Supporting Information

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SI Materials and Methods

Plant Transformation. *Agrobacterium*-mediated plant transformation and selection of transformants were carried out by using the floral-dip method (1), and transformants were selected by spraying with 0.3 mg/mL ammonium-glufosinate.

Isolation of T-DNA Insertion Lines. The presence of T-DNA insertions in *ate1-2*, *ate2-1*, and *prt6-5* was verified by PCR using primer pairs LB2/At1, LB2/At3, and LB2/At121, respectively (see Table S1 for the sequence of primers used). For detection of the wild-type alleles, primer pairs At1/At2-3 (*AtATE1*), At3/At4-2 (*AtATE2*), and At120/At121 (*PRT6*) were used. The *PRT6* alleles *prt6-1* and *prt6-2* (2, 3) were obtained from the Nottingham *Arabidopsis* Stock Centre (Loughborough, U.K.), and the presence of a T-DNA in *PRT6* was verified by using primer pairs At121/LB_SAIL and At231/GK8760, respectively. To detect the wild-type allele of *PRT6*, primer pairs At120/At121 and At231/At232 were used for *prt6-1* and *prt6-2*, respectively.

Construction of Mutant Lines. After crossing, F_1 plants were selfed, and plants of the F_2 generation were genotyped. The *ate1-2*, *ate2-1*, and *prt6-5* were genotyped as described above. Plants carrying the *axr1-3* allele were identified by PCR amplification of an *AXR1* fragment using primer pair At115/At118 and subsequent PvuII digestion. Plants carrying *bp-1* were identified by the appearance of the characteristic *bp* phenotype and by PCR using primer pair At206/At207, which resulted in a product only in the presence of a wild-type allele. Because *bp-1* is in the Landsberg *erecta* accession, we selected *ate1 ate2 bp-1* triple-mutant plants in the F_2 that did not carry the *erecta* mutation and reisolated *bp-1* and *ate1 ate2* mutants. Plants containing *as1-1* and *se-1*, respectively, were identified in the F_2 generation based on their characteristic phenotypes and were genotyped for *ate1-2* and *ate2-1*. The absence of phenotypic variation in the F_3 population indicated that the parent isolated in the F_2 generation was indeed homozygous for *as1-1* and *se-1*, respectively. The presence of different GUS reporter genes was confirmed in the F_2 population by using primer pair At221/At222. Segregation ratios were followed in the F_3 generation to identify plants homozygous for the respective GUS reporter.

In Vitro Enzymatic Assays of R-Transferases. Seven-day-old seedlings grown on 0.5 × MS plates under continuous light were frozen and ground in liquid nitrogen. Extraction buffer [50 mM Tris·HCl, pH 7.4; 150 mM KCl; 10 mM $MgCl_2$; 0.1% (vol/vol) Nonidet P-40; 15 mM β-mercaptoethanol; 10% (vol/vol) glycerol; and plant protease inhibitors (200-fold dilution; Sigma–Aldrich)] was added to the ground tissue, and soluble proteins were obtained by centrifugation at 100,000 × *g* for 30 min. The soluble fraction was loaded onto a 10DG gel filtration column (Bio-Rad) preequilibrated in ATE1 buffer [50 mM Tris·HCl, pH 7.4; 300 mM KCl; 15 mM β-mercaptoethanol; and 10% (vol/vol) glycerol]. Protein concentration was measured by using Bradford reagent (Bio-Rad). Before an assay, the extracts were diluted to 2.5 mg/mL with ATE1 buffer.

