Supporting Information

Conditional destabilization of the TPLATE complex impairs endocytic internalization.

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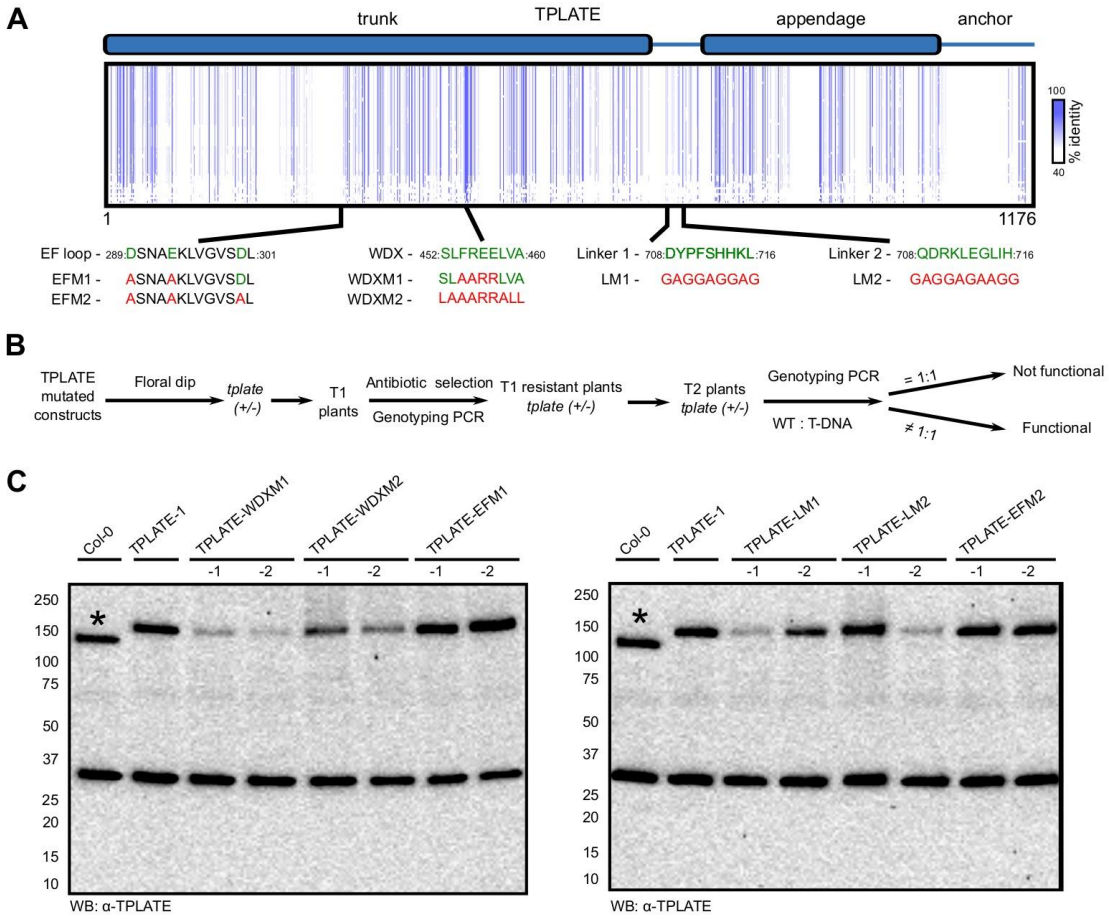

Figure S1. Identification and functionality assay of selected TPLATE motif substitution mutants.

(A) Schematic representation of TPLATE domain organization consisting of a trunk domain, a linker domain separating the trunk from the appendage domain and an anchor domain. *Arabidopsis* TPLATE was aligned against various eukaryotic species. The schematic view of the amino acid alignment data normalized on the *Arabidopsis* TPLATE sequence shows highly conserved amino acids as vertical blue lines. The four motifs selected for mutagenesis in the trunk and linker domains are shown. The conserved original amino acids of these motifs are highlighted in green and the corresponding substituted amino acids are indicated in red. Numbers represent the amino acid positions of these elements within *Arabidopsis* TPLATE.

(B) Schematic diagram employed to screen for functionality of the generated TPLATE mutants. The male sterility phenotype of the *tplate* T-DNA mutant line causes it to segregate in a 1-to-1 ratio of heterozygous and homozygous wild type plants (Aa:AA = 1:1). The segregation ratio of WT:T-DNA among T2 transgenic *tplate* lines expressing the complementation construct of TPLATE, mutated in the selected motifs, was used to initially evaluate the functionality of these constructs.

