Supplementary Information for

KARRIKIN UPREGULATED F-BOX 1 (KUF1) imposes negative feedback regulation of karrikin and KAI2 ligand metabolism in Arabidopsis thaliana

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Extended Description of Materials and Methods

Plant propagation and transformation
Plants were propagated in Sungro Professional Growing Mix under white light (~110 µmol m\(^{-2}\) s\(^{-1}\); MaxLite LED T8 16.5W 4000k light-emitting diode bulbs) with 16 h light/8 h dark photoperiod at ~21-24°C. Soil was supplemented with Gnatrol WDG, Marathon (imidacloprid), and Osmocote 14–14–14 fertilizer. Unless noted otherwise, seeds were surface-sterilized (5 min agitation in 70% EtOH with 0.05% (v/v) Triton X-100, followed by 70% and 95% EtOH washes and air drying) and grown on 0.5x Murashige-Skoog (MS) Medium with MES Buffer and Vitamins (Research Products International), pH 5.7, solidified with 0.8% (w/v) Bacto agar (BD). Floral dip transformation of Arabidopsis thaliana with Agrobacterium tumefaciens (GV3101 pMP90) was performed in 5% sucrose (w/v) with 0.025% (v/v) Silwet-77 (1).

Gene expression analysis
Arabidopsis seedlings were grown as described above, harvested after 4 d in red light, and immediately frozen in liquid N\(_2\) before storage at -80°C. Tissue was ground with a power drill and plastic pestle in a centrifuge tube in liquid N\(_2\). RNA was extracted from 50 to 100 mg of frozen tissue with Spectrum Plant Total RNA Kit (Sigma) including on-column DNase I digestion (Sigma). RNA concentrations were measured by Qubit RNA Broad-Range Assay kit and Fluorometer (Invitrogen). RNA integrity was tested by the 2100 Bioanalyzer (Agilent). First-strand cDNA synthesis was performed on 1 µg total RNA with Verso cDNA Synthesis Kit (Thermo-Fisher) and a 1:1 mixture of oligo dT and random hexamer primers, or iScript cDNA Synthesis Kit (Bio-Rad). Real-time quantitative PCR was performed on cDNA with Luna Universal qPCR Mastermix (New England Biolabs) in a CFX384 system (BioRad). Each 10 µl reaction used cDNA derived from 25 ng RNA and 0.25 µM of each primer. A two-step amplification-melt protocol was used with the following conditions: 95°C for 3 min; 40 cycles of 95°C for 15 s, 60°C for 60 s; melt-curve from 65°C to 95°C. Primer sequences for DLK2 (At3g24420), BBX20/STH7/BZS1 (At4g39070), IAA6 (AT1G52830), SMXL2 (At4g30350), and the reference gene CACS (At5g46630) are described in Table S1 and previously reported (2). Relative expression values were scaled relative to mock-treated wild type (Col-0).

For KUF1 analysis in lettuce, approximately 50 achenes (cv. Grand Rapids TBR, Stokes Seeds) were plated in the dark onto two layers of Whatman filter paper (Grade 1, 7 cm, Cytiva) soaked with 2.5 mL of 0.1% (v/v) acetone, or 1 µM KAR1, KAR2, or rac-GR24. Petri dishes were sealed with Parafilm and incubated at 20°C for 60 min in darkness, 10 min in far-red light (730 nm ±10 nm, 30 µmol m\(^{-2}\) s\(^{-1}\)), and 290 min in darkness. Achenes were frozen in liquid N\(_2\), stored at -80°C, and later ground with mortar and pestle in liquid N\(_2\). RNA extraction, cDNA synthesis, and qRT-PCR were performed as described for Arabidopsis above, except DNase I (New England Biolabs) digestion was not done on-column. LsKUF1a (Lsat_1_v5_gn_8_92780) was identified by BLASTp as one of three homologs of Arabidopsis KUF1. Expression of the other KUF1 homologs, Lsat_1_v5_gn_9_8360 and Lsat_1_v5_gn_1_85701, was too low for reliable detection. Primers for qRT-PCR analysis of LsKUF1a expression are described in Table S1. Actin primers were previously described (3).