Immediately before an assay, Arg-tRNA was produced by incubating total *Escherichia coli* tRNA (30 μg; Sigma–Aldrich), 40 units of *E. coli* aminoacyl-tRNA synthetase (Sigma–Aldrich), 2 μM Arg, and ≈85 nM [^{3}H]Arg (0.25 μCi) in reaction buffer (50 mM Tris·HCl, pH 8.0; 150 mM KCl; 10 mM $MgCl_2$; 50 mM β-mercaptoethanol; 5 mM ATP; 10 mM creatine phosphate; and 20 mg/mL creatine phosphokinase) for 15 min at 37 °C. The following components were then added: 0.2 mM puromycin (Sigma–Aldrich), 25 μg of bovine α-lactalbumin (Sigma–Aldrich), 150 μM MG132 (Calbiochem), and 0.4 mM Arg-Ala dipeptide (Sigma–Aldrich), and the mixture obtained was diluted 2-fold with H_2O to a final volume of 90 μL. The reaction was initiated by adding 25 μg of the crude extract and was carried out for 1.75 h at room temperature. For each reaction, three 40-μL aliquots were spotted on GF/C filters (Whatman) and treated as described previously (4).

IAA Content Measurement. Apices of short-day grown plants, including young leaves (≈100 mg per sample) were collected in 100% methanol (HPLC grade; Sigma–Aldrich). After the addition of 30 pmol of $(^2H)_2$-IAA standard (Campro Scientific) to each sample and heating at 60 °C for 5 min, the samples were incubated at room temperature for 1 h with occasional vortexing. The samples were then taken to complete dryness in vacuo. The determination of endogenous IAA levels was conducted as described previously (5). In brief, dried residues were dissolved in 30 μL of methanol to which 200 μL of diethyl ether was added. The samples were then applied to custom-made microscale aminopropyl solid-phase extraction cartridges. After washing with 250 μL of chloroform–2-propanol [2:1, (vol/vol)], IAA was eluted with 400 μL of acidified diethyl ether [2% acetic acid, (vol/vol)]. Eluates were dried, redissolved in 20 μL of methanol, and methylated with ethereal diazomethane. Excessive diazomethane and remaining solvent were removed in a gentle stream of nitrogen before samples were taken up in 15 μL of chloroform, and an aliquot of 1 μL of each sample was analyzed by GC-MS^2. All spectra were recorded on a Varian Saturn 2000 ion-trap mass spectrometer connected to a Varian CP-3800 gas chromatograph (Varian), equipped with a ZB-50 fused silica capillary column (Phenomenex).

Scanning Electron Microscopy. Tissue was fixed overnight in a 2% glutaraldehyde solution prepared in a 50 mM K-PO_4 (pH 7.0) buffer, then washed 5 times with 50 mM K-PO_4 (pH 7.0) buffer and dehydrated with an ethanol series on ice. Samples were kept in 100% ethanol until critical-point drying.

GUS Staining. GUS staining was performed as detailed previously (6).

Leaf Clearings. Leaves were collected and incubated overnight in a solution containing 85% (vol/vol) ethanol and 15% (vol/vol) acetic acid. Cleared leaves were then transferred to 100% ethanol and kept at 4 °C.

Constructions of GUS Translational Reporters. pRITA1 (7) was digested with SacI and blunted, followed by HindIII digestion. A 2-kb fragment containing GUS was isolated and ligated into pBJ36 (7), which was digested with NheI (blunted) and HindIII to generate pBJ36-GUS (pEG288; see Table S2 for the list of constructs generated in this study). The genomic regions downstream of *AtATE1* and *AtATE2* were amplified by using primers At58 and At59, and At65 and At66, respectively. The PCR fragments obtained for *AtATE1* and *AtATE2* were ligated into SpeI-blunted pBJ36-GUS, yielding pEG292 and pEG291, respectively. The genomic regions upstream of *AtATE1* or *AtATE2* were amplified by using primers At54 and At55, and At61 and At62, respectively. The resulting PCR fragments were digested

with XmaI/SphI and cloned into XmaI/SphI-digested pUC18, generating pEG286 (*AtATE1*) and pEG287 (*AtATE2*).

