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Supplementary Materials for

Peripheral membrane proteins modulate stress tolerance by safeguarding cellulose synthases

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The PDF file includes:

Other Supplementary Material for this manuscript includes the following:

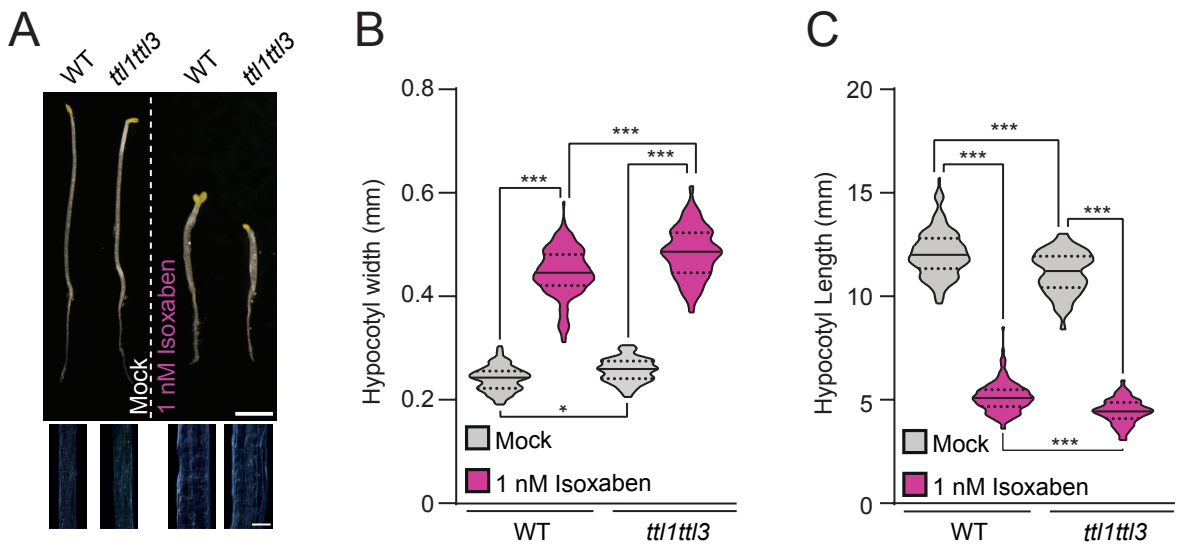

Fig. S1. *ttl1ttl3* seedlings show increased sensitivity to isoxaben.
(**A**) 5-day-old etiolated WT (Col-0) and *ttl1ttl3* seedlings germinated on control or media supplemented with 1 nM isoxaben (upper panels). Magnifications show distinctive hypocotyl width in *ttl1ttl3* double mutant plants in 1nM isoxaben, indicative of defective anisotropic growth (lower panels). Scale bar=5 mm (upper image) and 200 µm (close-up hypocotyls). (**B**) and (**C**) Quantification of hypocotyl width (B) and length (C) of seedlings as depicted in (A). Violin plots: centerlines show the medians; dotted lines indicate the 25th and 75th percentiles; N≥11 seedlings from 3 independent experiments. Unpaired t-test; **p*-value≤0.05, ***p*-value≤0.01, ****p*-value≤0.001.

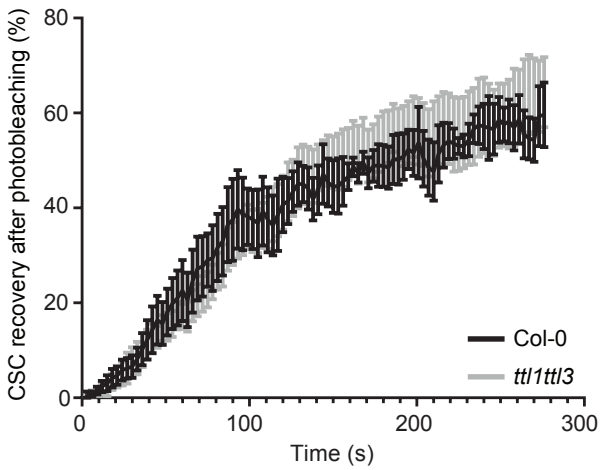

Fig. S2. *ttl1ttl3* mutant is not affected in CESA delivery to the plasma membrane.
Fluorescence Recovery After Photobleaching (FRAP) of 3-day-old WT (Col-0) and *ttl1ttl3* etiolated hypocotyl epidermal cells expressing YFP-CESA6. Graph displays re-population of the plasma membrane at indicated time points with fluorescent CESA foci as percentage of CESA density at t=0 (just before FRAP). Two-way ANOVA analysis of CESA recovery; p=0.84 (genotype), $p \leq 0.001$ (time), $p > 0.99$ (genotype × time). N≥7 cells from at least five seedlings and three independent experiments. Values are mean±SEM.