(C) Anti-TPLATE western blot detecting the presence of endogenous TPLATE in Col-0 (marked with an asterisk) as well as full-length of GFP fusions of TPLATE and various independent motif

substitutions mutants in the complemented *tplate* (-/-) background devoid of endogenous TPLATE.

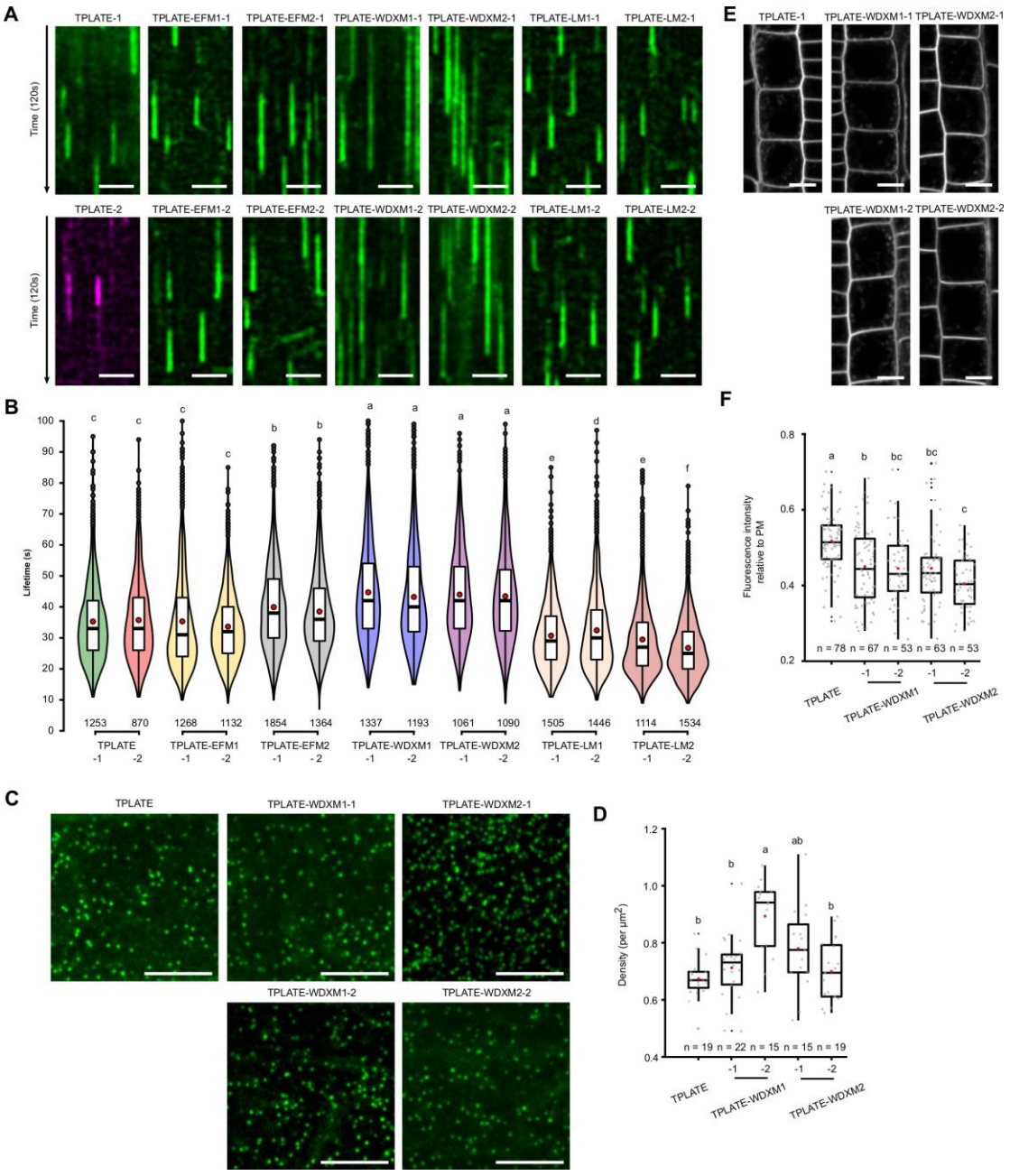

Figure S2. Functional substitutions in TPLATE affect endocytosis.

