Supporting Information

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SI Materials and Methods

Plant Materials, Growth Conditions, and Treatments. *A. thaliana* (L.) Heynh. (Columbia ecotype, Col-0; referred to *Arabidopsis*) plants were grown in soil (Metro Mix 366) in a growth room with 23 °C, 45% relative humidity, 85 $\mu E \cdot m^{-2} \cdot s^{-1}$ light and a photoperiod of 12 h light/12 h dark for 4 wk before protoplast isolation. To grow *Arabidopsis* seedlings on medium the seeds were surface-sterilized with 50% bleach for 15 min, washed with sterilized double-distilled H_2O (ddH_2O) and then placed on the plates with half-strength Murashige and Skoog medium (1/2 MS) containing 0.5% sucrose, 0.8% agar, and 2.5 mM MES at pH 5.7. The plates were first stored at 4 °C for 3 d in the dark for seed stratification and then moved to the growth room for different periods of time depending on the experiments.

For kymograph analysis of BRI1 dwell time in the PM seeds were plated on 1/2 MS medium containing 0.8% agar and 1% sucrose, adjusted to pH 5.8 with 20 mM MES, and grown in the darkness for 5 d after 4 h of light. For microsomal protein preparation plants were grown for 6 d on plates. For BR growth assay (hypocotyl growth) plants were grown for 5 d under continuous-light condition. For BRI1 internalization assay and BRI1 transcript analysis plants were grown for 5 d on plates under a 16-h/8-h light–dark cycle. BL (10 mM stock in DMSO), BRZ (20 mM stock in DMSO), and CHX (50 mM stock in DMSO) were used at the concentrations indicated in the figure legends.

WB Analysis and Immunoprecipitation. For BES1 dephosphorylation analysis and BRI1 detection 5-d-old seedlings were homogenized in liquid nitrogen. Total proteins were extracted with buffer containing 20 mM Tris·HCl, pH 7.5, 150 mM NaCl, 1% SDS, 100 mM DTT, and EDTA-free protease inhibitor mixture complete (Roche). For blocking and antibody dilutions 5% BSA powder in Tris-buffered saline was used. For protein detection the following antibodies were used: monoclonal α-GFP horseradish peroxidase-coupled (1/5,000; Miltenyi Biotech), α-ubiquitin P4D1 (1/2,500; Millipore), polyclonal α-BES1 (1) (1/4,000), polyclonal α-BRI1 kindly provided by Michael Hothorn, Department for Botany and Plant Biology, University of Geneva (1/5,000), monoclonal α-Tubulin (1/10,000; Sigma-Aldrich), and α-pT/pS antibodies (1/2,000; Millipore,). ProQ was purchased from Invitrogen. For BES1 dephosphorylation assay the ratio of the dephosphorylated BES1 to the total BES1 proteins was quantified based on the signal intensity. The loading was adjusted to an equal level based on the amount of Tubulin. For microsomal fraction isolation, 6-d-old seedlings were ground in liquid nitrogen and resuspended in ice-cold sucrose buffer [100 mM Tris, pH 7.5, 810 mM sucrose, 5% (vol/vol) glycerol, 10 mM EDTA, pH 8.0, 10 mM EGTA, pH 8.0, 5 mM KCl, and protease inhibitor]. The homogenate was transferred to a polyvinylpolypyrrolidone pellet, mixed and left for 5 min. Samples were then centrifuged for 5 min at 600 × *g* at 4 °C. The supernatant was collected. The extraction was repeated two more times. The supernatant was filtered with miracloth mesh. The clear supernatant was combined with same amount of water and centrifuged at 4 °C for 2 h at 21,000 × *g* to pellet microsomes (2). The pellet was resuspended in IP buffer (25 mM Tris, pH 7.5, 150 mM NaCl, 0.1% SDS, and protease inhibitor). Immunoprecipitations were carried out on solubilized microsomal proteins using GFP-Trap-A (Chromotek) according to the manufacturer's protocol. For quantification of ubiquitinated BRI1 the ratios of signal intensity obtained with α-GFP and α-Ub antibodies were determined using Image Lab.

In Vitro Pull-Down Assay. Fusion proteins were expressed from a bacterial protein expression vector in *Escherichia coli* BL21 strain using LB medium supplemented with 0.25 mM isopropyl β-D-1-thiogalactopyranoside. GST fusion proteins were purified with Pierce glutathione agarose (Thermo Scientific), and MBP fusion proteins were purified using amylose resin (New England Biolabs) according to the standard protocols from the manufactures. About 10 μg of GST or GST-fused proteins were incubated with 5 μL of prewashed glutathione agarose beads in 1 mL of pull-down buffer (20 mM Tris·HCl, pH 7.5, 1 mM β-mercaptoethanol, 3 mM EDTA, 150 mM NaCl, and 1% Nonidet P-40) for 30 min at 4 °C with gentle shaking. The beads were harvested by centrifugation at 700 × *g* for 1 min and then inoculated with 10 μg of MBP or MBP-fused proteins in 1 mL of pull-down buffer for 1 h at 4 °C with gentle shaking. The beads were harvested and washed three times with 1 mL of pull-down buffer and once with 1 mL of 50 mM Tris·HCl, pH 7.5. Bound proteins were released from beads by boiling in 20 μL of 2× SDS/PAGE sample loading buffer for 5 min and analyzed by WB with an α-HA antibody.