Yeast two-hybrid analysis
Y2HGold and Y187 yeast strains were transformed with either pGBKTK7 or pGADT7 vectors containing the indicated bait and prey inserts. Selection was performed on solid SD -Trp and SD -Leu media for 2-3 d at 30°C. Single colonies were inoculated in 1 mL of liquid SD-Trp or SD-Leu media and grown overnight at 30°C. Yeast cultures were diluted to an OD\(_{600}\)=0.5 with sterile water, then 25 µL of each desired bait and prey culture was mated in 25 µL of liquid YPDA. Yeast were grown overnight at 30°C and selected on solid SD -Leu/Trp media for 2-3 days. Three diploid yeast colonies for each bait and prey combination were grown in 1 mL SD -Leu/Trp overnight at 30 C, diluted 1:10 in 1 mL -Leu/Trp and grown overnight again. Cultures were diluted down to OD\(_{600}\)=0.3
before preparation of 1:10 serial dilutions. 5 μL of each serial dilution was spotted on solid SD-Leu/Trp, SD-Leu/Trp/His, and SD-Leu/Trp/His/Ade medium, grown 2 d at 30°C, and photographed. Similar results were obtained for all three colonies.

Western blot analysis
Col-0 and kuf1-1 seedlings were grown in triplicate for four days under continuous red light (30 μmol m⁻² s⁻¹) at 21°C. Whole seedlings (300-400 mg) were ground under liquid N₂ to a fine powder. Soluble proteins were extracted with 120 μL of lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% Tween-20, 1 mM PMSF, 1 mM DTT and 1× Complete protease inhibitors [Roche]) per 100 mg of tissue. Lysates were centrifuged twice at 20,000 g for 10 min each to pellet insoluble material. 60 μg total soluble protein were separated on a 12% SDS-PAGE gel containing 1% (v/v) 2,2,2-trichloroethanol (Sigma T54801) to allow visualisation of total protein with a UV transilluminator and ChemiDoc XRS+ system (Bio-Rad) to determine RbcL levels. Proteins were blotted onto PVDF membrane by semi-dry transfer using a Trans-Blot Turbo transfer system (Bio-Rad). Membranes were blocked in 1×TBS-T (pH 7.5) and 2% (w/v) BSA for 1 h at 22°C with rocking. Primary antibodies (anti-KAI2 raised in rabbit (4), and anti-actin raised in mouse, Sigma A0480) were diluted at 1:2500 in 10 mL 1×TBS-T + 0.2% (w/v) BSA, and membranes were incubated overnight at 4 °C (anti-KAI2) or for 1 h at 22 °C (anti-actin) with gentle rocking. Secondary antibodies were diluted at 1:1000 (goat anti-rabbit IgG-HP conjugate, ThermoFisher 32460) or 1:5000 (goat anti-mouse IgG-AP conjugate, ThermoFisher 31321) in the same buffer, and membranes were incubated for 1 h each at 22°C. Between antibody incubations, membranes were washed four times with 1×TBS-T, for 5 min per wash. HRP activity was detected with Clarity Western ECL substrate (Bio-Rad), and AP activity was detected with Immun-star AP substrate (Bio-Rad). Membranes were probed sequentially with anti-KAI2, then with anti-actin, with an additional 1-h blocking step in between. Chemiluminescence images (16-bit) were acquired and analysed using an Amersham Imager 680 system and built-in software. Image processing involved band detection, followed by background subtraction, and then determination of band volume (pixel count × pixel intensity). Band volume data were imported into Microsoft Excel where KAI2 values were normalised to corresponding actin values, or RbcL band volumes from the UV gel image. Finally, normalised values were scaled to the mean normalised value for Col-0.

SMAX1 and KAI2 analysis in Nicotiana benthamiana
A full-length wild-type KUF1 coding sequence was amplified from genomic DNA, Gateway-cloned into pDONR221A, and sequence-verified. Primers are described in Table S1. The kuf1Δ mutant coding sequence was derived from restriction digestion of pDONR221A-KUF1 with Bgl II, which caused a 517 bp internal deletion. This introduces a premature stop codon after the first 47 aa at the N-terminus. KUF1 and kuf1Δ were transferred into pGW402 (5), which drives transgene expression from a 35S promoter, by an LR clonase II (Invitrogen) reaction. pRATIO1212-SMAX1 was transiently co-expressed with pGW402, pGW402-KUF1, or pGW402-kuf1Δ via agroinfiltration in leaves of three-week-old Nicotiana benthamiana plants as described previously, with 0.5 OD₆₅₀ for each Agrobacterium tumefaciens culture (6, 7). pRATIO1212-SMAX1 was carried in a GV3101 strain of Agrobacterium that also carries the p19 plasmid, which suppresses post-transcriptional gene silencing. After 3 d, leaf discs were excised, selected based on adequate reference protein (Venus) expression, and treated with 0.02% (v/v) acetone or 10 μM KAR; for 16 h before fluorescence analysis (6). KAI2 was analyzed similarly, but in the pRATIO3212 vector.