(A-B) Representative kymographs and violin plot graphs show that motif substitution mutants of TPLATE display variable dwell-times at the PM. Among the motif substitutions mutants, both TPLATE-WDXM1 and TPLATE-WDXM2 displayed the most pronounced delay in average lifetime on the PM. Scale bar = 25 µm. The number of events analyzed for each independent line is indicated at the bottom of the graph. At least 12 movies from 4 seedlings were imaged for each independent transgenic line. Red circles represent the mean. Letters represent significantly different groups ($p < 0.001$) evaluated by the Tukey multiple pairwise-comparisons

test.

(C) Spinning disc images of TPLATE-WDXM1 and TPLATE-WDXM2 recruited to endocytic spots on the PM. Scale bar = 7 µm.

(D) Quantification of the endocytic spot density in TPLATE as well as in two independent TPLATE-WDXM1 and TPLATE-WDXM2 complemented lines. The “Find Maxima” tool of ImageJ was employed to quantify the density using time-projected images of 10 consecutive time-lapse frames. Numbers of quantified cells from independent seedlings (≥ 4 seedlings) are indicated.

(E-F) Representative confocal images and quantification showing that both TPLATE-WDXM1 and TPLATE-WDXM2 complemented lines display reduced FM4-64 uptake in root epidermal cells. Scale bar = 10 µm. Numbers represent the number of cells analyzed from at least 8 independent roots for each transgenic line.

Red circles represent the mean in panel (D) and (F). Letters represent significantly different groups ($p < 0.001$), evaluated by the Tukey multiple pairwise-comparisons test.

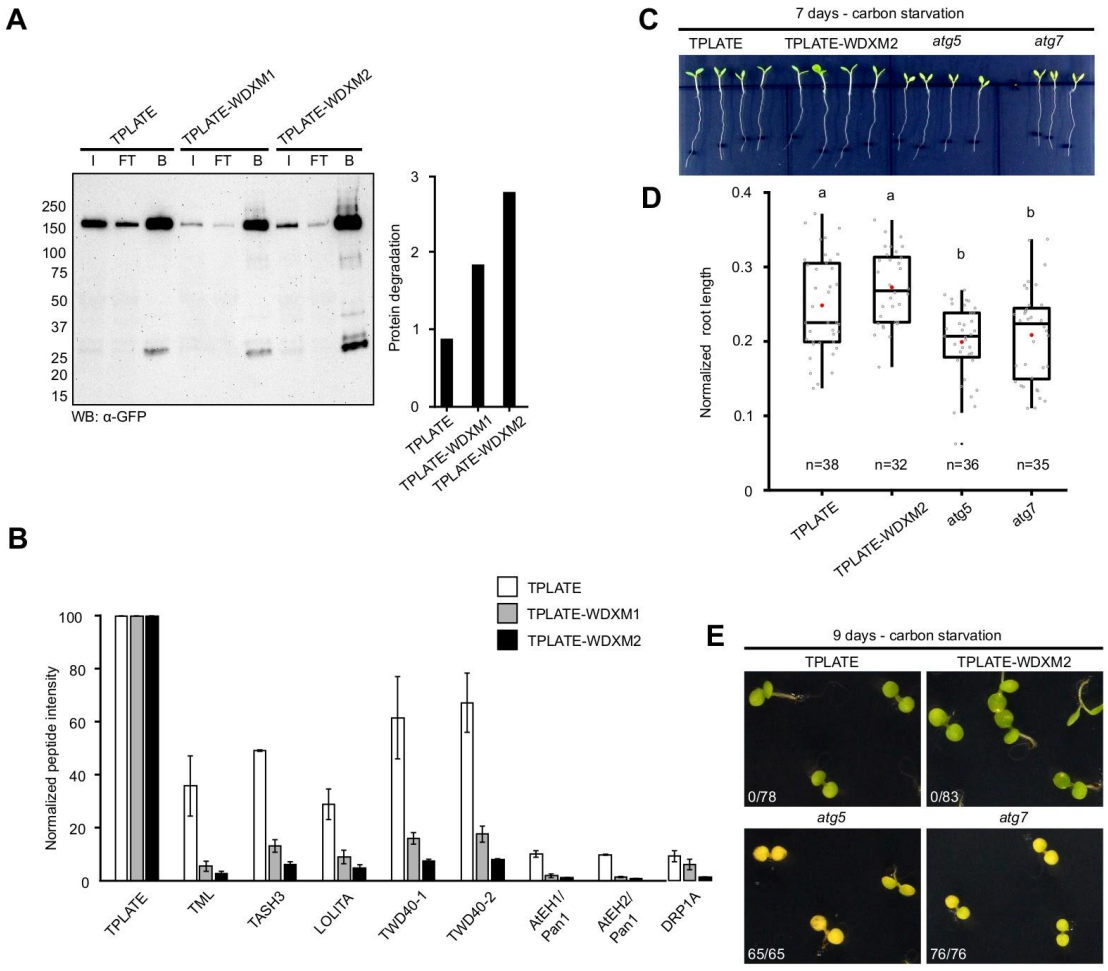

Figure S3. WDX domain substitutions destabilize TPC but do not cause hypersensitivity to nutrient depletion.

