Materials and Methods

Plant material and growth conditions

Wild-type (Col-0), *fls2*, *bak1-4*, *pub12-1*, *pub12-2*, *pub13* and *pub12/pub13* mutant *Arabidopsis* plants were grown in a growth room at 23°C, 60% relative humidity, 75 μE light with a 12 hr photoperiod for 30 days before protoplast isolation or bacterial inoculation. The *pub12-1* (SAIL_35_G10), *pub12-2* (WiscDsLox497_01), and *pub13* (SALK_093164) mutants were obtained from Arabidopsis Biological Resource Center (ABRC), and confirmed by PCR and RT-PCR analyses. The *pub12/pub13* double mutant was generated by the genetic cross between *pub12-2* and *pub13,* and confirmed by PCR and RT-PCR analyses. Seedlings were grown on ½MS plates with 0.5% sucrose and 0.9% agar at 23°C and 75 μE light with a 12 hr photoperiod for 12 days. Seedlings were transferred to 2 ml H_2O in the 6-well tissue culture plates one day before flg22 treatment for RT-PCR analysis or protein extraction. All experiments were repeated 3-4 times with reproducible results. Typical results with statistical analyses are shown.

cDNA library construction and yeast two-hybrid screen

Total RNA was isolated from 4-week-old *Arabidopsis* plants inoculated with DC3000 *hrcC* at the concentration of 10^8 cfu/ml for 2 hr and 6 hr. The mRNA was obtained with PolyAtract mRNA isolation system (Promega). The first- and second-strand cDNA synthesis was performed by standard molecular cloning protocols with an oligo (dT)-XhoI primer and EcoRI adaptor. The cDNA was XhoI digested, size-fractionated, and ligated with EcoRI/XhoI digested modified pGADT7 vector (Clontech). About 100,000 clones were screened for interaction with the BAK1 kinase domain in a DNA-binding domain fusion vector pBridge (Clontech) in the medium SD-T-L-H-A. The positive clones were further confirmed with additional yeast two-hybrid assays with empty vector controls, and the gene identity was revealed by sequencing and BLAST search.

Plasmid constructs, protoplast transient assay, and generation of transgenic plants.

Arabidopsis BAK1, *FLS2*, and *BIK1* constructs were reported previously (*11*). *PUB12*, *PUB13*, *PUB14* and *PUB29* genes were amplified by PCR from Col-0 cDNA, and introduced into a plant expression vector with an HA or FLAG epitope-tag at the C terminus. UBQ10 was cloned into a plant expression vector with an FLAG epitope-tag at the N terminus. Based on the cDNA

sequences of our PUB12 clones and the expressed sequence tag (EST) database, the C terminus of the protein was apparently mis-annotated in the database of The Arabidopsis Information Resource (TAIR). Figure S1 shows the correct sequences of PUB12 with 65% identity and 79% similarity with PUB13 at the amino acid level. PUB13 and FLS2 point mutations were generated by a site-directed mutagenesis kit (Stratagene). The primer sequences for different PUBs, PUB13 and FLS2 point mutations and truncations are listed in Supplemental Experimental Procedures. Full-length PUBs, cytosolic domain or kinase domain of BAK1 and FLS2 were sub-cloned into a modified GST or MBP fusion protein expression vector pGEX4T-1 (Pharmacia) or pMAL-C2 (New England Biolabs) with BamHI/NcoI and StuI digestion. Protoplast transient assay was carried out as described (*29*). For Co-IP assays, 1 ml protoplasts were transfected with 200 μg of DNA. The *PUB13* transgenic plants in Col-0 and *pub13* mutant were generated by *Agrobacterium*-mediated transformation with the *PUB13* cDNA in pCB302 vector under the control of its native promoter with an FLAG tag. The PUB13 promoter was amplified from genomic DNA of Col-0 with primers 5'-CCGCTCGAGGAGCTCACTAGTTTCTTCATTGAGACCAATATC-3' and 5'-CATGCCATGGTGAATTGATTCTTCTCTG-3'.

Co-immunoprecipitation, *in vitro* phosphorylation and immunocomplex kinase assays.

For transgenic plants, twelve-day-old seedlings or leaves of 4-week-old soil-grown plants carrying the *pPUB13::PUB13-FLAG* transgene were ground with 1 ml of extraction buffer (10 mM HEPEs, pH7.5, 100 mM NaCl, 1 mM EDTA, 10% Glycerol, 0.5% Triton X-100 and protease inhibitor cocktail from Roche). For protoplasts, samples were lyzed with 0.5 ml of extraction buffer. After vortexing vigorously for 30 sec, the samples were centrifuged at 13,000 rpm for 10 min at 4°C. For the co-IP assay, the supernatant was incubated with an anti-HA or anti-FLAG antibody for 2 hr, and then protein-G-agarose beads for another 2 hr at 4°C with gentle shaking. The immunoprecipitated proteins were analyzed by Western blot with an anti-HA, -FLAG or -FLS2 antibody. The protein bands with appropriate molecular weight are shown.

Expression of the GST and MBP fusion proteins and affinity purification were performed as standard protocol, and *in vitro* phosphorylation and immunocomplex kinase assays were carried out as described (*11*).

In vitro ubiquitination assay

The *in vitro* ubiquitination assays were performed as described with some modifications (*30*). The reactions contain 500 ng of substrate protein, 250 ng of purified His_6-E1 (AtUBA1), 500 ng of purified His_6-E2 (AtUBC8), 1.25 μg of FLAG tagged ubiquitin (Boston Biochem) and 1 μg of purified GST-PUB in the ubiquitination buffer (0.1 M Tris-HCl pH 7.5, 25 mM $MgCl_2$, 2.5 mM dithiothreitol, 10 mM ATP) to a final volume of 30 μl. The reactions were incubated at 30°C for 2 hr, and then stopped by adding SDS sample buffer and boiled at 100°C for 5 min. The samples were then separated by 7.5% SDS–PAGE and the ubiquitinated substrates were detected by Western blotting analysis.