In Vivo Co-IP Assay. *Arabidopsis* protoplasts were transfected with a pair of constructs tested (empty vector carrying GFP as the control) and incubated for 12 h. The total proteins were isolated with 0.5 mL of extraction buffer (10 mM Hepes, pH 7.5, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% Triton X-100, and 1× protease inhibitor mixture from Roche). The samples were vortexed vigorously for 30 s and then centrifuged at 16,000 × *g* for 10 min at 4 °C. The supernatant was inoculated with α-FLAG agarose beads for 2 h at 4 °C with gentle shaking. The beads were collected and washed three times with washing buffer (10 mM Hepes, pH 7.5, 100 mM NaCl, 1 mM EDTA, 10% glycerol, and 0.1% Triton X-100) and once more with 50 mM Tris·HCl, pH 7.5. Bound proteins were released from beads by boiling in SDS/PAGE sample loading buffer and analyzed by WB with an α-HA antibody.

For co-IP assays with seedlings 14-d-old seedlings grown on 1/2 MS agar plates were transferred into ddH_2O for 24 h, treated with 50 μM MG132 for 5 h, and then with 1 μM BL for another 3 h. Seedlings were ground with liquid nitrogen. The total proteins from 50 seedlings were isolated with 1 mL of extraction buffer. The samples were centrifuged twice at 16,000 × *g* for 10 min at 4 °C to remove cell debris. The supernatant was subjected to IP assay using an α-GFP antibody and protein-G-agarose, and the immunoprecipitated proteins were analyzed by WB with an α-HA antibody.

In Vitro Phosphorylation Assay. Fusion proteins were isolated as in the in vitro pull-down assays. The phosphorylation reactions were performed in 30 μL of kinase buffer (20 mM Tris·HCl, pH 7.5, 10 mM $MgCl_2$, 5 mM EGTA, 100 mM NaCl, and 1 mM DTT) containing 10 μg of substrate proteins and 1 μg of kinases with 0.1 mM cold ATP and 5 μCi [^{32}P]-γ-ATP at room temperature for 3 h with gentle shaking. The reactions were stopped by adding 4× SDS loading buffer. Phosphorylation of fusion proteins was analyzed by autoradiography after separation with 12% SDS/PAGE.

In Vitro and in Vivo Ubiquitination Assays. The in vitro ubiquitination assay was performed as described with minor modifications (3, 4). The reactions contain 1 μg of substrate proteins (different RLK_{CD} domains), 1 μg of HIS_6-E1 (AtUBA1), 1 μg of HIS_6-E2 (AtUBC8), 1 μg of HIS_6-ubiquitin (Boston Biochem), and 1 μg of GST-PUB in the ubiquitination reaction buffer (0.1 M

Tris·HCl, pH 7.5, 25 mM $MgCl_2$, 2.5 mM DTT, and 10 mM ATP) to a final volume of 30 μL. The reactions were incubated at 30 °C for 2 h and then stopped by adding SDS sample loading buffer and boiled for 5 min. The samples were then separated by 7.5% SDS/PAGE and the ubiquitinated substrates were detected by WB analysis with different antibodies recognizing the substrate proteins.

For in vivo ubiquitination assays protoplasts were cotransfected with *FLAG-UBQ* and the constructs carrying the gene of interest with an HA tag (empty vector carrying GFP as the control) and incubated for 12 h as reported previously (4). The ubiquitinated proteins were detected with an α-HA antibody WB after IP with an α-FLAG antibody.

LC-MS/MS Analysis. The LC-MS/MS analysis was performed as reported previously (5). Briefly, the in vitro phosphorylation reaction using GST-$BRI1_{CD}$ as a kinase and GST-$PUB13_{ARM}$ as a substrate was performed in a 20-μL reaction (with cold ATP only) for 2 h at a room temperature. Six individual reactions were combined and separated by 10% SDS/PAGE gel. The gel was stained with Thermo GelCode Blue Safe Protein Stain and destained with distilled H_2O. The corresponding bands were cut for MS analysis. The gel bands were in-gel-digested with trypsin overnight and phosphopeptides were enriched for LC-MS/MS analysis with a LTQ Orbitrap XL mass spectrometer (Thermo Scientific). The MS/MS spectra were analyzed with Mascot (version 2.2.2; Matrix Science), and the identified phosphorylated peptides were manually inspected to ensure confidence in phosphorylation site assignment.

Real-Time qRT-PCR. Total RNA was extracted from 5-d-old seedlings using the RNeasy kit (Qiagen). For *HS::BRI1-YFP*/Col-0 and *HS::BRI1-YFP/pub12pub13*, 5-d-old seedlings were induced at 37 °C for 1 h followed by recovery at room temperature for 1 h before RNA extraction. iScript cDNA synthesis kit (Bio-Rad) was used to synthesize cDNA from RNA. qRT-PCR analysis was done with SYBR green I Master kit (Roche) on a LightCycler 480 (Roche). Expression of *BRI1* was normalized to the expression of *Actin4* gene. The gene specific primers are listed in Table S2.

Confocal, Spinning-Disk Microscopy, and Image Analysis. Root and hypocotyl were imaged by spinning-disk ultraview microscope (PerkinElmer) equipped with 60× water (for imaging root meristem epidermal cells) or 100× oil immersion objective (for imaging hypocotyl cells). The excitation wavelength used was 515 nm provided by diode laser excitation controlled by the Volocity software and emission light was collected with an emission filter Chroma ET 525/50. Time lapses were acquired during 3 min at 500-ms intervals and images were captured with a Hamamatsu electron-multiplying CCD camera. The videos of three independent experiments were then processed with ImageJ software. For kymograph analysis a walking average of 4 was applied. Kymographs were generated with a line thickness of 3. Images were converted to 8-bit in ImageJ for BRI1-mCitrine fluorescence signal intensity measurements. Regions of interest (ROIs) were selected based on the PM or cytosol localization. Histograms listing all intensity values per ROI were generated and the averages of the 100 most intense pixels were used for calculations.