(A) GFP-Trap analysis of TPLATE-GFP, TPLATE-WDXM1-GFP and TPLATE-WDXM2-GFP

complemented lines. Input (I), flow through (FT) and bound (B) fractions were analyzed using an anti-GFP antibody. Protein degradation was quantified as the ratio between full-length protein and all smaller bands. The data for TPLATE and TPLATE-WDXM2 is also presented in Figure 2 and expanded here with the independent TPLATE-WDXM1 line for comparison.

(B) MS analysis following co-IP on complemented TPLATE, TPLATE-WDXM1 and TPLATE-WDXM2 lines. For each TPC subunit, the intensity of peptides present in all experiments was averaged and normalized to the values of the bait protein. Standard deviations are based on three technical repeats. This panel also represents an expansion of the panel in Figure 2 and serves to confirm the data obtained with TPLATE-WDXM2 using the independent TPLATE-WDXM1 line.

(C-E) Seedling development phenotype in response to fixed carbon starvation of TPLATE and TPLATE-WDXM2 complemented plants compared with *atg5 and atg7* mutants. Seedlings were grown vertically or horizontally on ½ MS solid medium without sucrose at 20 °C for 5 days followed by 7-day (C) or 9-day (E) constant dark. For root growth measurements, root growth in the dark for 7 days was normalized to the 5-day root growth under light conditions (D). The numbers in the box plot represent the number of seedlings measured. Red circles represent the mean. Letters represent significantly different groups ($p < 0.001$) evaluated by the Tukey multiple pairwise-comparisons test. To quantify the yellowing of cotyledons better than in the vertically growing conditions seedlings were grown horizontally and visually scored after a 9-day carbon starvation treatment (E). Numbers represent the amount of seedlings showing yellowing cotyledons out of the total number of seedlings analyzed.

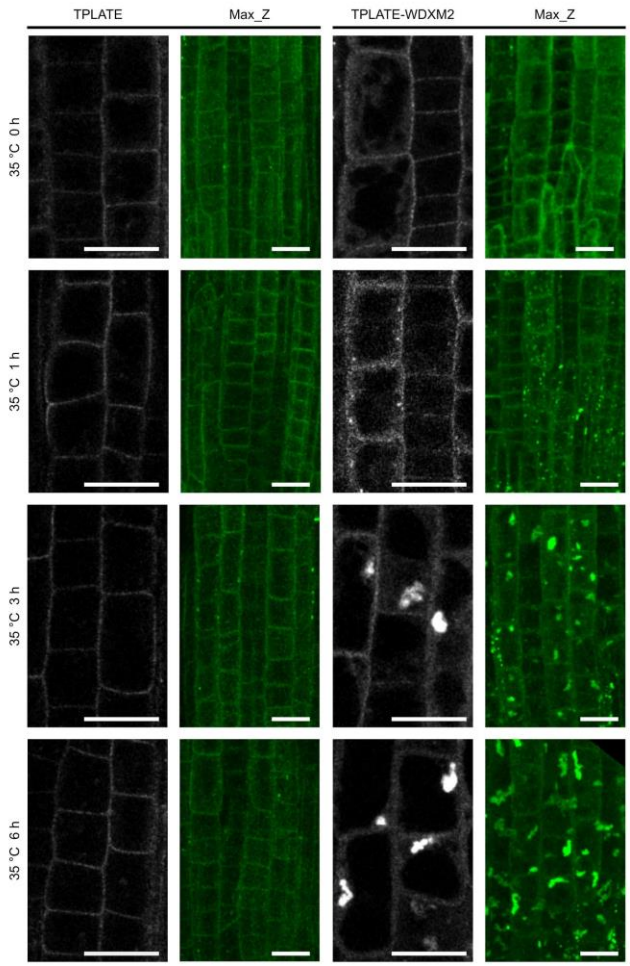

Figure S4. Short-term heat treatment induces an incremental aggregation of TPLATE-WDXM2 in the cytoplasm.