Measurement of ROS production

ROS burst was determined by a luminol-based assay as described with modifications (*31*). Four to five leaves of each five-week-old *Arabidopsis* plant were excised into leaf discs of 0.25 cm^2, following an overnight incubation in 96-well plate with 100 μl of H_2O to eliminate the wounding effect. H_2O was replaced by 100 μl of reaction solution containing 50 μM of luminol and 10 μg/ml of horseradish peroxidase (Sigma) supplemented with 100 nM of flg22. The measurement was conducted immediately after adding the solution with a luminometer (Perkin Elmer, 2030 Multilabel Reader, Victor X3), with a 1 min interval reading time for a period of 20 min. The measurement values for ROS production from 40 leaf discs per treatment were indicated as means of RLU (Relative Light Units). The experiments were repeated four times and similar results were obtained.

Callose deposition

Callose deposition was conducted as described by Wang et al. with modifications (*32*). Briefly, two to three leaves of 5-week-old Col-0 and *pub* mutant plants were infiltrated with 1 μM of flg22, and leaves were excised 6 and 12 hr after infiltration. Control treatments were infiltrated with H_2O. Excised leaves were immediately cleared in alcoholic lactophenol [95% ethanol: lactophenol (phenol : glycerol : lactic acid : H_2O=1:1:1:1) = 2:1] for overnight. Samples were subsequently rinsed with 50% ethanol and H_2O. Cleared leaves were stained with 0.01% aniline

blue in 0.15 M phosphate buffer (pH = 9.5) and the callose deposits were visualized under a UV filter using a fluorescence microscope. Callose deposits were counted using ImageJ 1.43U software (http://rsb.info.nih.gov/ij/). The number of deposits was expressed as the mean of three different leaf areas and analyzed using the general linear model of SAS (SAS Institute, Inc., Cary, NC), with mean separations by least significant difference (LSD). At least three independent experiments were conducted with similar results obtained.

Pathogen infection assays

P. syringae tomato DC3000 and *P. syringae maculicola* ES4326 strains were grown overnight at 28°C in the KB medium with 50 µg/ml rifampicin for DC3000 and 50 µg/ml streptomycin for ES4326. Bacteria were collected, washed and diluted to the desired density with H_2O. Four-week-old *Arabidopsis* leaves were infiltrated with bacteria at a concentration of $5x10^5$ cfu/ml using a needleless syringe. To measure bacterial growth, two leaf discs were ground in 100 µl H_2O and serial dilutions were plated on KB medium with appropriated antibiotic. Bacterial colony forming units (cfu) were counted 2 days after incubation at 28°C. Each data point is shown as triplicates (*33*).

29. L. Shan *et al.*, *Cell Host Microbe* **4**, 17 (Jul 17, 2008).
30. T. R. Rosebrock *et al.*, *Nature* **448**, 370 (Jul 19, 2007).
31. X. Gao *et al.*, *Plant J* **66**, 293 (Apr, 2011).
32. L. Wang *et al.*, *PLoS Pathog* **5**, e1000301 (Feb, 2009).
33. P. He *et al.*, *Cell* **125**, 563 (May 5, 2006).

Primers for construct cloning and point mutations

Primer Name	Sequences
PUB12-BamHI-F	CG GGATCC ATGGCGAAATCAGAGAAACAC
PUB12-Smal-R	TCCCCCGGGGATTAGGGAGATTTGATCTTCC
PUB13-Nco1-F	CATGCCATGGAGGAAG AGAAAGCTTC
PUB13-Stu1-R	GAAGGCCTAGTATCTGCAGCTTCTGTGG
PUB13-ARM-NcoI-F	CATGCCATGGAGCCT CCAAAGCCTCCGAG
PUB13 Promoter-XhoI/SacI/SpeI-F	CCG CTCGAG GAGCTC ACTAGT TTCTTCATTGAGACCAATATC
PUB13 Promoter-NcoI-R	CATGCCATGGTGAATTGATTCTTCTCTG
PUB14-NcoI-F	CATG CC ATGGGATTAACGAATTGTTG
PUB14-StuI-R	GAAGGCCTTGGAACAGTAGTTACTGC
PUB29-BamHI/NcoI-F	CG GGATCC ATGGGGAGAG ATGAAACAG
PUB29-StuI-R	GAAGGCCTAAAAGGCATAATATGAGTAG
PUB13-C262A-F	TGATGATTTT CGCGCTCCGA TTTCGCTG
PUB13-C262A-R	CAGCGAAATCGGAGCGCGAAAATCATCA
PUB13-W289A-F	CATGTATTGA GAAAGCGATA GAAGGTGG
PUB13-W289A-R	CCACCTTCTATCGCTTTCTCAATACATG
UBQ10-BamH1-F	CGGGATCCATGCAGATCT TTGTTAAGAC TCTCACC
UBQ10-Stu1-R	AACTGCAGAGGCCTTTAACCACCACGGAGCCTGAGG
FLS2-T867V-F	TTGG AGCAAGCAGTAGATTCATTC AAC
FLS2-T867V-R	GTTGAATGAATCTACTGCTTGCTCCAA
FLS2-K898M-F	GATTGCAGTAATGGTATTGAATCTAAAG
FLS2-K898M-R	CTTTAGATTCAATACCATTACTGCAATC
FLS2-P1076A-F	GACGAAAC AGAGAGCAAC TTCGTTGAAT G
FLS2-P1076A-R	CATTCAACGAAGTTGCTCTCTGTTTCGTC

Primers for RT-PCR analysis

PUB12-F	CAAGCTCCACCTGTTCTTTAAGT
PUB12-R	CCTGGTGTTGGAGAGAGATCA
PUB13-F	GATTGCTGCGATTTCTGA
PUB13-R	TTATCATCCTCCGTCTGC

Primers for confirming T-DNA insertions in pub knockout

pub12-2-LP	TAACCACAGCTACCCAAAACG
pub12-2-RP	TAATTTCCTAATTTGGCCGTG
pub13-LP	AAGAGGTATGGCTCCAGCTTC
pub13-RP	ACGTGCTTTGTTTTGCTATGG

Supplemental data

Fig. S1. The alignment of the amino acid sequences of PUB12 and PUB13. The UND domain, U-box and ARM repeats are indicated. The conserved cystein (C) and tryptophan (W) residues are highlighted in yellow.