The genomic regions encoding *AtATE1* and *AtATE2* (including introns) were amplified by PCR using primers At56 and At57, and At63 and At64, respectively. The PCR fragment obtained for *AtATE1* was digested with NcoI/MfeI and cloned into NcoI/MfeI-digested pEG286 (yielding pEG289), whereas the PCR fragment generated for *AtATE2* was restricted with NcoI/BclI and then ligated into pEG287, which was treated with the same enzymes, yielding pEG290. pEG289 and pEG290 were digested with XmaI/NcoI, and the promoter regions followed by the coding regions of *AtATE1* and *AtATE2* were ligated into XmaI/NcoI-digested pEG292 and pEG291, respectively, to yield the *AtATE1* (pEG294) and the *AtATE2* (pEG296) translational reporters. For *Agrobacterium*-mediated plant transformation, these reporter constructs were transferred to pML-BART by using NotI digestion, yielding pEG305 (*AtATE1*) and pEG301 (*AtATE2*). The coding regions of *AtATE1* and *AtATE2* were sequenced. These sequenced regions were used to generate the rescuing constructs.

***AtATE1* and *AtATE2* Rescuing Constructs.** To rescue *ate1 ate2* plants with a genomic fragment encoding *AtATE1*, a PCR-produced fragment encoding the downstream region of *AtATE1* (using primers At56 and At59) was digested with XbaI/SpeI and then ligated into pBJ36 treated with the same enzymes. The fragment containing the putative *AtATE1* promoter and the coding region was excised from pEG289 by using XmaI/*Bst17*ZI and was subcloned into a pBJ36 derivative containing the downstream region of *AtATE1* by using the same enzymes (yielding pEG327). pEG327 was digested with NotI, and the genomic fragment encoding *AtATE1* was cloned into NotI-digested pML-BART (pEG329).

To rescue *ate1 ate2* double-mutant plants with *AtATE2*, pEG290 was digested with NcoI and blunted, followed by XmaI digestion, and the fragment containing the *AtATE2* promoter and the coding region was cloned into pBJ36 that had been digested with ClaI, followed by blunting and digestion with XmaI. This plasmid was digested with SacII/SpeI and used to clone the downstream region of *AtATE2*, obtained by PCR using primers At50 and At66, followed by SacII/SpeI digestion. This plasmid (pEG328) was treated with NotI, and the *AtATE2* genomic fragment was cloned into NotI-digested pML-BART (pEG330), which was used for *Agrobacterium*-mediated plant transformation.

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2. Garzon M, et al. (2007) PRT6/At5g02310 encodes an Arabidopsis ubiquitin ligase of the N-end rule pathway with arginine specificity and is not the CER3 locus. *FEBS Lett* 581:3189–3196.
3. Holman TJ, et al. (2009) The N-end rule pathway promotes seed germination and establishment through removal of ABA sensitivity in Arabidopsis. *Proc Natl Acad Sci USA* 106:4549–4554.
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5. Pollmann S, Duchting P, Weiler EW (2009) Tryptophan-dependent indole-3-acetic acid biosynthesis by 'IAA-synthase' proceeds via indole-3-acetamide. *Phytochemistry* 70:523–531.
6. Weigel D, Glazebrook J (2002) *Arabidopsis–A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).
7. Gleave AP (1992) A versatile binary vector system with a T-DNA organisational structure conducive to efficient integration of cloned DNA into the plant genome. *Plant Mol Biol* 20:1203–1207.