Confocal images and Z-projections of root cells from TPLATE and TPLATE-WDXM2 complemented lines subjected to short-term heat treatment. 5-day old seedlings, grown vertically on ½ MS media at 21 °C were subjected to 35 °C for 0 h, 1 h, 3 h and 6 h and imaged immediately following the treatment. The images present single optical sections (greyscale) as well as maximum Z-projections (green) at lower magnification. Scale = 20 µm.

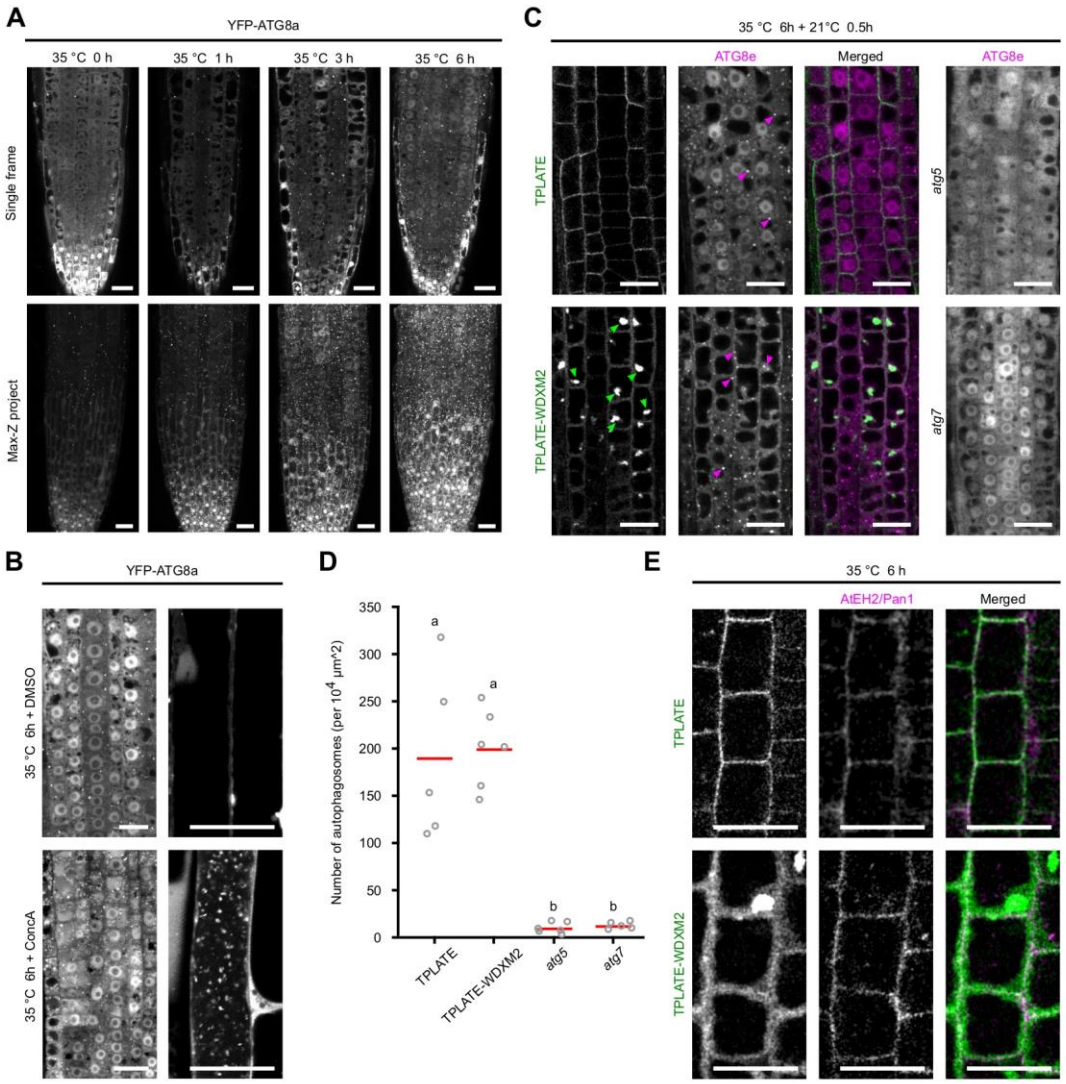

Figure S5. Destabilizing TPC does not inhibit autophagosome formation under heat stress.

(A) Confocal images (top) and Z-projections (below) of root cells from a YFP-ATG8e expressing line during a time series of heat treatment. 5-day old seedlings, grown vertically on ½ MS media at 20 degrees were subjected to 35 °C from 0 to 6 hours and were imaged immediately following the treatment. Scale bars = 20 µm.