Supplemental data

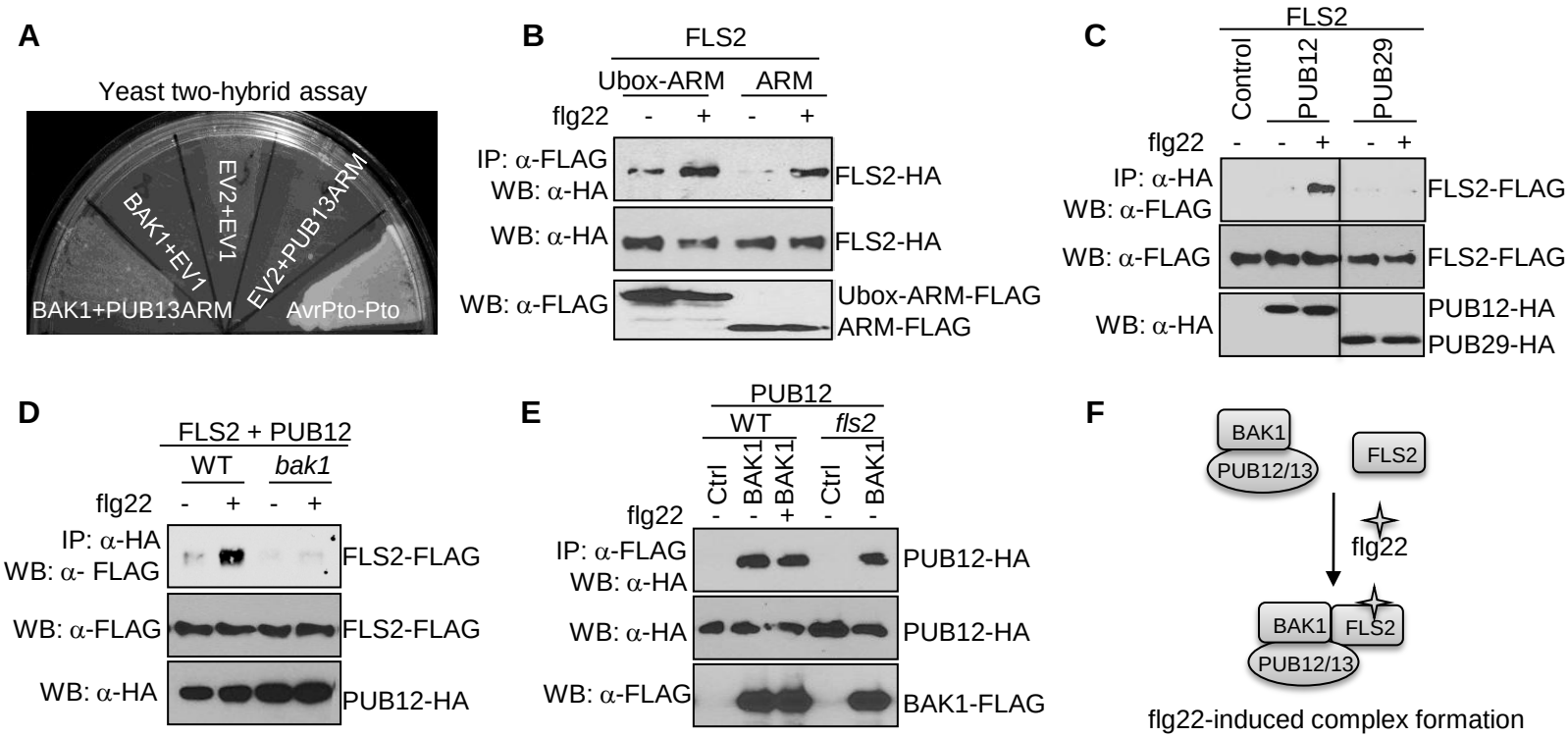

Fig. S2. Association of PUB12/13 with FLS2 and BAK1. (A) BAK1 kinase domain interacts with the PUB13 ARM domain in a yeast two-hybrid assay. EV, empty vector. The interaction of AvrPto and Pto is a positive control. The yeast colonies were grown on a SD-L-T-A-H plate at 30˚C for 4 days. **(B)** The ARM domain of PUB13 is sufficient to associate with FLS2. 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 FLS2 co-immunoprecipitates with PUB13 ARM domain or Ubox-ARM domain. The middle and bottom panels show the protein expression of FLS2-HA and FLAG tagged PUB13Ubox-ARM or PUB13ARM. Protoplasts were stimulated with 1 μM flg22 for 10 min. **(C)** flg22 induces FLS2-PUB12, not FLS2-PUB29 association. **(D)** flg22-induced FLS2-PUB12 association depends on BAK1. FLS2-FLAG and PUB12-HA were co-expressed in WT or *bak1-4* mutant protoplasts. **(E)** BAK1-PUB12 interaction is independent of FLS2. The above experiments were repeated at least three times with similar results. (**F**) A model of flg22-induced FLS2/BAK1/PUB12/13 complex formation.