1. Yin Y, et al. (2002) BES1 accumulates in the nucleus in response to brassinosteroids to regulate gene expression and promote stem elongation. *Cell* 109:181–191.
2. Abas L, Luschnig C (2010) Maximum yields of microsomal-type membranes from small amounts of plant material without requiring ultracentrifugation. *Anal Biochem* 401:217–227.
3. Lu D, et al. (2011) Direct ubiquitination of pattern recognition receptor FLS2 attenuates plant innate immunity. *Science* 332:1439–1442.
4. Zhou J, He P, Shan L (2014) Ubiquitination of plant immune receptors. *Methods Mol Biol* 1209:219–231.
5. Li F, et al. (2014) Modulation of RNA polymerase II phosphorylation downstream of pathogen perception orchestrates plant immunity. *Cell Host Microbe* 16:748–758.

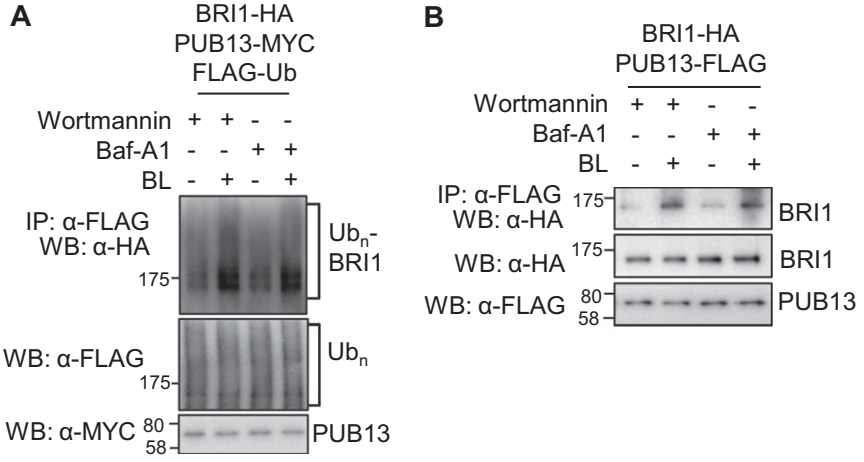


Fig. S1. BL-induced BRI1 ubiquitination (*A*) and BRI1–PUB13 association (*B*) in the presence of vacuolar degradation inhibitors. (*A*) *Arabidopsis* protoplasts were cotransfected with *FLAG-Ub*, *BRI1-HA*, and *PUB13-MYC* and incubated for 10 h followed by the treatment with 1 μM BL for 3 h in the presence of 1 μM bafilomycin A1 (Baf-A1) or 1 μM wortmannin. The ubiquitinated BRI1 was detected with an α-HA WB after IP with α-FLAG antibody (*Top*). The total ubiquitinated proteins were detected by an α-FLAG WB and PUB13 proteins were detected by an α-MYC WB. (*B*) *Arabidopsis* protoplasts were cotransfected with *BRI1-HA* and *PUB13-FLAG* and incubated for 10 h. Protoplasts were pretreated with 1 μM Baf-A1 or 1 μM wortmannin for 2 h before 1 μM BL treatment for 3 h. The association of BRI1–PUB13 was detected by an α-HA WB after α-FLAG IP.