(B) Confocal images of root cells from YFP-ATG8e expressing lines upon 6h heat treatment. 5-day old seedlings, grown vertically on ½ MS media at 20 degrees were subjected to 35 °C with or without 0.5 µM ConcA treatment for 6h and then imaged. Higher up in the root (right side images), the accumulation of autophagosomes inside the vacuole upon ConcA treatment indicates that autophagic flux is not impaired by the treatment. Scale bars = 25 µm.

(C-D) Confocal images and quantification of mCherry-ATG8e positive autophagosomes in TPLATE and TPLATE-WDXM2 complemented lines as well as in *atg* mutants. Purple arrowheads point to autophagosomes, green arrowheads point to heat-aggregated TPLATE-WDXM2. 5-day old seedlings grown at 20 °C were treated with 35 °C for 6 hours and then allowed to recover shortly at 20 °C for 30 mins. Scale bar = 25 µm. The number of

autophagosomes in a region of interest (ROI) from at least 4 seedlings for each group were analyzed with Image J using the “Find maxima tool”. Red lines represent the mean. Letters represent significantly different groups ($p < 0.001$) evaluated by the Tukey multiple pairwise-comparisons test.

(E) Confocal images of AtEH2/Pan1-mRuby3 in TPLATE and TPLATE-WDXM2 complemented lines after 6h heat treatment. 5-day old seedlings grown at 20 °C were treated with 35 °C for 6 hours and then were imaged. Scale bar = 25 µm.

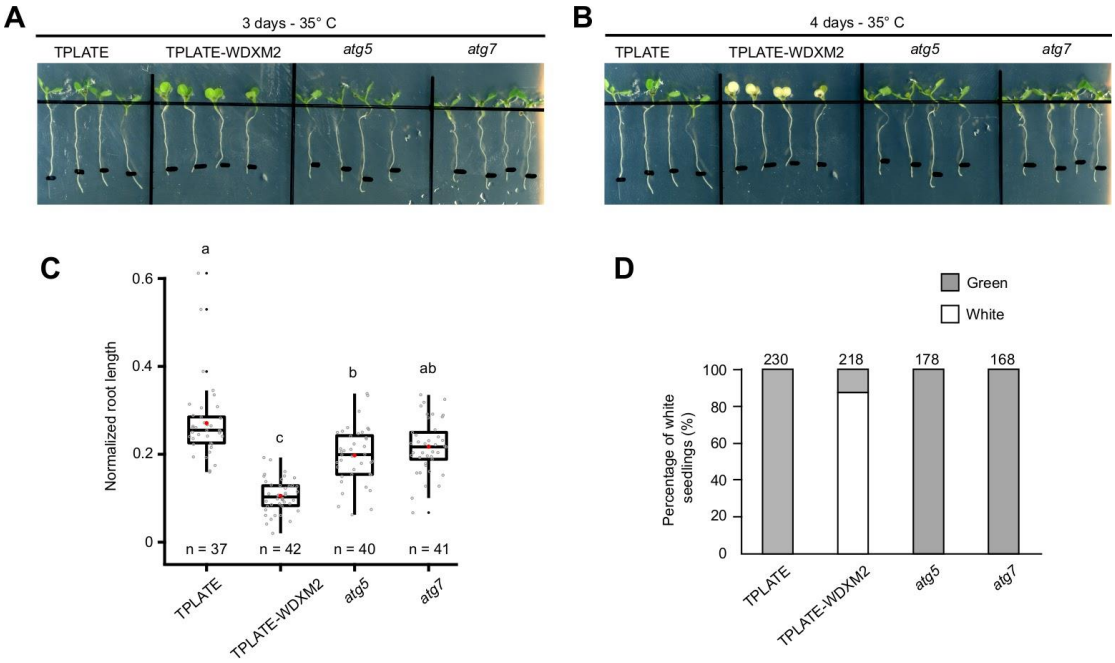

Figure S6. Destabilizing TPLATE-WDXM2 does not phenocopy *atg* mutants under long-term heat stress.

(A-B) Phenotypic comparison between TPLATE, TPLATE-WDXM2 complemented lines and *atg5* and *atg7* mutants in response to heat stress. Seedlings were grown vertically at 20 °C for 5 days and then moved to 35 °C for 3 (A) or 4 days (B). Panel (A) and panel (B) show the same plants after 3- or 4-day heat treatment.