Fig. S2

Supplemental data

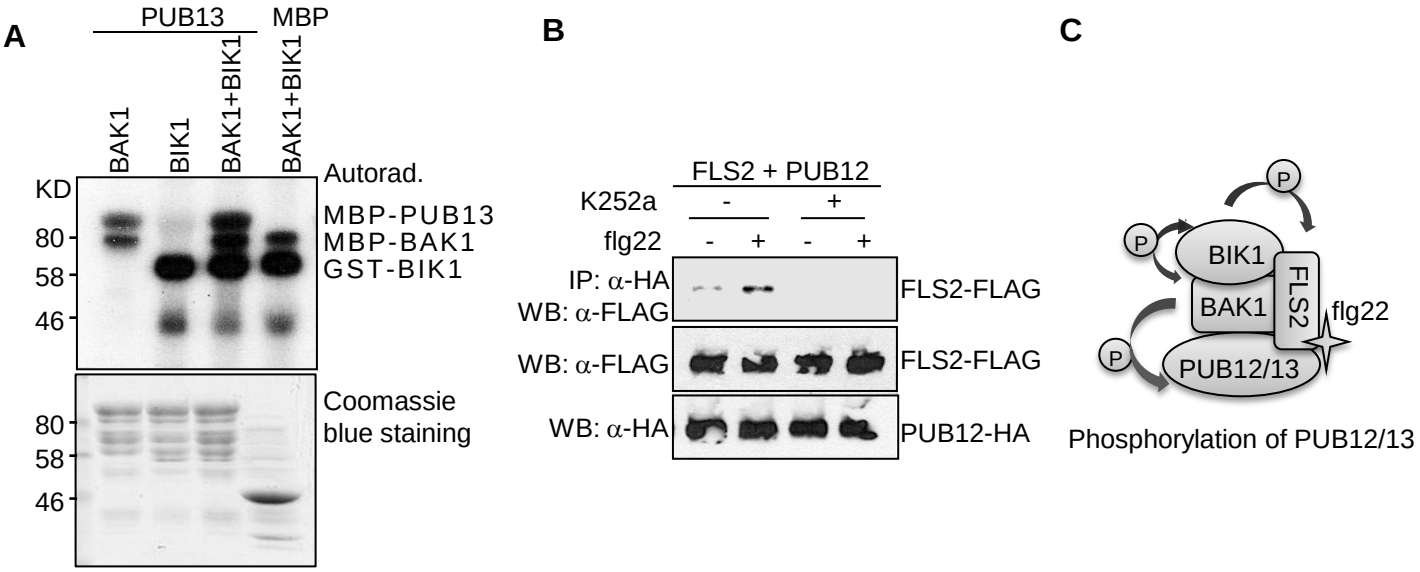

Fig. S3. Phosphorylation events in FLS2/BAK1/BIK1/PUB12/13 complex. **(A)** BIK1 enhances BAK1 phosphorylation of PUB13. MBP-PUB13, MBP-BAK1 and GST-BIK1 were used in an *in vitro* kinase assay. **(B)** Kinase inhibitor K252a suppresses FLS2 and PUB12 association. The above experiments were repeated three times with similar results. **(C)** A model of phosphorylation events in FLS2/BAK1/BIK1/PUB12/13 complex.

Supplemental data

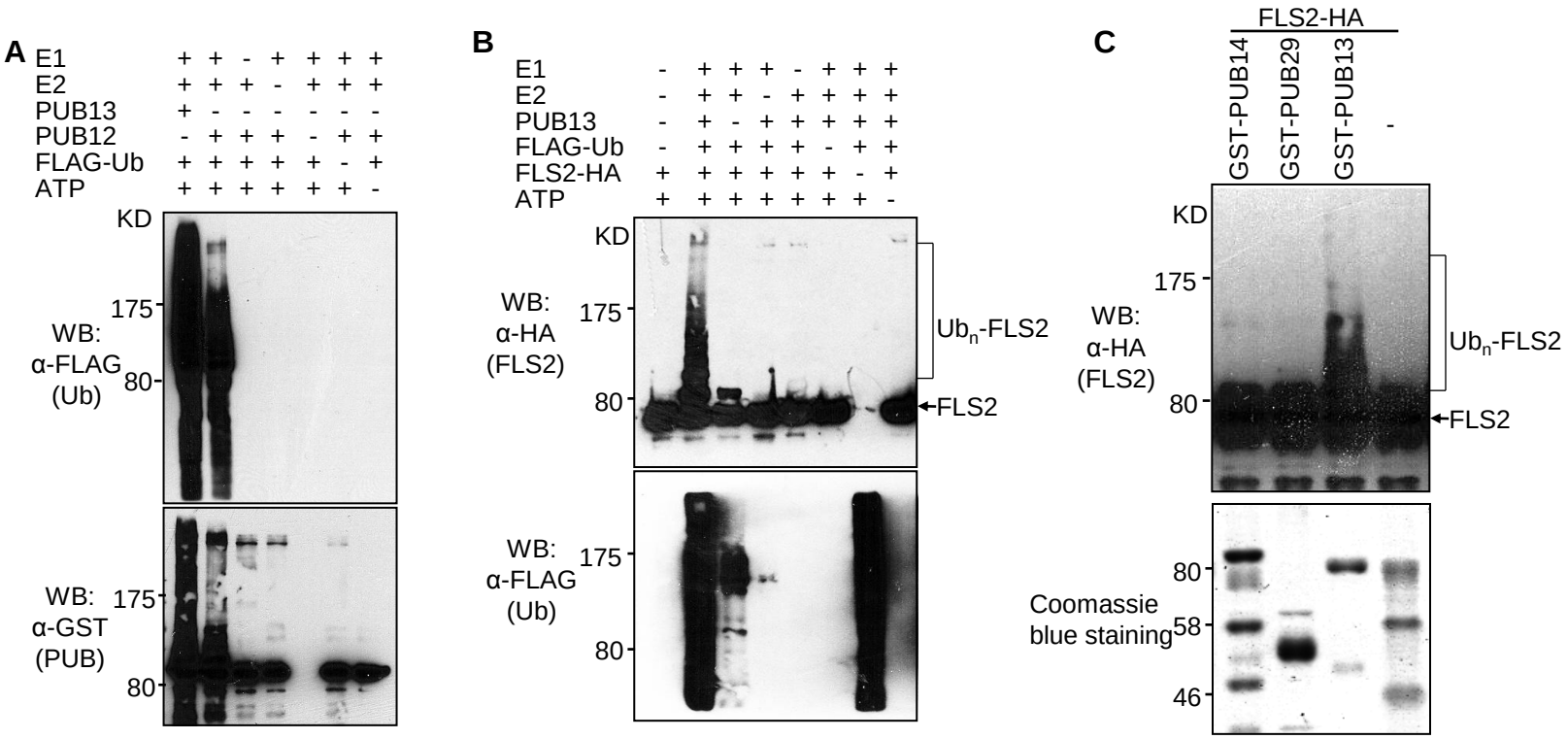

Fig. S4. Auto-ubiquitination of PUB12/13 and the specificity of PUB13 ubiquitination on FLS2. (A) PUB12 and PUB13 possess E3 ubiquitin ligase activity. An *in vitro* ubiquitination assay was performed with GST-PUB12 or GST-PUB13 in combination with E1, E2, FLAG-Ub and ATP. Protein ubiquitination was detected by Western blot with an anti-FLAG or anti-GST antibody. **(B)** FLS2 ubiquitination by PUB13 in an *in vitro* ubiquitination assay with the components as indicated. **(C)** An *in vitro* ubiquitination assay was performed with MBP-FLS2-HA, E1, E2, FLAG-Ub, ATP and different GST-PUBs. The bottom panel shows the protein control of different GST-PUBs. The above experiments were repeated three or four times with similar results.

