Supplementary Methods

Gene expression analysis

Arabidopsis thaliana ecotype Columbia-0 (Col-0) plants, designated wild type (WT), were grown on MS medium containing various sugar concentrations under each light/dark condition at 22˚C after seeds were surface-sterilized and incubated for 2 days at 4˚C in the dark. For gene expression analysis, total RNA was extracted from Arabidopsis seedlings using Trizol-reagent (Invitrogen) and treated with RQ1 RNase-free DNase (Promega), followed by cDNA synthesis using Rever Tra Ace (TOYOBO) and oligo(dT) primer (Promega). The gene specific primers are listed in Supplementary Table S1.

In vitro ubiquitination assay

To generate recombinant ATL8 protein, the coding sequence of the N-terminal truncated ATL8 fragment, from Val71 to Phe185, was PCR amplified using the primers listed in Supplementary Table S1, and introduced into the pENTR/D-TOPO vector (Invitrogen) to generate the plasmid pENTR/D-TOPO/ΔTMATL8. This pENTR/D-TOPO/ΔTMATL8 plasmid was transferred to the pDEST-mal destination vector, followed by construction of the MBP-ATL8 expression vector using an LR reaction. To prepare the recombinant ATL8 protein mutated in Cys123 (MBP-ATL8C123S), the mutated fragments were amplified by the primers listed in Supplementary Table S1 and cloned into the pENTR/D-TOPO vector (pENTR/D-TOPO/ΔTMATL8C123S)). *In vitro* ubiquitination assay was performed as previously described (Sato et al., 2011, Plant J.).

Subcellular localization analysis

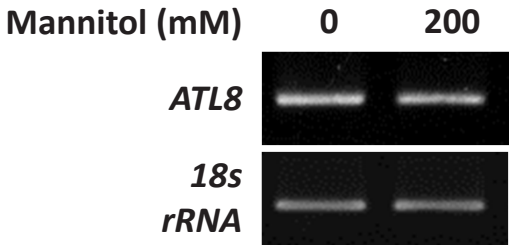
For the subcellular localization analysis, the full-length *ATL8* cording sequence containing the Cys123Ser mutation was amplified using the primers listed in Supplementary Table S1. The amplified cDNA was cloned into the pENTR/D-TOPO vector (pENTR/D-TOPO/ATL8C123S) (Invitrogen) and transferred to the pGWB5 binary vector as described by the manufacturer (Invitrogen). Constructed vectors were introduced into *Agrobacterium tumefaciens* GV3101 (pMP90) by electroporation and supplied for transient expression in *N. benthamiana* leaves as previously described (Yasuda et al., 2017, Mol. Plant). Subcellular localization was observed with confocal laser-scanning microscopy Zeiss LSM510 as previously described (Aoyama et al., 2017, BBRC). For the transient expression in mesophyll protoplast cells, pENTR/D-TOPO/ATL8C123S was transferred to pUGW5 vector and expressed as previously described (Yoo et al., 2007, Nature Protocols). The *Agrobacterium* transformed by pGWB5/ATL8C123S construct was also supplied to generate transgenic Arabidopsis plants continuously expressing ATL8C123S-GFP by floral dip method (Clough and Bent, 1998, Plant J.). To prepare the water-soluble and membrane fractions, we extract total protein from 2-week-old *ATL8C123S-GFP* plants with 1.5 ml extraction buffer with or without Triton-X 100 and subjected to ultracentrifugation as describe (Sato et al., 2009, Plant J.). The membrane fraction was resolved with 1% Triton-X 100, and each fraction was immunoprecipitated with anti-GFP beads (MBL). Proteins were detected by western blotting with anti-GFP (MBL) and anti-H^+-ATPase (Agrisera) antibodies.

Split-ubiquitin yeast two-hybrid assay

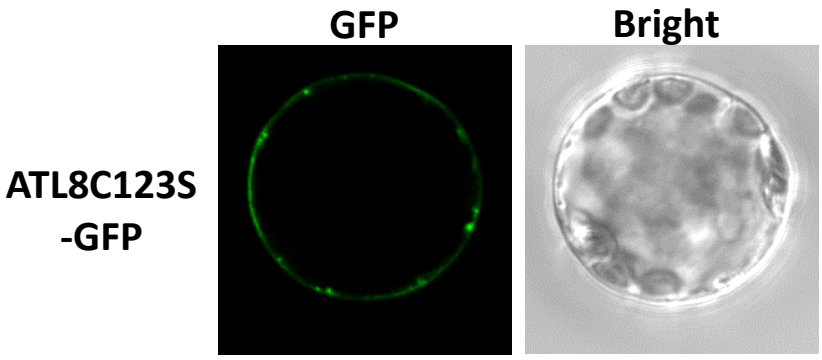
Yeast strain L40ccua (*MATa his3Δ200 trp1-901 leu2-3,112 LYS2*::$(lexAop)_4$-*HIS3 ura3*:: $(lexAop)_8$-*lacZ ADE2*::$(lexAop)_8$-*URA3 gal80* can^R $cyh2^R$) was used for split ubiquitin yeast-two hybrid assays. The full-length coding sequence of *ATL8* with or without a C123S substitution was cloned into pMetYC_GW destination vector as a C-terminally the C-terminal half of ubiquitin (Cub)-fused construct, and the full-length coding sequences of SS4 was cloned into pNX32_GW destination vector as N-terminally the N-terminal half of ubiquitin (Nub)-fused constructs (Jones et al., 2014, Science). As a control, the empty vector pNX32_GW was used. Yeasts were transformed using Frozen-EZ Yeast Transformation II Kit (Zymo Research) according to the manufacturer's protocol. Handling of yeast cultures and β-galactosidase assays were performed as described in the Yeast Protocols Handbook (Clontech).