(C-D) Phenotypic quantification of TPLATE, TPLATE-WDXM2 complemented lines and *atg5* and *atg7* mutants after 3- or 4-day heat treatment. (**C)** For each seedling, the primary root growth after 3-day heat treatment was normalized to the root growth before heat treatment respectively. Red circles represent the mean. Letters represent significantly different groups ($p < 0.001$) evaluated by the Tukey multiple pairwise-comparisons test. **(D)** The ratio of seedlings with whitening versus green cotyledons after 4-day heat treatment was visually quantified. The number of seedlings analyzed is indicated.

Table S1. Segregation analysis in the offspring of *tplate* (+/-) plants expressing various TPLATE motif substitution isoforms.

Segregation ratios of the progeny of heterozygous *tplate* mutants expressing various TPLATE motif substitution constructs are shown. The male sterility phenotype of the *tplate* T-DNA insertion line causes the ratio between T-DNA: WT among the offspring progeny of heterozygous *tplate* mutants to equal 1:1. The ratios of T-DNA: WT among T2 transgenic plants of *tplate* mutants expressing TPLATE motif substitution constructs were identified by genotyping PCR to preliminary evaluate the functionality of these TPLATE isoforms. At least 3 individual transgenic lines carrying TPLATE motif substitutions constructs were analyzed. $\chi 2$ 0.05 (1) = 3.841.

Mutant plants	Line number	T-DNA	WT	Total	χ2 (1:1)
TPLATE-EFM1	**-1**	12	10	22	0,182
	-2	15	9	24	1,500
	-3	17	7	24	4,167
	Total	44	26	70	4,620
TPLATE-EFM2	**-1**	16	8	24	2,667
	-2	22	2	24	16,667
	-3	17	7	24	4,167
	Total	55	17	72	20,056
TPLATE-WDXM1	**-1**	18	6	24	6,000
	-2	20	4	24	10,667
	-3	18	6	24	6,000
	Total	56	16	72	22,220
TPLATE-WDXM2	**-1**	21	3	24	13,500
	-2	22	2	24	16,667
	-3	18	6	24	6,000
	Total	61	11	72	34,720
TPLATE-LM1	**-1**	18	6	24	6,000
	-2	19	5	24	8,167
	-3	18	6	24	6,000
	Total	55	17	72	20,050
TPLATE-LM2	-1	16	8	24	2,667
	-2	20	4	24	10,667
	-3	21	3	24	13,500
	Total	57	15	72	24,500

Table S2. Primers used for mutagenesis PCR.

Primers		Sequence
Sewing primer	Fwd	ggggacaagtttgtacaaaaaagcaggctATGGACATTCTTTTTGCTCAGATCC
	Rev	ggggaccactttgtacaagaaagctgggtTGTTAACTTTGGTATATTTTCTATCTTTGCA
TPLATE-EFM1	Fwd	ATGCCACCGTTGTGGCCTCCAACGCCGCGAAGCTGGTTGG
	Rev	CCAACCAGCTTCGCGGCGTTGGAGGCCACAACGGTGGCAT
TPLATE-EFM2	Fwd	TCTCAGCGTTGGTTACCCATTTGGCGCCATTCTTGG
	Rev	ACCAACGCTGAGACGCCAACCAGCTTCGCG
TPLATE-WDXM1	Fwd	TTAGCAGCTAGACGACTGGTGGCAATGCTTGTGGAAAGC
	Rev	CAGTCGTCTAGCTGCTAAGCTCTTACTGGTAACACTATTGAGATCATCTTTAATCCTTG
TPLATE-WDXM2	Fwd	TTAGCGGCAGCTAGACGAGCATTATTGATGCTTGTGGAAAGCTGCTTCCAGTTG
	Rev	CAATAATGCTCGTCTAGCTGCCGCTAACTTACTGGTAACACTATTGAGATCATCTTTAATCCTTGC
TPLATE-LM1	Fwd	GGTGCAGGCGGTGCTGGAGGTGCAGGAACTGTTCAGTTTTACGAACCATCAGCTGC
	Rev	TCCTGCACCTCCAGCACCGCCTGCACCTCCTCTTGTCTCACTAACTCCAGCCCAC
TPLATE-LM2	Fwd	GGTGCAGGCGGTGCTGGAGCTGCAGGTGGAAAGGCCATTCTCGAGCTTTGGAGG
	Rev	TCCACCTGCAGCTCCAGCACCGCCTGCACCAGCAGCTGATGGTTCGTAAAACTGAACAG