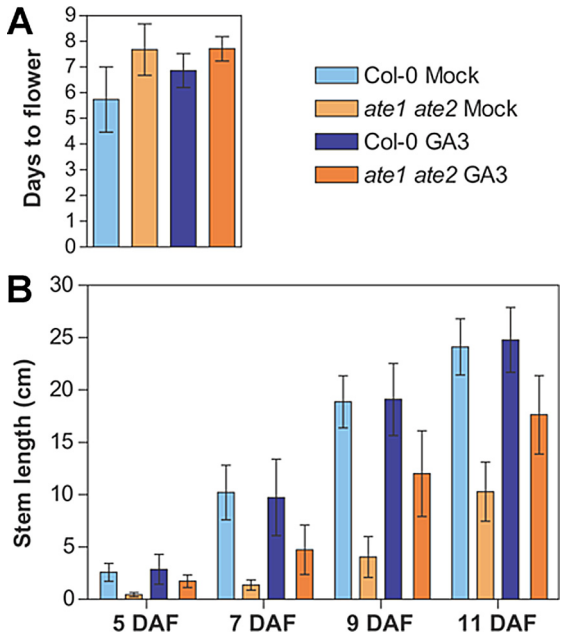

Fig. S1. GA_3 treatment partially rescues stem elongation defects of *ate1 ate2* mutant plants. Wild-type and *ate1 ate2* mutant plants were grown in short-day conditions for 2 months and transferred to continuous light for a synchronous induction of flowering. After transfer to continuous light, plants were treated with 100 μM GA_3 or a mock solution every 3 days. (*A*) Floral transition after transfer to continuous light was assessed under the microscope by the appearance of flower buds. (*B*) The length of stems was measured at the indicated times after the appearance of flower buds. Error bars represent standard deviations of 1 representative experiment. DAF, days after flower bud appearance.

Fig. S2. Complementation of the *ate1 ate2* double mutant by genomic fragments containing either *AtATE1* or *AtATE2*. T2 and T3 plants were characterized in short-day conditions to determine whether the mutant phenotypes were rescued by introduction of genomic fragments encoding either R-transferase. Leaf phenotypes of *ate1 ate2* plants and stem elongation defects (see arrows) were rescued by introduction of either gene. Pictures were taken ≈3 months after germination. AtATE1c and AtATE2c indicate *ate1 ate2* transformed with a genomic region encoding *AtATE1* and *AtATE2*, respectively. Col-0, wild-type accession Columbia-0.

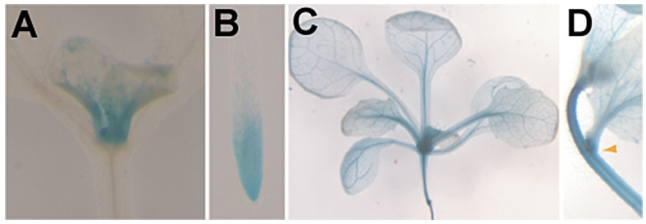

Fig. S3. Expression pattern of *AtATE2*. Wild-type plants were transformed with a construct driving the expression of a translational GUS reporter for *AtATE2*. Seedlings were stained to detect GUS activity. Similarly to *AtATE1*, *AtATE2* is expressed in the shoot (*A*) and the root apex (*B*) of 5-day-old seedlings grown in long-day conditions. In older plants, *AtATE2* is also expressed in leaves and in the hypocotyl (*C*), as well as in the stem and in the axils of cauline leaves (indicated by arrowhead) (*D*).

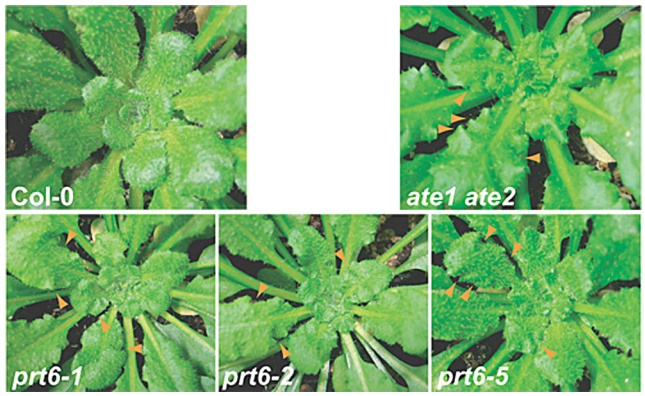

Fig. S4. Leaf morphology defects of *prt6-1*, *prt6-2*, *prt6-5*, and *ate1 ate2* mutant plants (as indicated). Plants were grown for 2 months in short-day conditions. Arrowheads point to serrations and wavy margins.