SMAX1₀₂-LUC degradation assays in Arabidopsis thaliana
To monitor SMAX1₀₂ degradation, 9-day-old wt or kuf1 plants expressing UBO10:SMAX1₀₂-LUC2-*F2A-mScarlet-I (7) were grown in a white 96-well plate (Perkin Elmer OptiPlate 96) containing 200 μL 0.5x MS agar medium. Seedlings were sprayed with 2 mM D-luciferin and incubated 3 h before treatment to equilibrate. 10 μM KAR₁, KAR₂, rac-GR24, or mock control (0.1% v/v acetone) was then sprayed along with 2 mM D-luciferin. Luminescence was measured using a CLARIOStar plate reader (BMG Labtech) under controlled 21°C temperature. For normalization of untreated seedlings (Fig. 6B), total leaf and cotyledon surface area was measured in ImageJ.
β-glucuronidase (GUS) reporter analysis

The -2967 bp region upstream of the KUF1 translational start codon was Gateway-cloned into plant transformation vector pGWB533 (5), which enables C-terminal fusions to β-glucuronidase (GUS). Three independent, homozygous, single-insertion transgenic lines carrying the pGWB533-KUF1p T-DNA were grown as described for seedling growth assays (above) and stained for GUS activity after 4 d in red light (8). Tissues from 5-week-old adult plants grown in soil in 16 h white light: 8 h dark conditions were analyzed by the same protocol.

Determination of root hair density and length

*Arabidopsis thaliana* seeds were surface-sterilized by washing with 1 ml of 70% (v/v) ethanol and 0.05% (v/v) Triton X-100 with gentle mixing by inversion for 6 minutes at room temperature, followed by one wash with 96% ethanol and 3 washes with sterile distilled water. Seedlings were grown in axenic conditions on 12×12 cm square Petri dishes containing 60 mL of 0.5x MS medium, pH 5.8 (Duchefa), supplemented with 1% (w/v) sucrose and solidified with 1.5% (w/v) agar. Seeds were stratified at 4°C for 3 d in the dark, and then transferred to a growth cabinet at 22°C and placed vertically under fluorescent white light (120 μmol m⁻² s⁻¹) for 5 d. Images of 10 seedling roots per genotype and treatment were taken at 5 d post-germination with a Zeiss Stereo Discovery V8 microscope equipped with a Zeiss Axiocam 503 color camera. Root hair density was determined by counting the root hairs between 2 and 3 mm from the root tip on each root, and root hair length was measured for 10 root hairs per root using Fiji (https://imagej.net/Fiji/Downloads) as described previously (9).
Fig. S1. *KUF1* expression in constitutive KAR/KL and SL signaling mutants.

Expression of *KUF1* relative to CACS reference transcripts in seedlings grown 4 d in red light on 0.5x MS medium supplemented with 0.1% (v/v) acetone (gray) or 1 μM rac-GR24 (blue), measured by quantitative real-time RT-PCR. Relative expression values scaled to Col-0. (*n* = 4-5 pools of seedlings; mean ± SD) Letters indicate statistical groups, *p*<0.05, Tukey’s multiple comparisons test.
Fig. S2. Characterization of KUF1 domains.

(A) Yeast two-hybrid interactions between GAL4 DNA-binding domain (BD) fusions to KUF1, kuf1ΔF-box, or nothing, and GAL4 activation domain (AD) fusions to Arabidopsis Skp1 (ASK1) or nothing. The kuf1ΔF-box bait has a 46 aa deletion of aa 34-79. Cultures of diploid yeast colonies formed after mating pGBKT7 in Y2HGold with pGADT7 in Y187 were serially diluted and tested for growth on selective –His or –His/Ade media after two days incubation at 30°C. (B) AlphaFold v2.0 model AF-Q8GX29-F1 of KUF1/SKIP25. Structure visualization and coloring was performed in ChimeraX. Boundaries of blade-forming Kelch repeats were set based on the structure. Beta sheets forming the propeller had very high (pLDDT > 90) model confidence. Model confidence was typically lower for loops connecting blades, and the N-terminal region upstream of the F-box motif had very low (pLDDT < 50) model confidence.
Fig. S3. Hypocotyl elongation of *kuf1* heterozygotes and rescued *kuf1*.