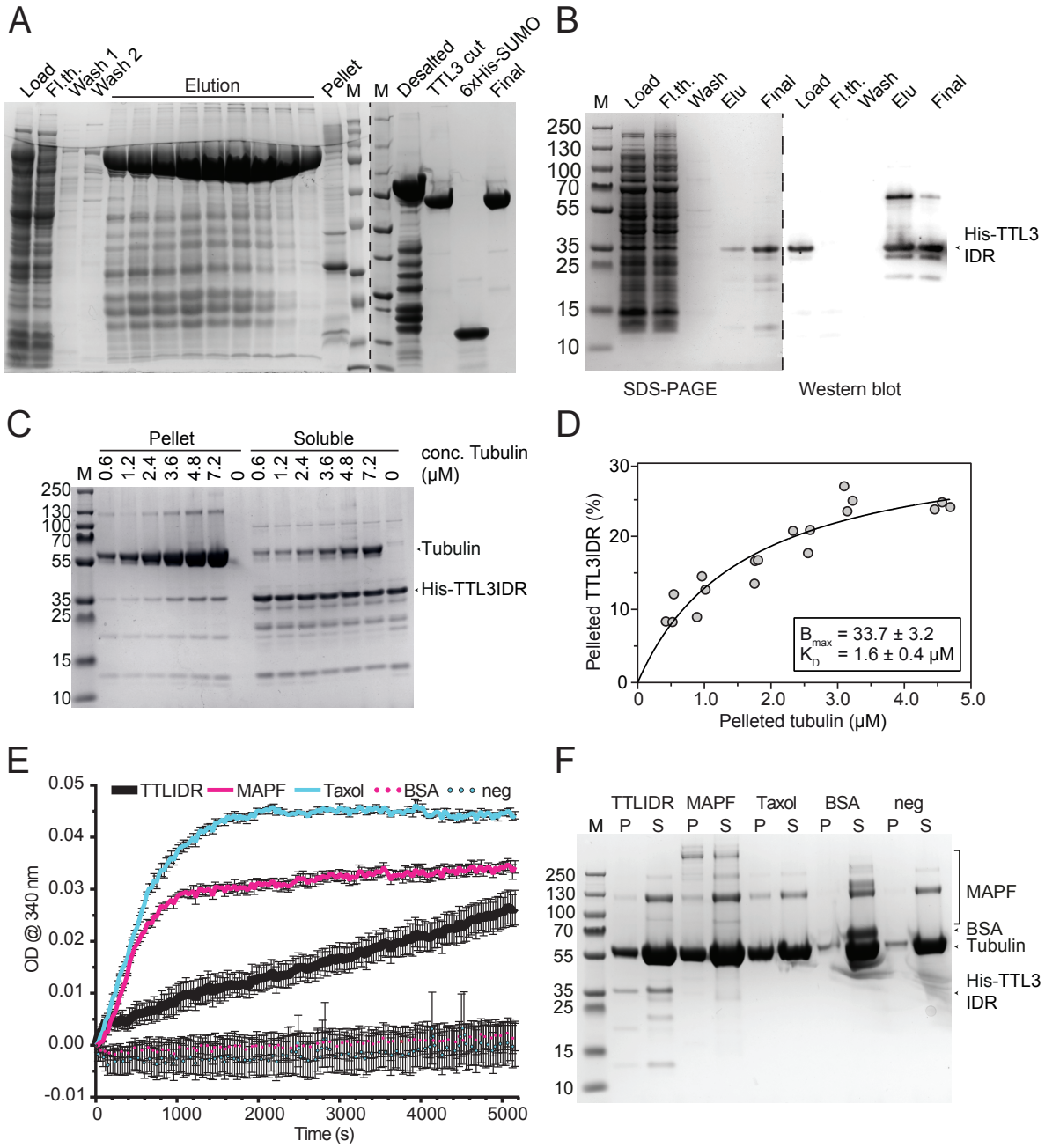

Fig. S3. TTL3IDR interacts with and promotes microtubule formation.
(**A**) Expression and purification of 6xHis-SUMO-TTL3. SDS-PAGE gel of the purification steps. M = Molecular marker; Load = crude protein extract; Fl.th. = flowthrough after Ni-NTA Agarose binding; TTL3 cut = TTL3 after digestion with SUMO protease and gel filtration; Final = Desalted, cut and concentrated protein. (**B**) Expression and purification of 6xHis-TTL3IDR. Left panel: Coomassie-stained SDS-PAGE gel of the purification steps. M = Molecular marker; Load = crude protein extract; Fl.th. = flowthrough after Ni-NTA Agarose binding; Elu = Imidazole elution; Final = Desalted and concentrated protein. Right panel: Representative image of a Western blot to detect 6xHis-TTL3IDR. (**C**) Representative Coomassie gel used to estimate the dissociation constant for 6xHis-TTL3IDR and microtubules. Tubulin and 6xHis-TTL3IDR molecular sizes are indicated by