Supplemental data

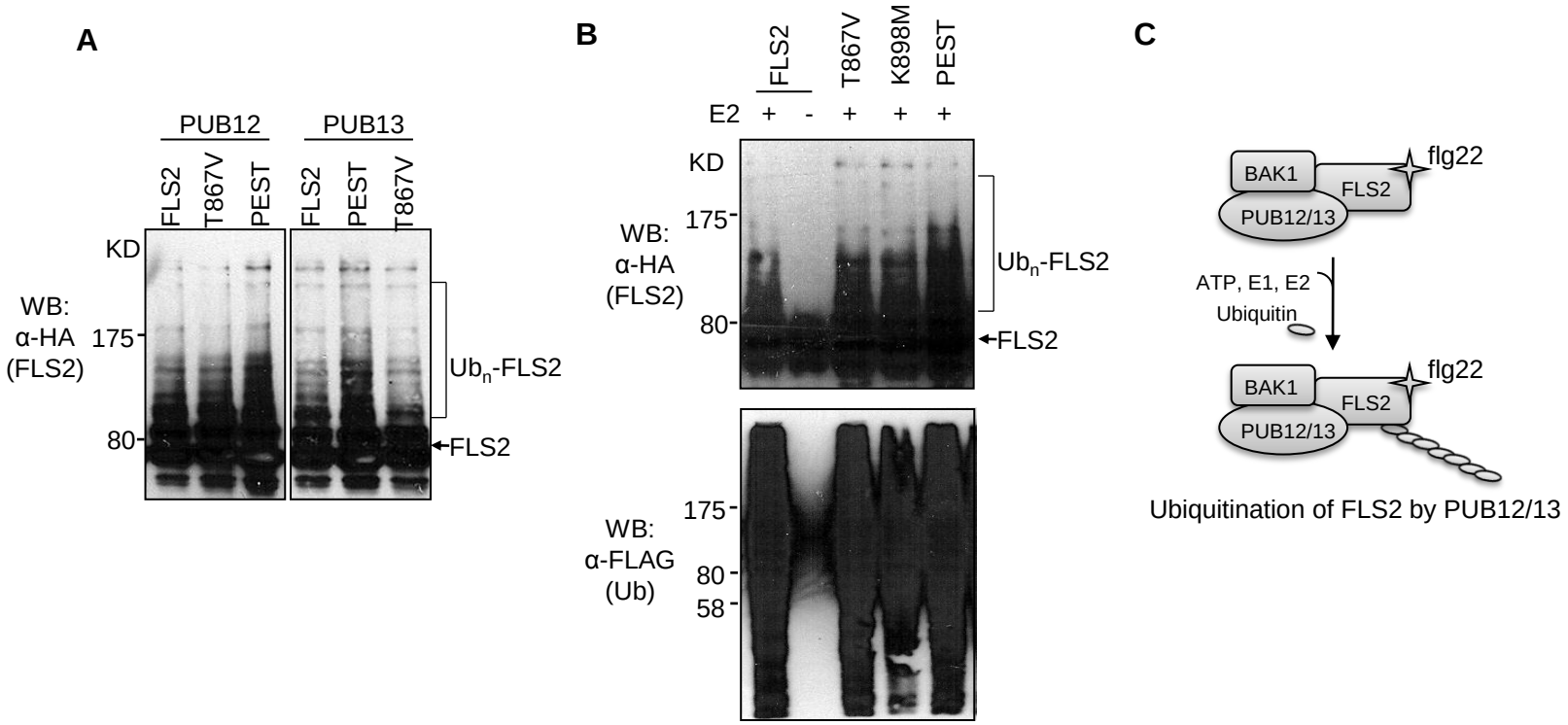

Fig. S5. FLS2 ubiquitination by PUB12/13. (A) PEST and T867 are not required for FLS2 ubiquitination by PUB12 or PUB13. **(B)** Kinase activity is not required for FLS2 ubiquitination by PUB13. The ubiquitination of MBP-FLS2-HA, MBP-FLS2T867V-HA, MBP-FLS2K898M-HA or MBP-FLS2PEST(P1076A)-HA was tested with GST-PUB12 or GST-PUB13 in an *in vitro* ubiquitination assay. The above experiments were repeated at least three times with similar results. **(C)** A model of FLS2 ubiquitination by PUB12 and PUB13.