(A) Hypocotyl lengths of progeny from a selfed, heterozygous *kuf1* parent grown 4 d in red light on 0.5x MS medium. Seedlings in the segregating population were genotyped individually after measurement. \( n = 10-22; \text{mean } \pm \text{ SD} \) *, \( p < 0.01 \), Dunnett’s T3 multiple comparisons test, comparison to Col-0

(B) Hypocotyl lengths of seedlings grown 4 d in red light on 0.5x MS medium supplemented with 0.1% (v/v) acetone, 1 µM KAR1, or 1 µM rac-GR24. Box plots have Tukey whiskers. Percent growth inhibition relative to the mock-treated control for each genotype is indicated below box plots of experimental treatments. \( n = 50-75 \)
Fig. S4. Hypocotyl growth inhibition and cotyledon expansion of kuf1 seedlings in response to KAR and rac-GR24 treatments.

(A) Hypocotyl growth inhibition of wild-type, kuf1, and two rescued KUF1p:KUF1 transgenic lines (3-6 and 19-8) grown 4 d in red light on 0.5x medium supplemented with 0.1% (v/v) acetone, 1 μM KAR1, 1 μM KAR2, or 1 μM rac-GR24. Growth inhibition for each treated seedling was calculated relative to the average length of mock-treated seedlings. These growth inhibition data are derived from the same measurements of hypocotyl length shown in Fig. 4B. Box plots have Tukey whiskers. (n = 100) *, p<0.01, Dunnett’s multiple comparisons test, compared to Col-0 with same treatment. #, p<0.01, Dunnett’s multiple comparisons test, KAR1 vs. KAR2 within genotype. 

(B) Relative luminescence from 9-d-old SMAX1D2-LUC seedlings in wt or kuf1 backgrounds after treatment with 0.1% (v/v) acetone (mock) or 10 μM KAR1, KAR2, or rac-GR24. The relative light units (RLU) percentage indicates the amount of SMAX1D2-LUC luminescence remaining at each time point. It is measured as the amount of luminescence from each seedling at each time point compared to its luminescence at time 0 h. Seedlings were grown in 16 h white light: 8 h dark. (n = 9-10) *, p < 0.01, Bonferroni’s multiple comparisons test of kuf1 vs. wt for each treatment and time point.
**Fig. S5. Hypocotyl elongation of kuf1 under different light intensities**

(A) Hypocotyl length of wild-type and kuf1 seedlings grown 4 d under the indicated intensities of red light on 0.5x MS medium supplemented with 0.1% (v/v) acetone or 1 μM KAR1. For each light fluence, the percent growth inhibition relative to the mock-treated control for each genotype is indicated below box plots of KAR1 treatments. (n = 60) (B) Hypocotyl length of wild-type and kuf1 seedlings grown 5 d in the dark after stratification (to match age of seedlings grown 4 d under red light) on 0.5x MS medium supplemented with 0.1% (v/v) acetone, 1 μM KAR1, or 1 μM KAR2. (n = 80) Box plots have Tukey whiskers. (C) Expression of KUF1 relative to CACS reference transcripts in seedlings grown 4 d in red light or darkness on 0.5x MS medium, measured by quantitative real-time RT-PCR. (n = 5 pools of seedlings; mean ± SD) Letters indicate statistical groups, p<0.05, Dunnett’s T3 multiple comparisons test.
Fig. S6. KUF1 effects on SMAX1 degradation in *N. benthamiana*