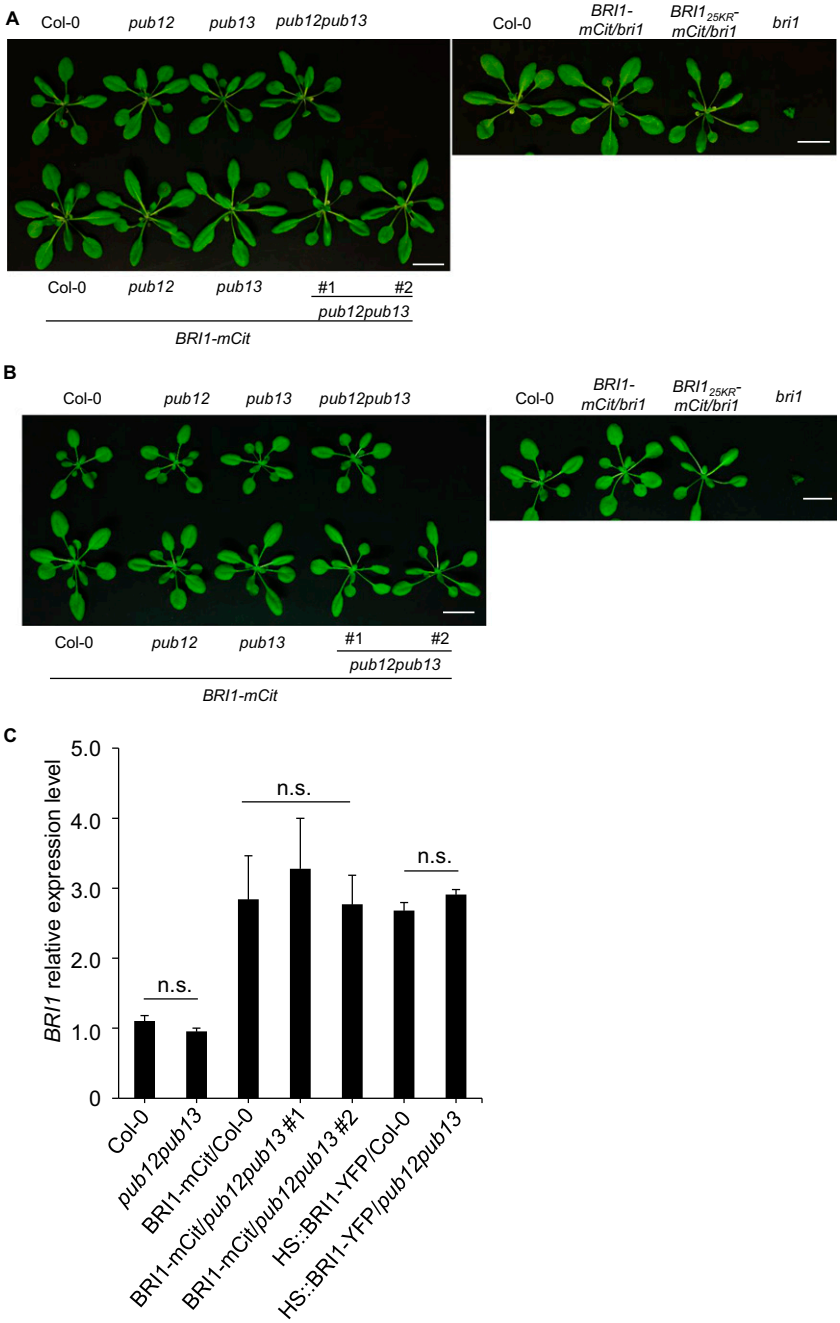


Fig. S2. Characterization of BRI1-mCit/*pub12pub13* transgenic plants. (*A* and *B*) Growth phenotypes of soil-grown plants under different conditions. Plants were grown under 22 °C, 56% relative humidity, 16 h light/8 h dark (*A*) or under 22 °C, 58% relative humidity, 12 h light/12 h dark (*B*) for 4 wk. (Scale bars, 2 cm.) (*C*) Transcriptional analysis of *BRI1*. Total RNA was isolated from 5-d-old seedlings, followed by cDNA synthesis and qRT-PCR analysis of *BRI1* ($n = 3$). No significant differences (n.s.) were observed between Col-0 and *pub12pub13*, between BRI1-mCitrine (mCit)/Col-0 and BRI1-mCit/*pub12pub13* lines, or between *HS::BRI1-YFP*/Col-0 and *HS::BRI1-YFP*/*pub12pub13* by using *t* test. Error bars indicate SD. *ACTIN4* gene was used as an internal control.

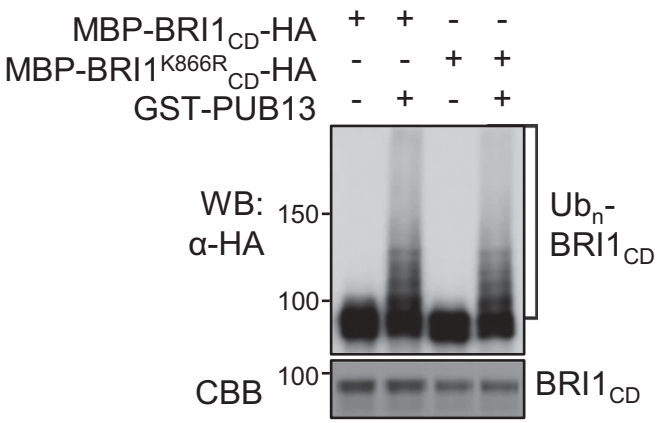


Fig. S3. PUB13 ubiquitinates BRI1$^{K866R}_{CD}$ to a level similar to BRI1$_{CD}$. The ubiquitination of MBP-BRI1$_{CD}$-HA or MBP-BRI1$^{K866R}_{CD}$-HA by GST-PUB13 was detected by an α-HA WB after an in vitro ubiquitination assay. The protein inputs are shown by CBB staining.