Supplemental data

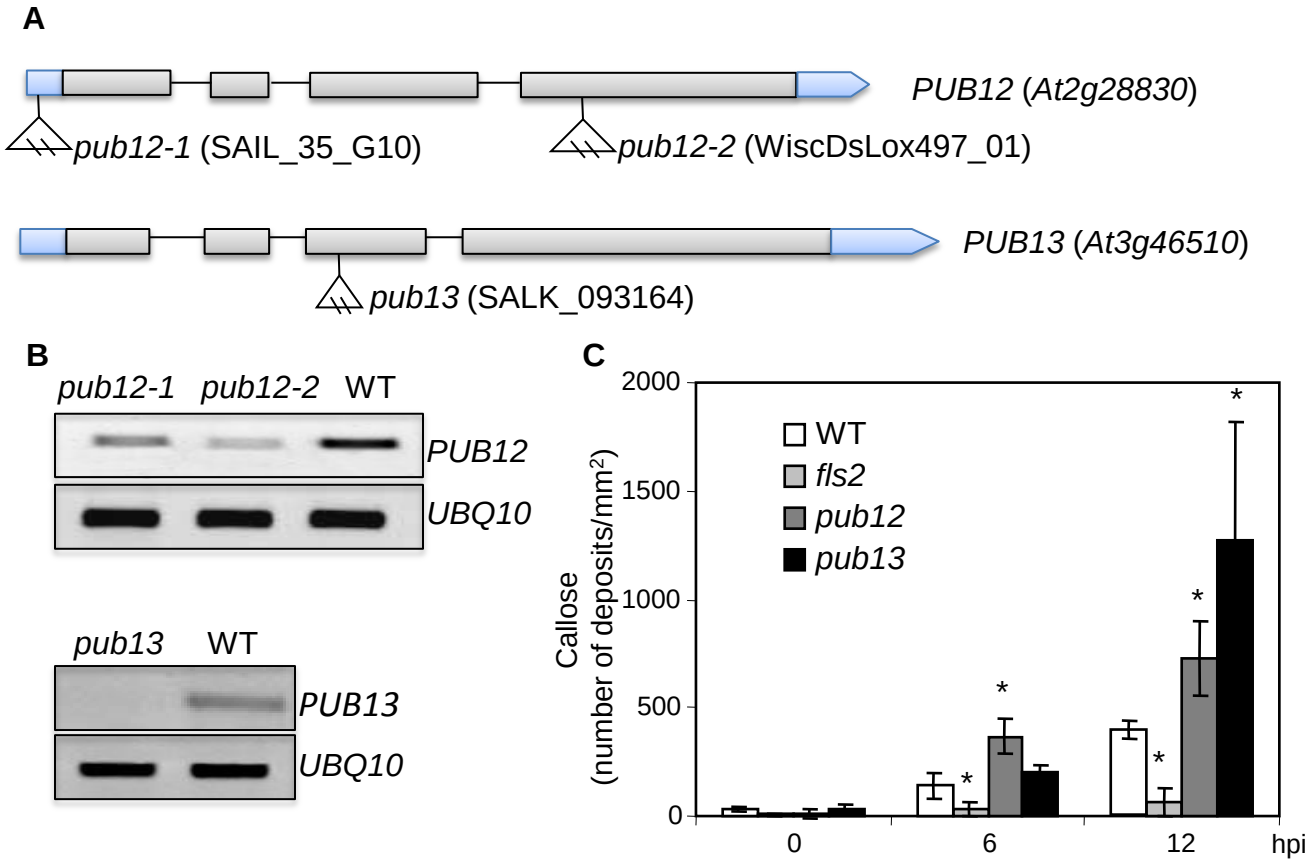

Fig. S6. Analysis of *pub12* and *pub13* mutants. (A) T-DNA insertion sites in *pub* mutants with exons (gray boxes). **(B)** RT–PCR analysis of *PUB12*, *PUB13* and *UBQ10* (control) in WT and *pub* mutant plants. **(C)** Statistical analysis of callose deposition in WT, *pub12* and *pub13* mutant plants. The above experiments were repeated three times with similar results.

Fig. S6

Supplemental data

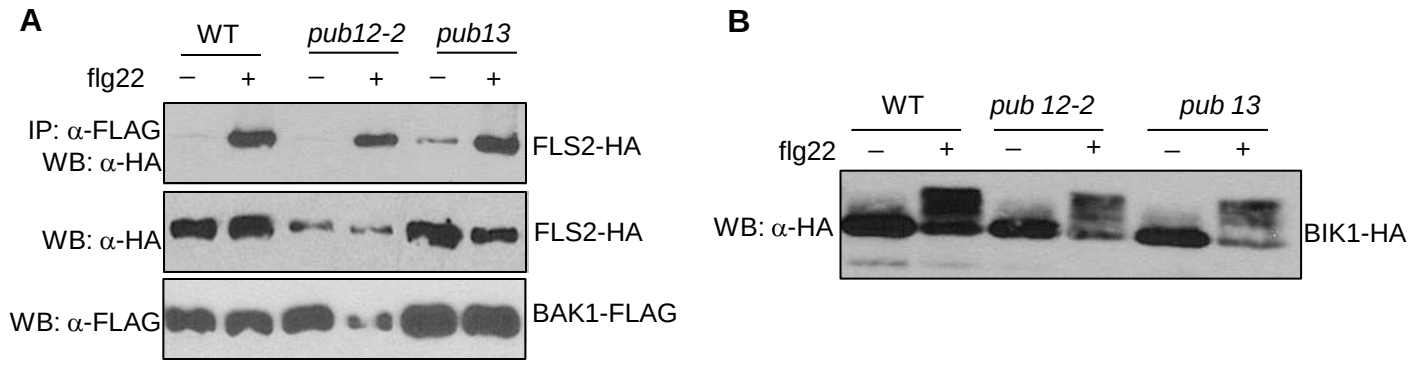

Fig. S7. PUB12 and PUB13 are not required for FLS2/BAK1 association and BIK1 phosphorylation. The protoplasts were isolated from WT, *pub12-2* and *pub13* plants. **(A)** FLS2-HA and BAK1-FLAG were co-expressed in protoplasts for 6 hr before 1 μM flg22 treatment for 10 min for Co-IP assay. **(B)** BIK1-HA was expressed in protoplasts for 6 hr before 1 μM flg22 treatment for 10 min for Western blot. The above experiments were repeated three times with similar results.