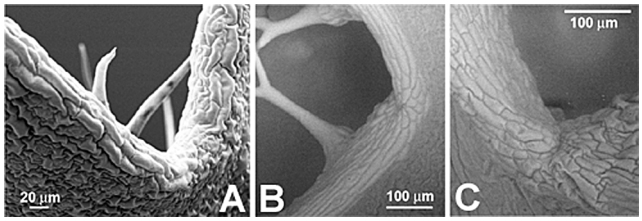

Fig. S5. Scanning electron micrographs of leaf margins. The sinus regions of leaves from plants grown in short-day conditions were examined for the presence of ectopic stipules, a characteristic of BP overexpression. Although leaves of *as1-1* (*B*) and *ate1 ate2 as1-1* (*C*) showed the presence of a notch in the sinus region where ectopic stipules would be expected, leaves of *ate1 ate2* double mutants (*A*) did not show the presence of either ectopic stipules or a notch.

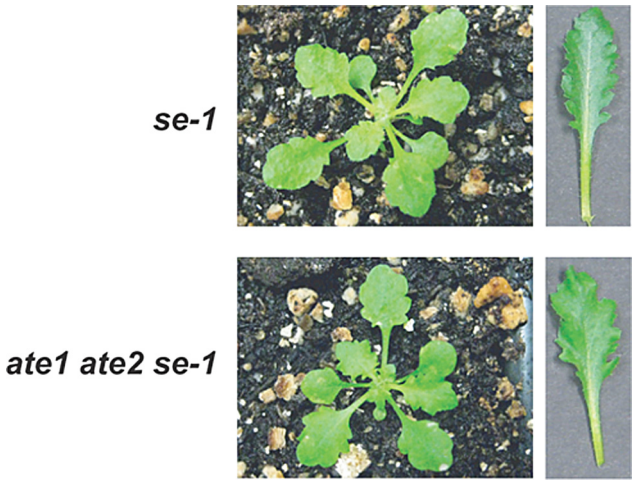

Fig. S6. Additive genetic interaction between *ate1 ate2* and *se-1* mutants. Plants were grown in short-day conditions. The overall morphology of 1-month-old *se-1* and *ate1 ate2 se-1* plants was similar, although leaves of the triple-mutant plants were slightly more serrated than those of the *se-1* single mutant. Mature leaves of 2-month-old *se-1* and *ate1 ate2 se-1* plants are morphologically similar, except that leaves of the triple-mutant plants are wavier than those of *se-1* plants.