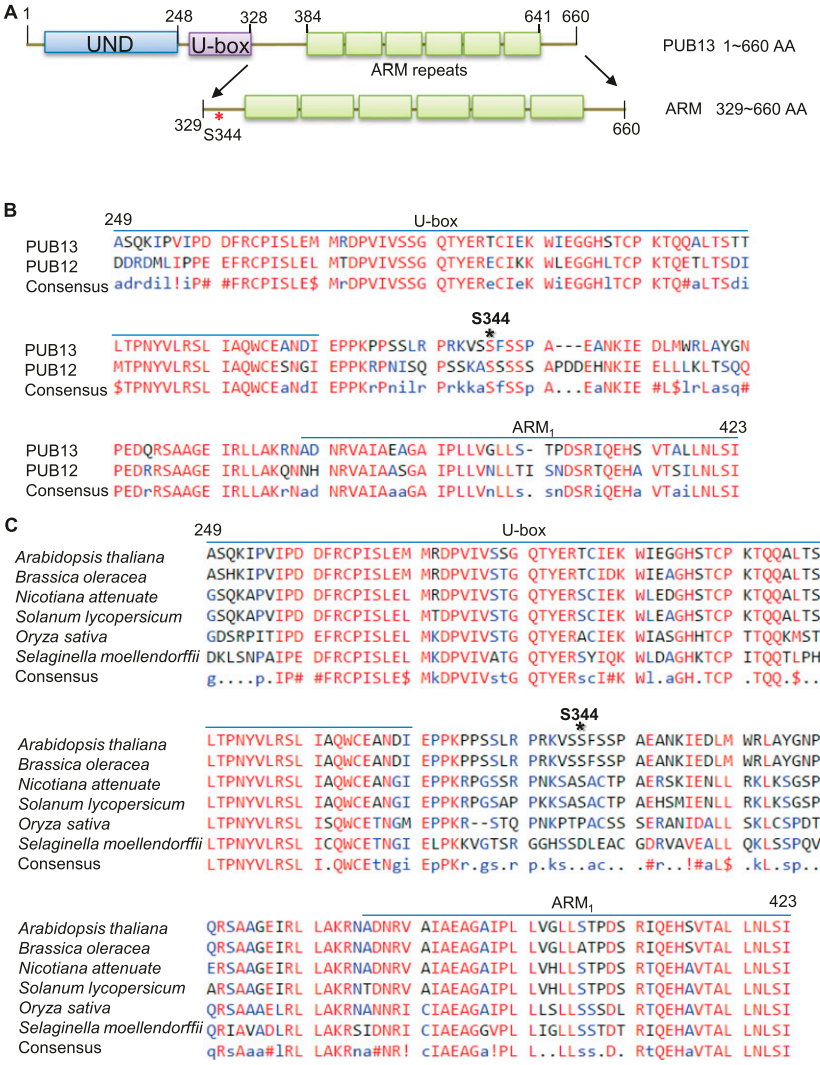


Fig. S4. (*A*) A schematic protein domain structure of *Arabidopsis* PUB13. PUB13 contains a UND, a U-box domain, and an ARMADILLO (ARM) repeat domain. The amino acid position is labeled on the top and the S344 site is labeled at the bottom. (*B*) The alignment of *Arabidopsis* PUB12 and PUB13. The amino acid position of PUB13 is labeled on the top. The first ARM repeat is labeled as ARM$_1$ based on annotation from www.uniprot.org/uniprot/Q9SNC6. (*C*) The alignment of PUB13 from *Arabidopsis thaliana*, *Brassica oleracea* (*XP_013603034*), *Nicotine attenuate* (*XP_019246036*), *Solanum lycopersicum* (*XP_004250960*), *Oryza sativa* (*OsI_037539*), and *Selaginella moellendorffii* (*XP_002986139*).

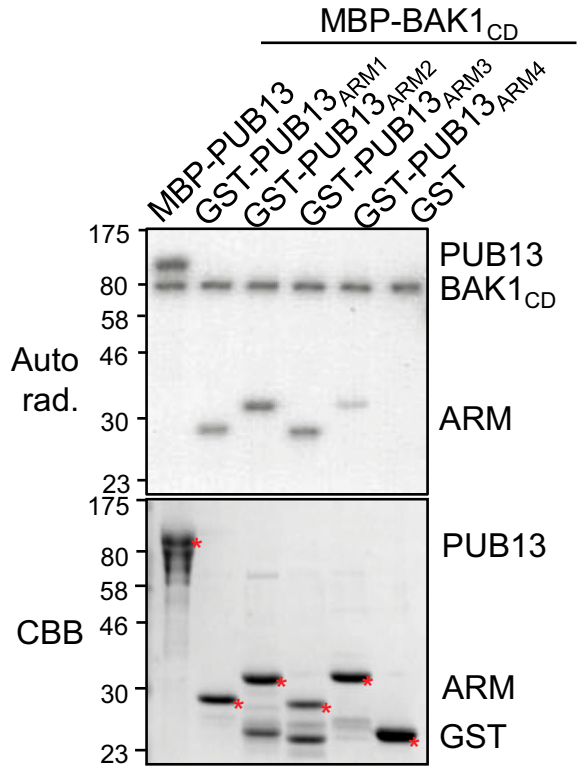


Fig. S5. Different PUB13 ARM truncations are phosphorylated by $BAK1_{CD}$. GST-fused PUB13 ARM truncation proteins (10 μg) were used as substrates and MBP-$BAK1_{CD}$ (1 μg) as the kinase in an in vitro kinase assay. Phosphorylation was detected by autoradiography, and the protein loading is shown by CBB staining. The amino acid positions of different ARM truncations are as follows: ARM1, 329–390; ARM2, 391–495; ARM3, 496–564; and ARM4, 565–660.

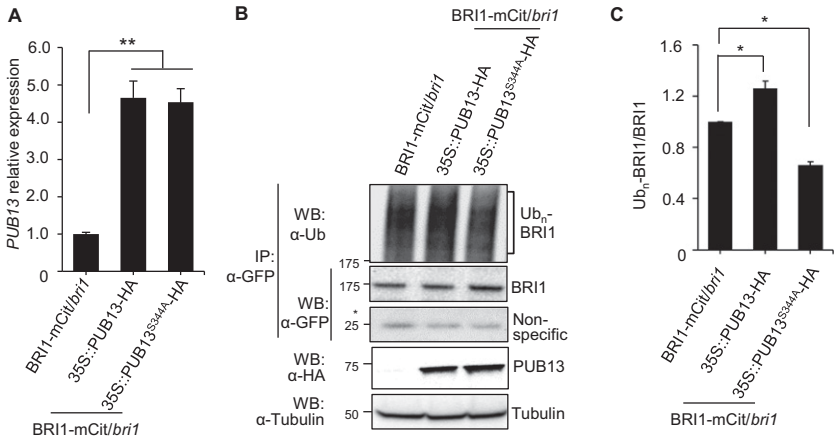


Fig. S6. $PUB13^{S344}$ is required for BRI1 ubiquitination. (*A*) qRT-PCR analysis of *PUB13* ($n = 3$). Total RNA was isolated from 5-d-old seedlings of BRI1-mCitrine (mCit)/*bri1* and *35S::PUB13-HA*/BRI1-mCit/*bri1*. (*B*) BRI1 ubiquitination was reduced in mutated $PUB13^{S344A}$ overexpression lines. IP was performed using α-GFP antibodies on solubilized microsomal fraction protein extracts from BRI1-mCitrine/*bri1* or *35S::PUB13-HA*/BRI1-mCit/*bri1* lines and subjected to immunoblotting with α-Ub (P4D1) (*Top*) or α-GFP (*Middle*). The asterisk indicates nonspecific signals from the same gel used as loading controls. Total proteins were isolated from 5-d-old seedlings and detected by WB using α-HA antibody to detect PUB13-HA protein accumulation (*Bottom*). The protein inputs were equilibrated by WB using α-Tubulin antibodies. (*C*) Quantification of BRI1 ubiquitination profiles detected in *B*. Error bars represent SD ($n = 3$).