Supplemental data

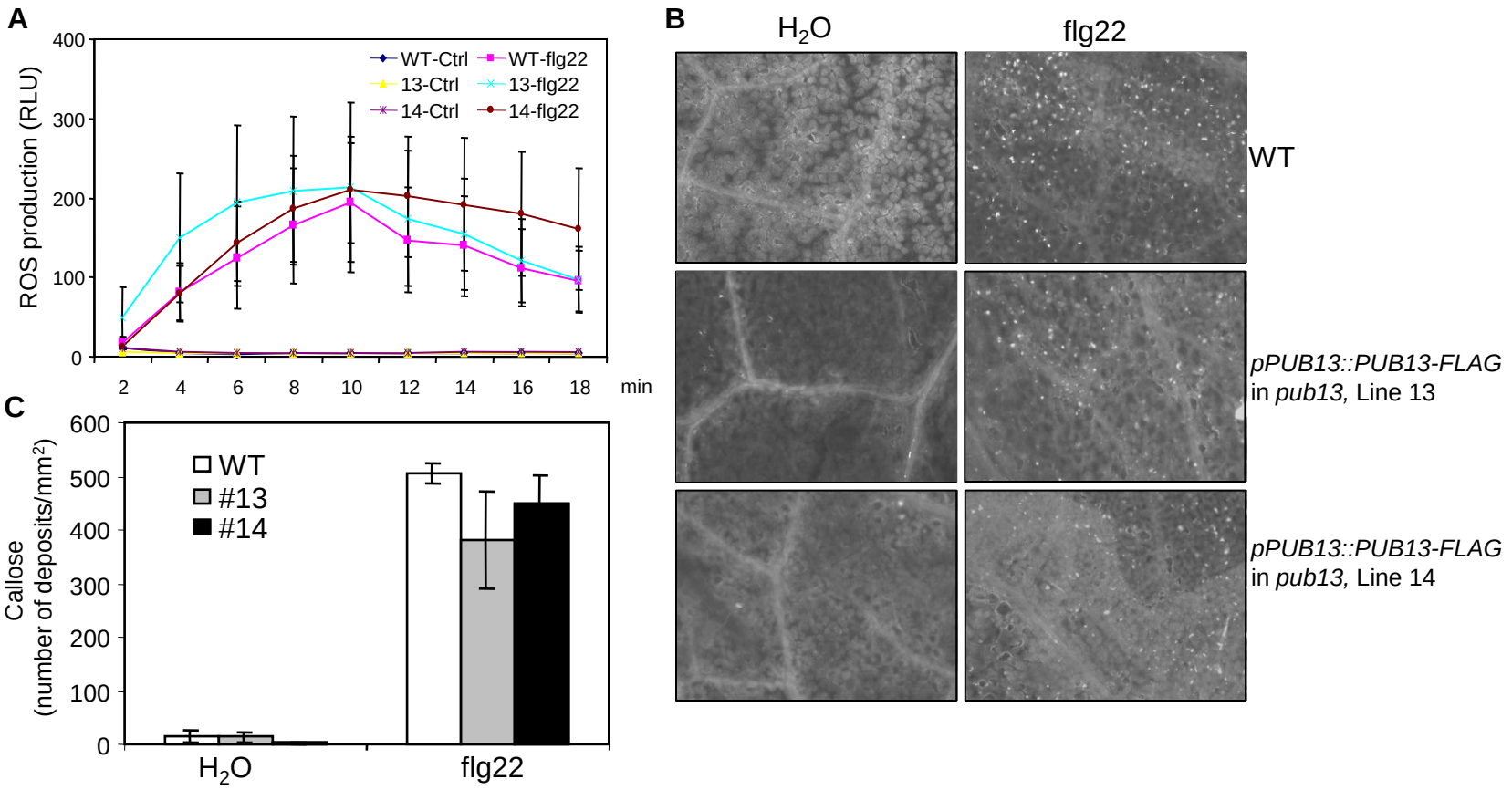

Fig. S8. The *pPUB13::PUB13-FLAG* construct complements *pub13* mutants. (A) flg22-triggered ROS burst in WT and *pPUB13::PUB13-FLAG/pub13* plants. 13 and 14 are two independent transgenic lines. **(B)** flg22-induced callose deposition in WT and *pPUB13::PUB13-FLAG/pub13* plants. Callose deposits were detected 12 hr after 1μM flg22 treatment by aniline blue staining. **(C)** Statistical analysis of callose deposition in WT and *pPUB13::PUB13-FLAG/pub13* plants. The above experiments were repeated three times with similar results.

Fig. S8

Supplemental data

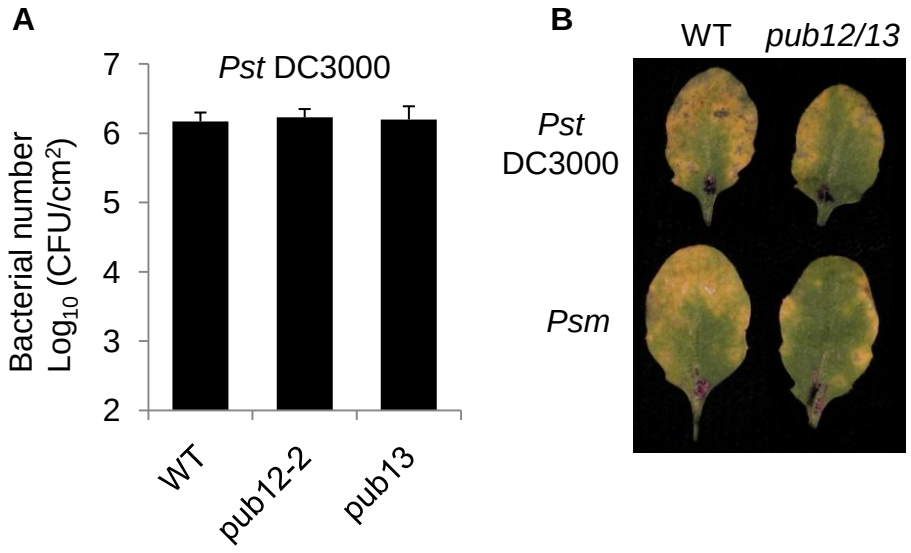

Fig. S9. Bacterial growth assay in *pub12*, *pub13 and pub12/13* mutants. (A) Four-week-old *Arabidopsis* plants were inoculated with *Pst* DC3000 at a concentration of 5 x10^5 cfu/ml. The bacterial counting was performed 3 days post-inoculation (dpi). The data are shown as means ± standard errors from 3 replicates. **(B)** Phenotype of bacterial infection in WT and *pub12/13* mutants. Four-week-old *Arabidopsis* plants were inoculated with *Pst* DC3000 and *Psm* at a concentration of 5 x10^5 cfu/ml. The picture was taken 6 days post-inoculation. The above experiments were repeated four times with similar results.

