and proteolytic degradation of TLR4 and TLR9, appears to be analogous to PUB12/13 in *Arabidopsis* FLS2 signaling (25). However, the regulation and activation of TLR ubiquitination remain unknown. The mechanism of activating FLS2 ubiquitination is also unique and distinct from RTK signaling (Fig. S11). The ubiquitination of RTKs is regulated by ligand-induced RTK phosphorylation (26). It appears that FLS2 phosphorylation is not required for its ubiquitination (Figs. S5A & S5B). Instead, phosphorylation appears to be required for flg22-induced FLS2-PUB12/13 association (Fig.

2C & S3B). It is plausible that phosphorylation of PUB12/13 promotes their association with FLS2 *in vivo* (Fig. S11). More interestingly, ubiquitination only occurs on FLS2 not BAK1, a common signaling partner of multiple membrane receptors involved in immunity and development. This suggests a mechanism of how the specificity of the signal output is determined for a shared signaling component.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

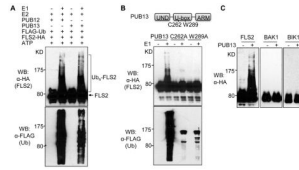## Acknowledgments

References and Notes

1. Takeuchi O, Akira S. Cell. Mar 19.2010 140:805. [PubMed: 20303872]

2. Boller T, Felix G. Annu Rev Plant Biol. 2009; 60:379. [PubMed: 19400727]

3. Jones JD, Dangl JL. Nature. Nov 16.2006 444:323. [PubMed: 17108957]

4. Dodds PN, Rathjen JP. Nat Rev Genet. Aug.2010 11:539. [PubMed: 20585331]

5. Gomez-Gomez L, Boller T. Mol Cell. Jun.2000 5:1003. [PubMed: 10911994]

6. Chinchilla D, et al. Nature. Jul 26.2007 448:497. [PubMed: 17625569]

7. Heese A, et al. Proc Natl Acad Sci U S A. Jul 17.2007 104:12217. [PubMed: 17626179]

8. Li J, et al. Cell. Jul 26.2002 110:213. [PubMed: 12150929]

9. Nam KH, Li J. Cell. Jul 26.2002 110:203. [PubMed: 12150928]

10. Schulze B, et al. J Biol Chem. Mar 26.2010 285:9444. [PubMed: 20103591]

11. Lu D, et al. Proc Natl Acad Sci U S A. Jan 5.2010 107:496. [PubMed: 20018686]

12. Zhang J, et al. Cell Host Microbe. Apr 22.2010 7:290. [PubMed: 20413097]

13. Robatzek S, Chinchilla D, Boller T. Genes Dev. Mar 1.2006 20:537. [PubMed: 16510871]

14. Yee D, Goring DR. J Exp Bot. 2009; 60:1109. [PubMed: 19196749]

15. Azevedo C, Santos-Rosa MJ, Shirasu K. Trends Plant Sci. Aug.2001 6:354. [PubMed: 11495788]

16. Gohre V, et al. Curr Biol. Dec 9.2008 18:1824. [PubMed: 19062288]

17. Levkowitz G, et al. Mol Cell. Dec.1999 4:1029. [PubMed: 10635327]

18. Craig A, Ewan R, Mesmar J, Gudipati V, Sadanandom A. J Exp Bot. 2009; 60:1123. [PubMed: 19276192]

19. Trujillo M, Shirasu K. Curr Opin Plant Biol. Aug.2010 13:402. [PubMed: 20471305]

20. Yang CW, et al. Plant Cell. Apr.2006 18:1084. [PubMed: 16531496]

21. Gonzalez-Lamothe R, et al. Plant Cell. Apr.2006 18:1067. [PubMed: 16531490]

22. Trujillo M, Ichimura K, Casais C, Shirasu K. Curr Biol. Sep 23.2008 18:1396. [PubMed: 18771922]

23. Zeng LR, et al. Plant Cell. Oct.2004 16:2795. [PubMed: 15377756]

24. Wang YS, et al. Plant Cell. Dec.2006 18:3635. [PubMed: 17172358]

25. Chuang TH, Ulevitch RJ. Nat Immunol. May.2004 5:495. [PubMed: 15107846]

26. Lemmon MA, Schlessinger J. Cell. Jun 25.2010 141:1117. [PubMed: 20602996]
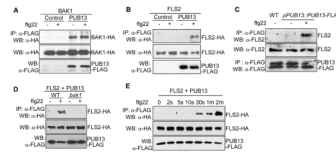
Fig. 1.