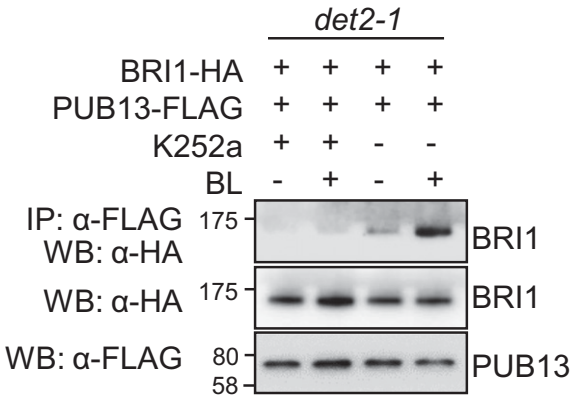


Fig. S7. K252a inhibits BRI1–PUB13 association. Protoplasts from the *det2-1* mutant were transfected with *BRI1-HA* and *PUB13-FLAG*, incubated for 10 h, and treated with 1 μM K252a for 1 h followed by 1 μM BL treatment for 3 h. The association of BRI1 and PUB13 was detected by an α-HA WB after an α-FLAG IP (*Top*), and the input BRI1 and PUB13 proteins were detected by α-HA (*Middle*) or α-FLAG (*Bottom*) WB, respectively.

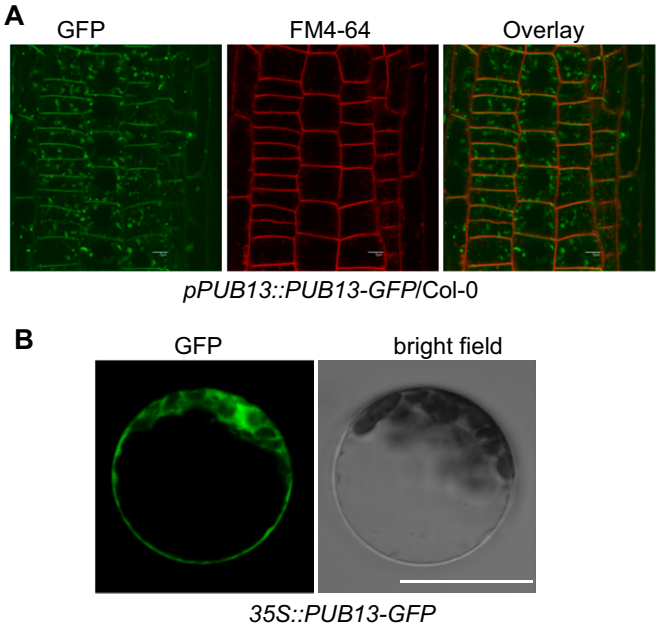


Fig. S8. PUB13-GFP signals could be detected at the PM. (*A*) PUB13 localizes at PM and intracellular compartments. Root epidermal cells of *Arabidopsis* seedlings expressing *pPUB13::PUB13-GFP* were treated with 2 μM FM4-64 for 20 min and then imaged under a confocal microscope. (Scale bar, 5 μm.) (*B*) *Arabidopsis* protoplasts expressing *35S::PUB13-GFP* were imaged under a confocal microscope. (Scale bar, 50 μm.)

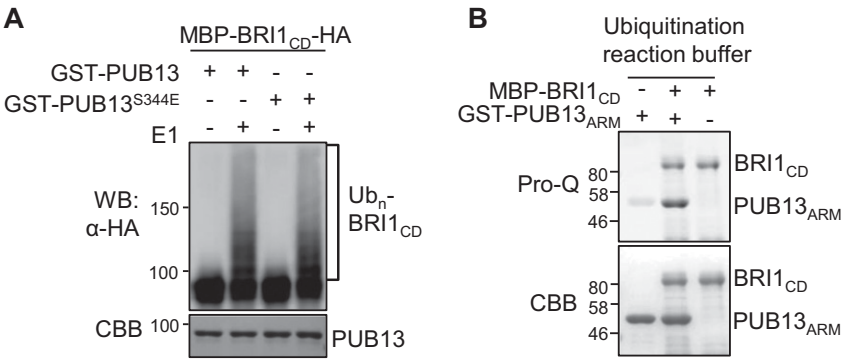


Fig. S9. (*A*) PUB13^{S344E} ubiquitinates BRI1 $_{CD}$ to a level similar to wild-type PUB13. The ubiquitination of MBP-BRI1$_{CD}$-HA by GST-PUB13 or GST-PUB13^{S344E} was detected by an α-HA WB after an in vitro ubiquitination assay. The protein inputs are shown by CBB staining. (*B*) PUB13 is phosphorylated by BRI1 in the ubiquitination reaction buffer. The phosphorylation reactions were performed in 30 μL of ubiquitination reaction buffer containing 1 μg of substrate proteins and 1 μg of kinases at room temperature for 3 h with gentle shaking. The reactions were stopped by adding 4× SDS loading buffer. Phosphorylation of PUB13 was detected using Pro-Q Diamond staining (*Top*), and the protein loading is shown by CBB staining (*Bottom*).