Supplemental data

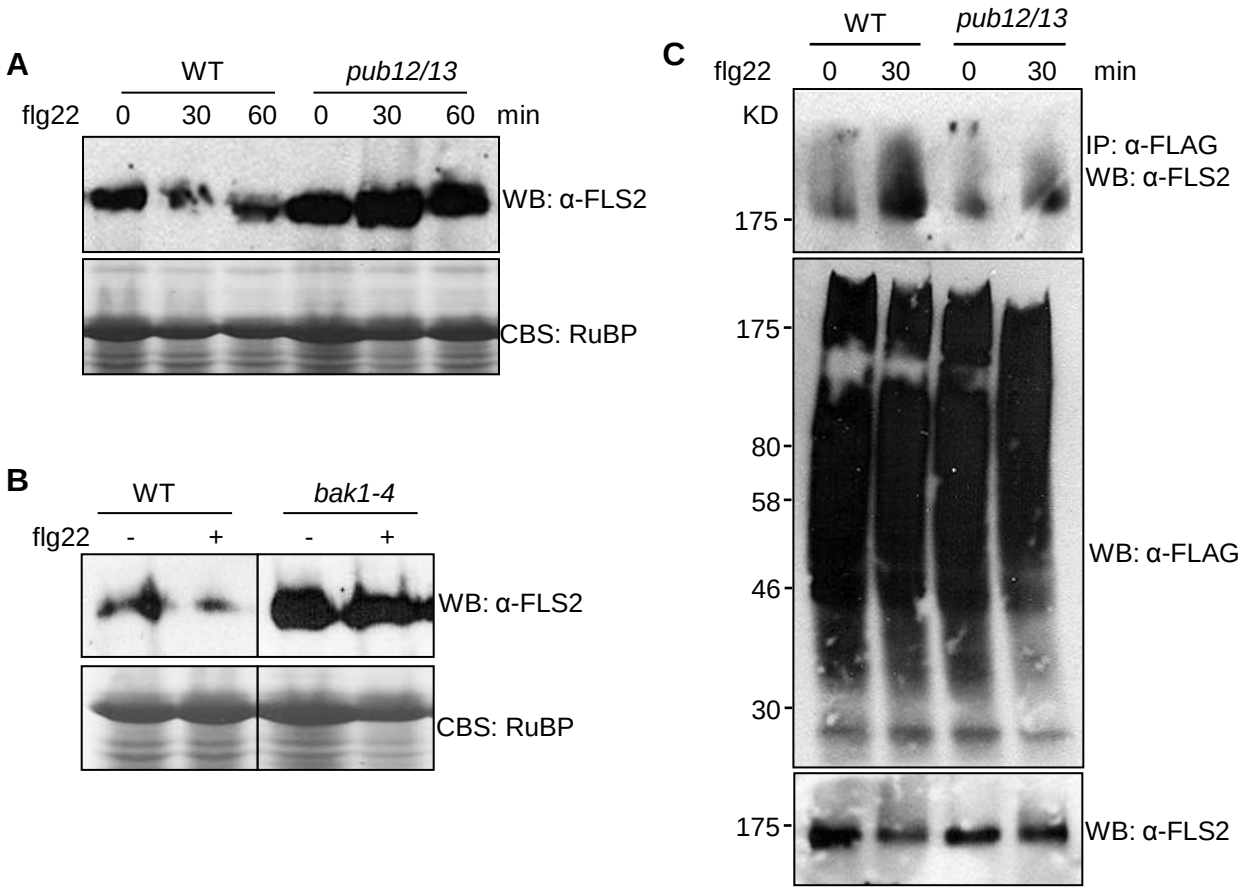

Fig. S10. FLS2 level in *pub12/13* and *bak1* mutants and *in vivo* FLS2 ubiquitination. (A) Four-week-old WT and *pub12/13 Arabidopsis* plants were inoculated with 1 μM flg22 for 30 and 60 min. **(B)** Four-week-old WT and *bak1-4 Arabidopsis* plants were inoculated with 1 μM flg22 for 20 min. The protein level of FLS2 was detected by an anti-FLS2 antibody (top panel). The equal protein loading was shown by Coomassie blue staining (CBS) for RuBisCo (bottom panel). **(C)** *in vivo* FLS2 ubiquitination in WT and *pub12/13* mutant. Protoplasts were transfected with N terminal FLAG-tagged UBQ10, and incubated for 10 hr before 1 μM flg22 treatment for 30 min. The ubiquitinated FLS2 was detected by an anti-FLS2 antibody after anti-FLAG antibody immunoprecipitation. The total ubiquitinated proteins were detected by an anti-FLAG antibody, and the expression of FLS2 was detected by anti-FLS2. The above experiments were repeated three to four times with similar results.

Fig. S10

Supplemental data

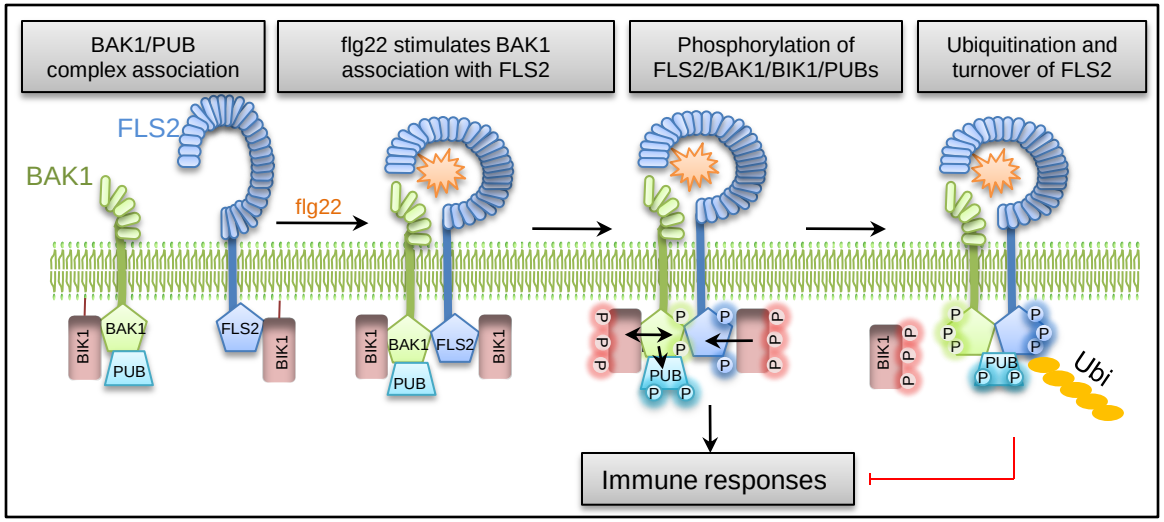

Fig. S11. A proposed model of PUB12/13-mediated ubiquitination of FLS2 in the attenuation of flagellin signaling.

Fig. S11

Supplemental data

Table S1. Putative BAK1-interacting proteins from a yeast two-hybrid screen

AGI	Annotation
AT1G01140	CIPK9 (CBL-interacting protein kinase 9)
AT1G20620	Catalase 3
AT1G66200	ATGSR2 (Arabidopsis thaliana glutamine synthase clone R2)
AT1G67880	Glycosyl transferase family 17 protein
AT3G15950	Unknown protein
AT3G46510	PUB13 (Plant U-box 13)
AT3G47910	Ubiquitin thiolesterase
AT4G30996	Unknown protein
AT4G39660	AGT2 (Alanine:glyoxylate aminotransferase 2)
AT5G18230	Transcription regulator NOT2/NOT3/NOT5 family protein