Ratio of SMAX1-mScarlet-I to Venus fluorescence in (A) wild-type and (B) *Nbd14a,b* *Nicotiana benthamiana* leaves co-infiltrated 3 d with the pRATIO1212-SMAX1 ratiometric reporter plasmid and the overexpression plasmids pGWB402-kuf1Δ, or pGWB402-KUF1, after 16 h treatment with 0.02% (v/v) acetone or 10 μM KAR1, KAR2, or rac-GR24 The kuf1Δ mutation has a deletion that causes premature truncation after the N-terminal 46 aa of KUF1. (n = 3 or 4 leaves; each leaf value is the mean relative fluorescence of 2 to 6 leaf discs; horizontal bar indicates the mean). *, p<0.05, Dunnett’s multiple comparisons test of KAR/GR24-treated vs. mock-treated samples for each KUF1 variant.
Fig. S7. *KUF1* expression patterns in adult *Arabidopsis* plants.
Representative images of β-glucuronidase staining (blue) in (A) rosette leaf, (B) rosette axillary bud, (C) flower buds, and (D) opened flower of five-week-old *KUF1p:GUS* plants grown in a 16 h white light: 8 h dark photoperiod. Scale bar = 5 mm for (A) and 1 mm for other panels.
**Fig. S8. KUF1 expression in lettuce achenes.**
Expression of LsKUF1a (Lsat_1_v5_gn_8_92780) relative to Actin reference transcripts in Lactuca sativa cv. Grand Rapids TBR achenes before and after 6 h imbibition in 0.1% (v/v) acetone (mock), 1 μM KAR1, 1 μM KAR2, or 1 μM rac-GR24, measured by quantitative real-time RT-PCR. Relative expression values scaled to dry, unimbibed achenes. (n = 4 pools of achenes; mean ± SD) Letters indicate statistical groups, p<0.05, Dunnett’s T3 multiple comparisons test.
| Primer | Sequence (5' > 3') | Notes |
|--------|--------------------|-------|
| AIKUF1-DT1-BsF | ATATATGTCCTGATGAAAATCTCCGTATTTAATCGAGTTT | dual gRNA cassette cloning for pHEE401E |
| AIKUF1-DT1-F0 | TGAAGACTCGCCTTTTATCGATTTTAGCTAGAAATAGC | |
| AIKUF1-DT2-R0 | AAGGTTGAACACATACACAACCGCAAATCTTACTGCGACTTAC | |
| AIKUF1-DT2-BsR | ATTATTGGTCTCAGAAGACCACTAAACACAACACATACACCGCAA | |
| AIKUF1CR_F | CCTCTCATTTGATTACACTTCACGC | genotyping kuf1-1 (711 bp WT, 511 bp mutant) |
| AIKUF1CR_R | CGACGGTTGATGAGAACTGAGAA | Arabidopsis qRT-PCR |
| CACS_qRT-F | ACTCAGGAAGGTGTACGGTCA | |
| CACS_qRT-R | TGCACTTGGAACAGGTTCGTT | |
| DLK2_qRT-F | GCTGCTTCTCCAAGGTATATAA | |
| DLK2_qRT-R | GAAATCAACCGCCAGCT | |
| IAA6_qRT-F | TCCGCGTCTCTGGATAGGAGGAGG | |
| IAA6_qRT-R | TTCAGATGTCAGCCCTTCTGTC | |
| KUF1_qRT-F | AACCGTGACTCGGTTTATTG | lettuce qRT-PCR |
| KUF1_qRT-R | AAGCAGGAGTACGGGAAGA | |
| SMXL2_qRT-F | CCACAGTTGTTGCAAGACGCTAA | |
| SMXL2_qRT-R | CCCGGTGAAGTATATCCGAATC | |
| STH7_qRT-F | CATCTCGGTGCTCTCTACTTCT | |
| STH7_qRT-R | CATCTCTGATAGATATGCTTCGTC | |
| LsKUF1a_qRT-F | TCTCCCCGCTTTTTCCGATCTCA | |
| LsKUF1a_qRT-R | GACAAATACGGCGGGCGTGAG | |
| LsActin_qRT-F | AGGGCAGTGTTCATAGATGGTG | |
| LsActin_qRT-R | CTCTTTTGGATGTGCCTCATCT | |
| KUF1rescue_F | GGGGACAAGTTTGTACAAAAAAGCAGGCTTC TCTCGATATCAT | cloning, attB1 and 5' end of promoter |
| KUF1_DNA_F | GGGGACAAGTTTGTACAAAAAAGCAGGCTTC TCTCGATATCAT | cloning, attB1 and 5' end of CDS |
| KUF1promoterBP_R | GGGGACCACCTTTGTACAAAGAAGCTGGCTTCTCTCTCTTTTTGTTT | cloning, 3' end of promoter and attB2 |
| KUF1rescue_WSC_R | GGGGACACCTTTGTACAAAGAAGCTGGCTTCTCTCTTTTTT | cloning, 3' end of CDS with stop and attB2 |
| KUF1rescue_NSC_R | GGGGACACCTTTGTACAAAGAAGCTGGCTTCTCTCTTTTTT | cloning, 3' end of CDS without stop and attB2 |
| KUF1resceueseq_1 | TCTGACGAGTGATCAATATCACGTTT | Sanger sequencing of KUF1 promoter and rescue constructs |
| KUF1resceueseq_2 | TCCACCTCTAACACATGTAATGCATT | |
| KUF1resceueseq_3 | TGAGATTATACACACTAACAGAAAATGT | |
| KUF1resceueseq_4 | TGACTGTAGTATGGTAGGTTCCTGA | |
| KUF1resceueseq_5 | GTATCCGCTTCTCTCTCACCA | |
| AIKUF1-RFBX FP | TGAAGACTCTACGCTTTTCTCGAGTTC | dropout KUF1 F-box from donor plasmid |
| AIKUF1-RFBX RP | AAGGAGGATAGGATTTTTCATATTCCGCTGAC | |
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