Flagellin induces BAK1-dependent FLS2-PUB13 complex association. **(A)** BAK1 interacts with PUB13 in a Co-IP assay. Protoplasts were co-expressed with BAK1-HA and PUB13-FLAG or a control vector. The Co-IP was carried out with an anti-FLAG antibody (IP: α-FLAG), and the proteins were analyzed using Western blot with an anti-HA antibody (WB: α-HA). The top panel shows that BAK1 co-immunoprecipitates with PUB13. The middle and bottom panels show the expression of BAK1-HA and PUB13-FLAG proteins. Protoplasts were stimulated with 1 μM flg22 for 10 min. **(B)** flg22 induces FLS2-PUB13 association in protoplasts. **(C)** flg22 induces FLS2-PUB13 association in *Arabidopsis* seedlings. Twelve-day-old seedlings of *pPUB13::PUB13-FLAG* transgenic plants were treated with 50 μM MG132 for 1 hr before H_2O or 1 μM flg22 treatment for 10 min. * indicates the specific band of PUB13-FLAG in *pPUB13::PUB13-FLAG* transgenic plants. **(D)** flg22-induced FLS2-PUB13 association depends on BAK1. **(E)** flg22 stimulates rapid association of FLS2-PUB13. The above experiments were repeated at least three times with similar results.

Fig. 2. BAK1 phosphorylates PUB12 and PUB13
(**A**) BAK1 phosphorylates PUB12 and PUB13 *in vitro*. An *in vitro* kinase assay was performed by incubating MBP fusion protein of BAK1 cytosolic domain (BAK1) or BAK1Km together with MBP, MBP-PUB12 or MBP-PUB13. Phosphorylation was analyzed by autoradiography (top panel), and the protein loading control was shown by Coomassie blue staining (bottom panel). (**B**) flg22 enhances PUB13 phosphorylation by BAK1. BAK1-FLAG or BAK1Km-FLAG was expressed in WT or *fls2* protoplasts for 8 hr followed by 1 μM flg22 treatment for 10 min. BAK1-FLAG was immunoprecipitated with an anti-FLAG antibody, and subjected to a kinase assay with MBP or MBP-PUB13 as substrate. The top band is phosphorylated PUB13 as indicated with an asterisk *, and the bottom band is BAK1 autophosphorylation. (**C**) Kinase inhibitor K252a suppresses FLS2 and PUB13 association. The above experiments were repeated at least three times with similar results.

Fig. 3. PUB12 and PUB13 ubiquitinate FLS2
(**A**) PUB12 and PUB13 ubiquitinate FLS2. The HA-tagged FLS2 cytosolic domain was purified as MBP fusion protein. The ubiquitination of FLS2 was detected by an anti-HA antibody. The overall ubiquitination was detected by an anti-FLAG antibody. (**B**) C262 and W289 are required for PUB13 E3 ligase activity. (**C**) PUB13 ubiquitinates FLS2, but not BAK1 or BIK1. The above experiments were repeated three times with similar results.
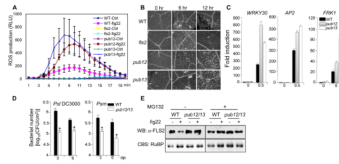
Fig. 4. Function of PUB12 and PUB13 in FLS2 signaling

(A) ROS burst in *Arabidopsis* leaves triggered by flg22. Leaf discs were treated with H_2O (Ctrl) or 100 nM flg22. The data are shown as means ± standard errors from 40 leaf discs. **(B)** flg22-induced callose deposition. Leaves were treated with 1μM flg22 for 6 and 12 hr. **(C)** flg22-induced gene induction. Twelve-day-old *Arabidopsis* seedlings were treated with 10 nM flg22 for 0.5 or 6 hr. The data are shown as means ± standard errors from 3 independent biological repeats. **(D)** The bacterial growth assay of DC3000 and *Psm*. Four-week-old *Arabidopsis* plants were inoculated with bacteria at a concentration of $5×10^5$ cfu/ml. The data are shown as means ± standard errors from 3 replicates. * indicates a significant difference with $p<0.05$ when compared with data from WT plants. **(E)** PUB12/13 promote FLS2 degradation. Twelve-day-old seedlings were treated with or without 50 μM MG132 for 1 hr before 1 μM flg22 treatment for 0.5 hr. The equal protein loading was shown by Coomassie blue staining (CBS) for RuBisCo (bottom panel). The above experiments were repeated three to four times with similar results.