Supplementary Table S1 List of primers used in this study.

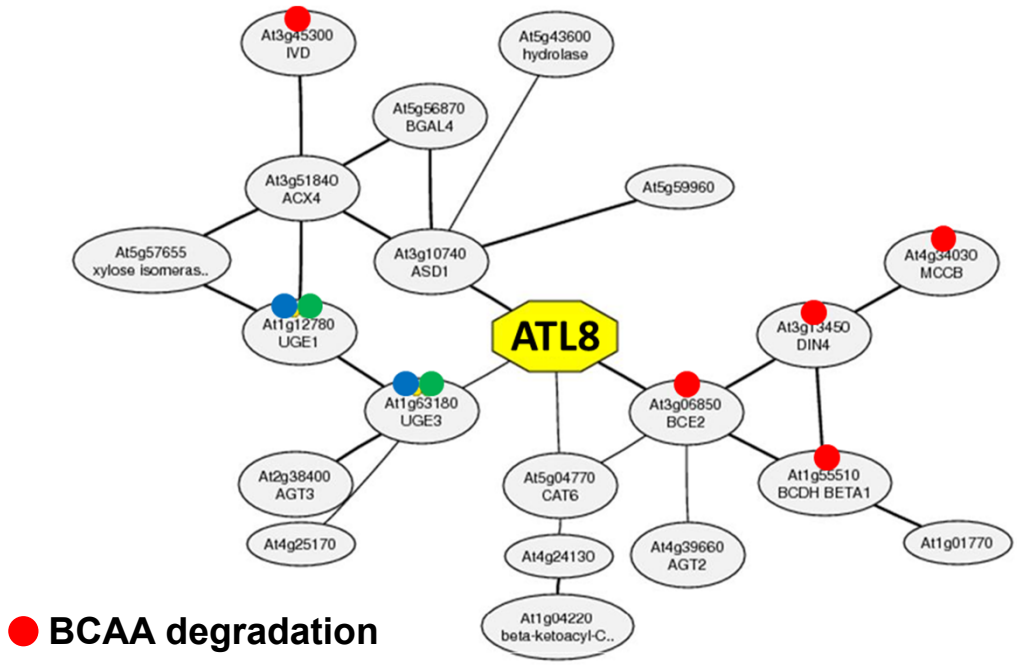
Gene	Sequence (Forward, 5'→3')	Sequence (Reverse, 5'→3')
Plasmid construction		
ATL8 CDS	CACCATGGCGCGCCTTCTC	AGGCAAGTTATCAGGACCATC
ATL8 CDS Val71-Phe185	CACCGTAGCTGCAGCCAACA	AGGCAAGTTATCAGGACCATC
ATL8 CDS C123S	TTGCCGCAGTCTGGACATGGT	ACCATGTCCAGACTGCGGCAA
SS4 CDS	CACCATGACGACGAAGCTATG	AGAGCTGTTCCTAATCGCACG
Gene expression analysis		
ATL8	CGGTTCTTCTTTGTGCACTG	GCAACACCCTAAGCTCGTCT
18s rRNA	CGGCTACCACATCCAAGGAA	GCTGGAATTACCGCGGCT
EF1α	GCTGTCCTTATCATTGACTCCACC	TCATACCAGTCTCAACACGTCC
ACT7	TTATCTGGTTCGTGGTGGTG	TCAATTCCCATCTCAACTAGGG



Supplementary Figure S1
ATL8 expression in response to osmotic treatment. WT seedlings were grown for 8 days in sugar-free MS medium and transferred to medium containing 0 or 200 mM mannitol for 1 h, and *ATL8* mRNA transcripts were determined by RT-PCR. *18s rRNA* was used as the internal control.



Supplementary Figure S2
ATL8C123S-GFP fusion protein was transiently expressed in mesophyll protoplast cells derived from Arabidopsis leaves. Confocal laser microscopy showing the subcellular localization of ATL8C123S-GFP.



Supplementary Figure S3
Co-expression network of *ATL8* predicted by ATTED II (http://atted.jp/).
Different colors indicate genes involved in different metabolic pathways.