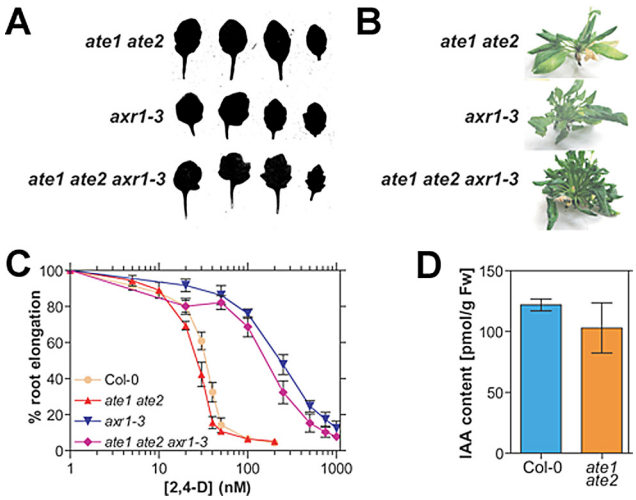

Fig. S7. Additive genetic interaction of *ate1 ate2* and *axr1-3*. (*A*) Leaves of *ate1 ate2 axr1-3* plants were more severely affected than those of *ate1 ate2* and *axr1-3* mutants. Leaves 3, 4, 5, and 6 of 18-day-old plants grown in continuous light are shown. (*B*) The outgrowth of axillary meristems in *ate1 ate2 axr1-3* triple mutants was enhanced compared with *ate1 ate2* and *axr1-3* mutant plants. Plants were grown in continuous light and were photographed 13 days after flowering. (*C*) 2,4-D-dependent inhibition of root elongation in wild-type, *ate1 ate2*, *axr1-3*, and *ate1 ate2 axr1-3* seedlings. Root length was measured 4 days after transfer to 2,4-D-containing medium. The percentage of root elongation relative to the same genetic background in the absence of 2,4-D was calculated. Error bars represent standard errors of 3 independent experiments, with $n = 12$ for each genetic background and for each experiment. (*D*) IAA levels in wild-type and *ate1 ate2* plants. Error bars represent standard deviations of 3 independent biological samples.

Table S1. Primers used in this study

Primer name	Primer sequence (5′ → 3′)
At1	GTGCAGCCCAGGGAACAAAGAGGTG
At2-3	CTTCCATCTAAAGAATTGGCTCCTCTC
At3	GCGAAGCCGAGTGAGCAGACAGA
At4-2	CCACAAAGAGGAATCTTTTCTTCATCATCAT
At120	AAAATTGATCCTTTCCATGCC
At121	CAACATAAGAATCTGCGGGAG
At231	TGTGGAAGTTTGTGTTCTCCC
At232	TCATTGGGATGTTTATCGACC
LB2	CCAAACTGGAACAACACTCAACCCTATCTC
LB_SAIL	GCTTCCTATTATATCTTCCCAAATTACCAATACA
GK8760	GGGCTACACTGAATTGGTAGCTC
At115	GGATTATTCAGGCGGAGGTCG
At118	GAAGATTCAATGTTGAAACTTGATAG**C**A**G**CT
At206	CTCCCAAAGCAAACGACGT
At207	CTGTTGTCGAGCCTCAAAGTC
At221	CGATGCGGTCACTCATTACG
At222	CCCAGTCGAGCATCTCTTC
At58	AAAATCGCGAAGCTTCAACAATACCAAGCTCCAT
At59	CGAGGACTAGTGATGATTGATATTTATATATCACGGAGAAT
At65	ACCACAGCTGCATTAAGCTTAAGTGCTTAACTCAAGC
At66	TTTTACTAGTTGATGGATAGGACGAGCTATGCA
At54	TTATTCCCGGGGAATCTTTCCCTGCTTCACCAGT
At55	AAGCTTGCATGCCCATGGAATCGCCGATTATTCATTCAGAAG
At61	TTATTCCCGGGTTATTAATTGTTCACAAGCATACATGG
At62	AAGCTTGCATGCCCATGGGATCGGAGAGAATCCACAACGA
At56	CCTTCATTTTGCCAATTGCATAACAG
At57	CGGACCATGGCACTACCGTTGATTTCATACACCATTCTCTCTGA
At63	ACTTCGTGTCGTTCATTTTGGTA
At64	CGGACCATGGCACTACCCCGGAGTTCATACACCATTATCTC
At50	GTCCAATATCAGTAAGGTTTCTTCATCATC
At66	TTTTACTAGTTGATGGATAGGACGAGCTATGCA

Point mutations are underlined and in bold.

Table S2. Plasmids used in this study

Plasmid name	Details
pEG288	pBJ36 GUS Cloning vector encoding the GUS gene
pEG305	pML-BART P_{AtATE1}:*AtATE1-GUS*:T_{AtATE1} *AtATE1* GUS translational fusion
pEG301	pML-BART P_{AtATE2}:*AtATE2-GUS*:T_{AtATE2} *AtATE2* GUS translational fusion
pEG329	pML-BART P_{AtATE1}:*AtATE1*:T_{AtATE1} *AtATE1* rescuing plasmid
pEG330	pML-BART P_{AtATE2}:*AtATE2*:T_{AtATE2} *AtATE2* rescuing plasmid