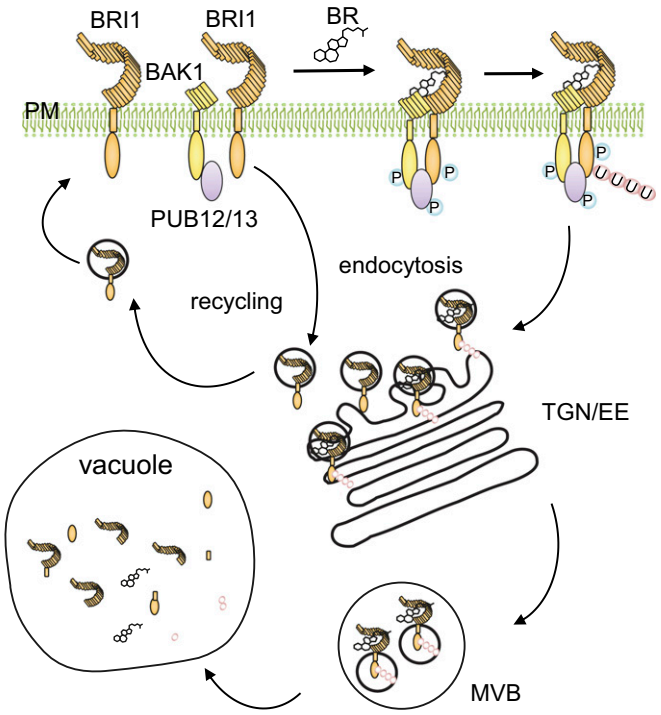


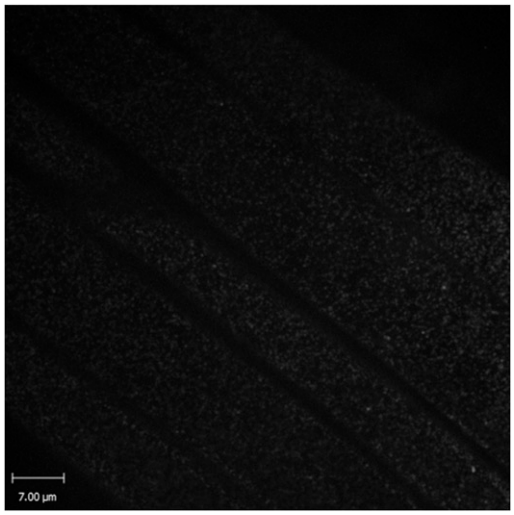
Fig. S10. Model for PUB12/PUB13-mediated BRI1 ubiquitination and internalization. BR hormones induce the phosphorylation of PUB12/PUB13 mediated by BRI1, and the phosphorylated PUBs further ubiquitinate BRI1. The ubiquitinated BRI1 is internalized and delivered to the vacuole for degradation to attenuate BR responses. The internalized ligand-free and inactive BRI1 is recycled back to the PM. BR-induced PUB12/PUB13-mediated ubiquitination of BRI1 may be a way to distinguish activated and inactivated BRI1 for internalization. MVB, multivesicular body.

Table S1. Primers for point mutations and gene cloning

Primer name	Sequences
Point mutations	
PUB13-C262A-F	5′-TGATGATTTT CGCGCTCCGA TTTCGCTG-3′
PUB13-C262A-R	5′-CAGCGAAATCGGAGCGCGAAAATCATCA-3′
PUB13-W289A-F	5′-CATGTATTGA GAAAGCGATA GAAGGTGG-3′
PUB13-W289A-R	5′-CCACCTTCTATCGCTTTCTCAATACATG-3′
PUB13 S343A-F	5′-GACCCAGAAAAGTAGCGTCCTTCTCATCTCCC-3′
PUB13 S343A-R	5′-GGGAGATGAGAAGGACGCTACTTTTCTGGGTC-3′
PUB13 S344A-F	5′-CCCAGAAAAGTATCGGCCTTCTCATCTCCC-3′
PUB13 S344A-R	5′-GGGAGATGAGAAGGCCGATACTTTTCTGGG-3′
PUB13 S346A-F	5′-CAGAAAAGTATCGTCCTTCGCATCTCCCGCAGAAG-3′
PUB13 S346A-R	5′-CTTCTGCGGGAGATGCGAAGGACGATACTTTTCTG-3′
PUB13 S347A-F	5′-GTATCGTCCTTCTCAGCTCCCGCAGAAGCG-3′
PUB13 S347A-R	5′-CGCTTCTGCGGGAGCTGAGAAGGACGATAC-3′
PUB13 S344E-F	5′-GACCCAGAAAAGTATCGGAATTCTCATCTCCCGCAG-3′
PUB13 S344E-R	5′-CTGCGGGAGATGAGAATTCCGATACTTTTCTGGGTC-3′
BRI1 K866R-F	5′-GCTGCTTTCGAGAGGCCATTGCGGAAG-3′
BRI1 K866R-R	5′-CTTCCGCAATGGCCTCTCGAAAGCAGC-3′
Gene cloning	
PUB13 ARM1-F	5′-CATGCCATGGAGCCTCCAAAGCCTCCGAG-3′
PUB13 ARM1-R	5′-GAAGGCCTTATGGCCACGCGGTTG-3′
PUB13 ARM2-F	5′-CATGCCATGGCCGAAGCTG GAGCCATA-3′
PUB13 ARM2-R	5′-GAAGGCCTATCTTTCTTGCCTCTTTG-3′
PUB13 ARM3-F	5′-CATGCCATGGCTGCTACTGCACTCTT-3′
PUB13 ARM3-R	5′-GAAGGCCTAACCAAACTTGGGACTG-3′
PUB13 ARM4-F	5′-CATGCCATGGAGTTTATCAGAACTG-3′
PUB13 ARM4-R	5′-GAAGGCCTAGTATCTGCAGCTTCTGTGG-3′