Table S3. Plant materials used in this study

Plants	Background	Identification	Source/Ref
tplate	*tplate (+/-), tplate (+/+)*	Genotyping PCR	(1)
atg5-1	*atg5-1 (-/-)*	Genotyping PCR	(2)
atg7-3	*atg7-3 (-/-)*	Genotyping PCR	(2)
LAT52p::TPLATE-GFP	*tplate (-/-)*	Genotyping PCR	(1)
LAT52p::TPLATE-tagRFP	*tplate (-/-)*	Genotyping PCR	(3)
LAT52p::TPLATE-EFM1-GFP	*tplate (-/-)*	Genotyping PCR	This study
LAT52p::TPLATE-EFM2-GFP	*tplate (-/-)*	Genotyping PCR	This study
LAT52p::TPLATE-WDXM1-GFP	*tplate (-/-)*	Genotyping PCR	This study
LAT52p::TPLATE-WDXM2-GFP	*tplate (-/-)*	Genotyping PCR	This study
LAT52p::TPLATE-LM1-GFP	*tplate (-/-)*	Genotyping PCR	This study
LAT52p::TPLATE-LM2-GFP	*tplate (-/-)*	Genotyping PCR	This study
UBQ10p::YFP-ATG8a	Col-0		(2)
UBQ10p::mCherry-ATG8e	Col-0		(2)
LAT52p::TPLATE-GFP x 35Sp::DRP1a-mRFP	*tplate (-/-)drp1a(+/-)*	Genotyping PCR	(3)
LAT52p::TPLATE-WDXM2-GFP x 35Sp::DRP1a-mRFP	*tplate (-/-)drp1a(+/-)*	Genotyping PCR	This study
UBQ10p::mCherry-ATG8e x *atg5-1*	*atg5-1 (-/-)*	Genotyping PCR	This study
UBQ10p::mCherry-ATG8e x *atg7-3*	*atg7-3 (-/-)*	Genotyping PCR	This study
UBQ10p::mCherry-ATG8e x LAT52p::TPLATE-GFP	*tplate (-/-)*	Genotyping PCR	This study
UBQ10p::mCherry-ATG8e x LAT52p::TPLATE-WDXM2-GFP	*tplate (-/-)*	Genotyping PCR	This study
LAT52p::TPLATE-GFP x H3.3p-EH2-mRuby3	*tplate (-/-) eh2(-/-)*	Genotyping PCR	(3)
LAT52p::TPLATE-WDXM2-GFP x H3.3p-EH2-mRuby4	*tplate (-/-) eh2(-/-)*	Genotyping PCR	This study

Table S4. Antibodies used in this study.

Antibody	dilution	Incubation time	Source/Ref
anti-TPLATE2	1/1000	1h	(4)
anti-PIN1	1/600		donated by Dr. Ranjan Swarup
anti-PIN2	1/600		(5)
anti-PIP2	1/600		Agrisera
anti-sheep-Alexa488	1/600		Dianova
anti-rabbit-Alexa555	1/600		Dianova
anti-GFP-HRP	1/1000	1h-o/n	Miltenyi Biotec GFP/HRP antibody (130-091-833)
anti-rabbit	1/10000	1h	Amersham ECL Mouse IgG, HRP-linked whole Ab (NA931)

Supplemental Data Source 1: Sequences of the multiple sequence alignment

This data source contains all sequences that were used to generate the schematic alignment shown in Figure 1 and Figure S1.

The alignment in Figure 1 and Figure S1The multiple sequence alignment was constructed with the MAFFT algorithm in einsi mode and visualized by the Jalview program.

Supplemental Data Source 2: MaxQuant result file containing the significantly identified peptide sequences in all experiments.

The first tab shows the peptides used for the quantification in Figure 2 and Figure S3 and their location on the protein sequence. The second tab shows the MaxQuant data. The third tab gives an overview of the column headers of the MaxQuant data.

For the quantification, only those peptides were used that were identified with all three bait proteins in all replicates. Those peptides are listed below here and mapped onto the protein sequence. Average intensities of those peptides were generated for each bait protein per replica. Similarly, this was done for all the other TPC subunits. The average intensities of the replicas of the subunits and their respective standard deviations were calculated and normalized to the average intensity of the three replicas of the bait and plotted in Figure 2 and Figure S3. These values were plotted in Figure 2 and Figure S3.

References

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4. Dejonghe W, et al. (2019) Disruption of endocytosis through chemical inhibition of clathrin heavy chain function. Nat Chem Biol 15(6):641-649.
5. Abas L, et al. (2006) Intracellular trafficking and proteolysis of the Arabidopsis auxin-efflux facilitator PIN2 are involved in root gravitropism. Nat Cell Biol 8(3):249-256.