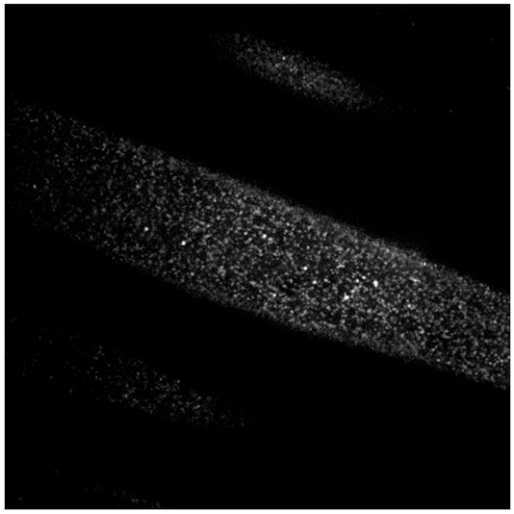
Table S2. Primers for genotyping and qRT-PCR

Primer name	Sequences
Genotyping	
pub12-2-LP	5′-TAACCACAGCTACCCAAAACG-3′
pub12-2-RP	5′-TAATTTCCTAATTTGGCCGTG-3′
pub13-LP	5′-AAGAGGTATGGCTCCAGCTTC-3′
pub13-RP	5′-ACGTGCTTTGTTTTGCTATGG-3′
qRT-PCR	
BRI1-fwd	5′-CCGTGTACTTTCGATGGCGTTA-3′
BRI1-rev	5′-GAGAGACAGGAGAGACGAGGAC-3′
Actin4-fwd	5′-AGCACTTGCACCAAGCAGCATG-3′
Actin4-rev	5′-ACGATTCCTGGACCTGCCTCATC-3′



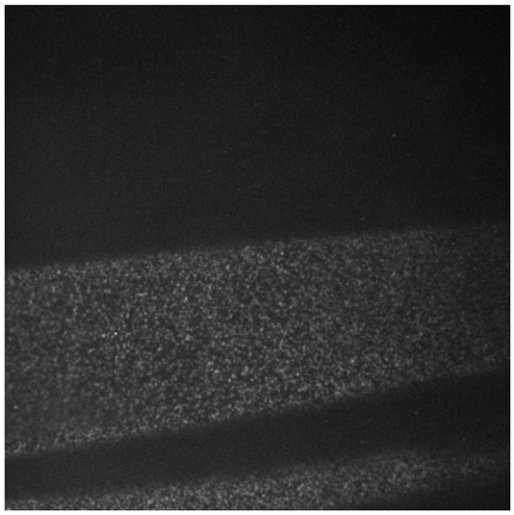
Movie S1. PM dynamics of BRI1-mCitrine in Col-0. The presented time series were acquired from a hypocotyl cell of an etiolated *Arabidopsis* seedling expressing BRI1-mCitrine in Col-0 using VAEM/spinning-disk confocal microscopy. Time-lapse was acquired for 3 min with 500-ms intervals. Movie is shown in frequency of seven frames per second.

Movie S1



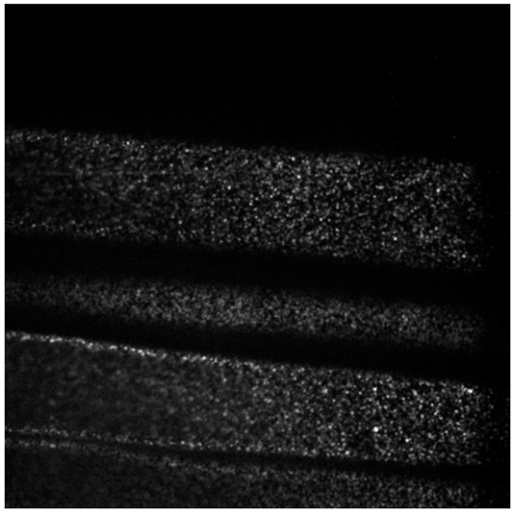
Movie S2. PM dynamics of BRI1-mCitrine in *pub12pub13*. The presented time series were acquired from hypocotyl cells of etiolated *Arabidopsis* seedlings expressing BRI1-mCitrine in *pub12pub13* (BRI1-mCitrine/*pub12pub13* #1) using VAEM/spinning-disk confocal microscopy. Time-lapses were acquired for 3 min with 500-ms intervals. Movie is shown in frequency of seven frames per second.

Movie S2



Movie S3. PM dynamics of BRI1-mCitrine in *pub12pub13*. The presented time series were acquired from hypocotyl cells of etiolated *Arabidopsis* seedlings expressing BRI1-mCitrine/*pub12pub13* #2 using VAEM/spinning-disk confocal microscopy. Time-lapses were acquired for 3 min with 500-ms intervals. Movie is shown in frequency of seven frames per second.

Movie S3



Movie S4. PM dynamics of $BRI1_{25KR}$-mCitrine in *bri1*. The presented time series were acquired from a hypocotyl cell of an etiolated *Arabidopsis* seedling expressing $BRI1_{25KR}$-mCitrine in *bri1* using VAEM/spinning-disk confocal microscopy. Time-lapse was acquired for 3 min with 500-ms intervals. Movie is shown in frequency of seven frames per second.

Movie S4