arrowheads. (**D**) The binding affinity of 6xHis-TTL3IDR to microtubules was determined by calculating their dissociation constant (K_D) (1.6±0.4 μM; best-fit values ± Std. Error). 6xHis-TTL3IDR levels were kept constant while the amount of microtubules was increased. K_D was calculated by fitting a saturation binding curve onto the data, obtained from images as in (F); N=3 independent experiments. **(E)** Microtubule turbidity assay. Tubulin was incubated with buffer (neg) or BSA as negative controls, a microtubule-associated protein fraction (MAPF) or taxol as positive controls, and the 6xHis-TTL3IDR. Microtubule formation was measured at 340 nm. Values are mean±SEM. N=3 technical replicates. **(F)** Reactions, as shown in (C), were spun down, separated in pellet and supernatant fractions, and analyzed by SDS-PAGE. While distinct tubulin bands at 55 kDa are visible in the pellet fraction of the positive controls (MAPF, taxol) and 6xHis-TTL3IDR, indicative of microtubule formation and tubulin stabilization, clear smearing of bands appears in the supernatant fractions of the negative controls (BSA, buffer), indicative of no microtubule formation and tubulin degradation. M = Molecular marker; P = Pellet fraction; S = Supernatant fraction.

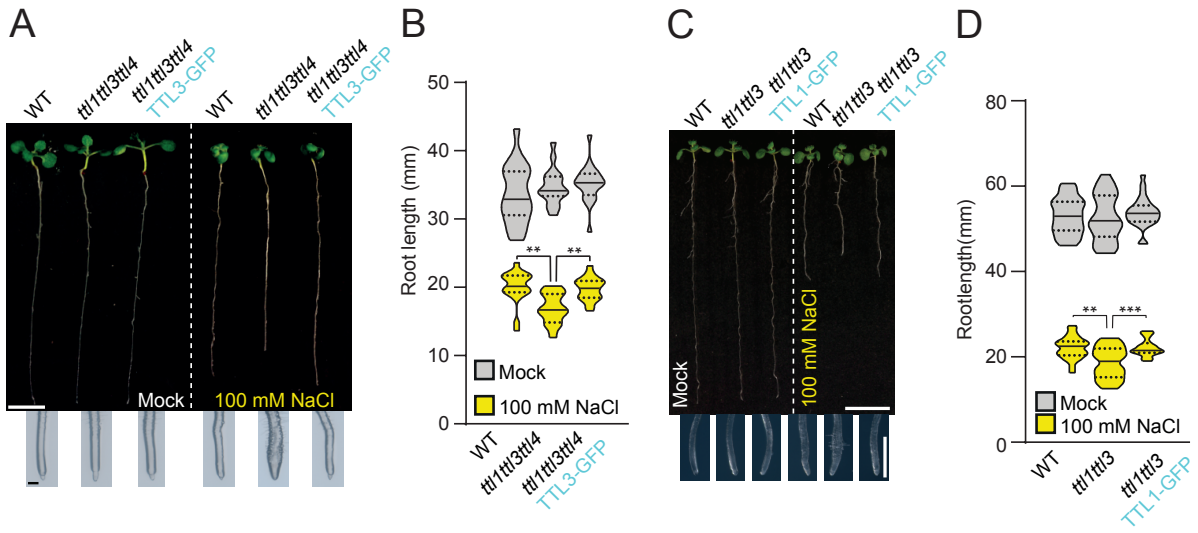

Fig. S4. *TTL3-GFP* and *TTL1-GFP* driven by their own promoters complement the root growth defects of *ttl* mutants in the presence of NaCl.

(**A**) The *TTL3-GFP* construct complements the root growth defects of the *ttl1tt3ttl4* mutant seedlings on NaCl. 3-day-old WT (Col-0), *ttl1ttl3ttl4*, and *ttl1ttl3ttl4* TTL3-GFP seedlings germinated and grown on control conditions were transferred to control and NaCl containing media and grown in the light for 8 additional days (upper panels). Magnifications show distinctive root tip swelling in *ttl1ttl3ttl4* triple mutant plants under salt stress, indicative of anisotropic growth failures (lower panels). Scale bars=5 mm (upper image) and 200 µm (close-up root tips). (**B**) Quantification of root length of seedlings as in (A). Violin plots: centerlines show the medians; dotted lines indicate the 25th and 75th percentiles; Welch's ANOVA between mock groups: *p*-value=0.29; Welch's ANOVA between NaCl treated groups: *p*-value≤0.001; Dunnett's T3 multiple comparison: ***p*-value≤0.01. N≥15 roots and three independent experiments. (**C**) The *TTL1-GFP* construct complements the root growth defects of the *ttl1tt3* mutant seedlings on NaCl. 3-day-old WT (Col-0), *ttl1ttl3,* and *ttl1ttl3* TTL1-GFP seedlings germinated and grown on control conditions were transferred to control and NaCl containing media and grown in the light for 8 additional days (upper panels). Magnifications show distinctive root tip swelling in *ttl1ttl3* triple mutant plants under salt stress, indicative of anisotropic growth failures (lower panels). Scale bars=1 cm (upper image) and 1 mm (close-up root tips) µm. (**D**) Quantification of root length of seedlings as in (C). Violin plots: centerlines show the medians; dotted lines indicate the 25th and 75th percentiles; Welch's ANOVA between mock groups: *p*-value=0.29; Welch's ANOVA between NaCl treated groups: *p*-value≤0.001; Dunnett's T3 multiple comparison: ***p*-value≤0.01, ****p*-value≤0.001. N≥15 roots and two independent experiments.

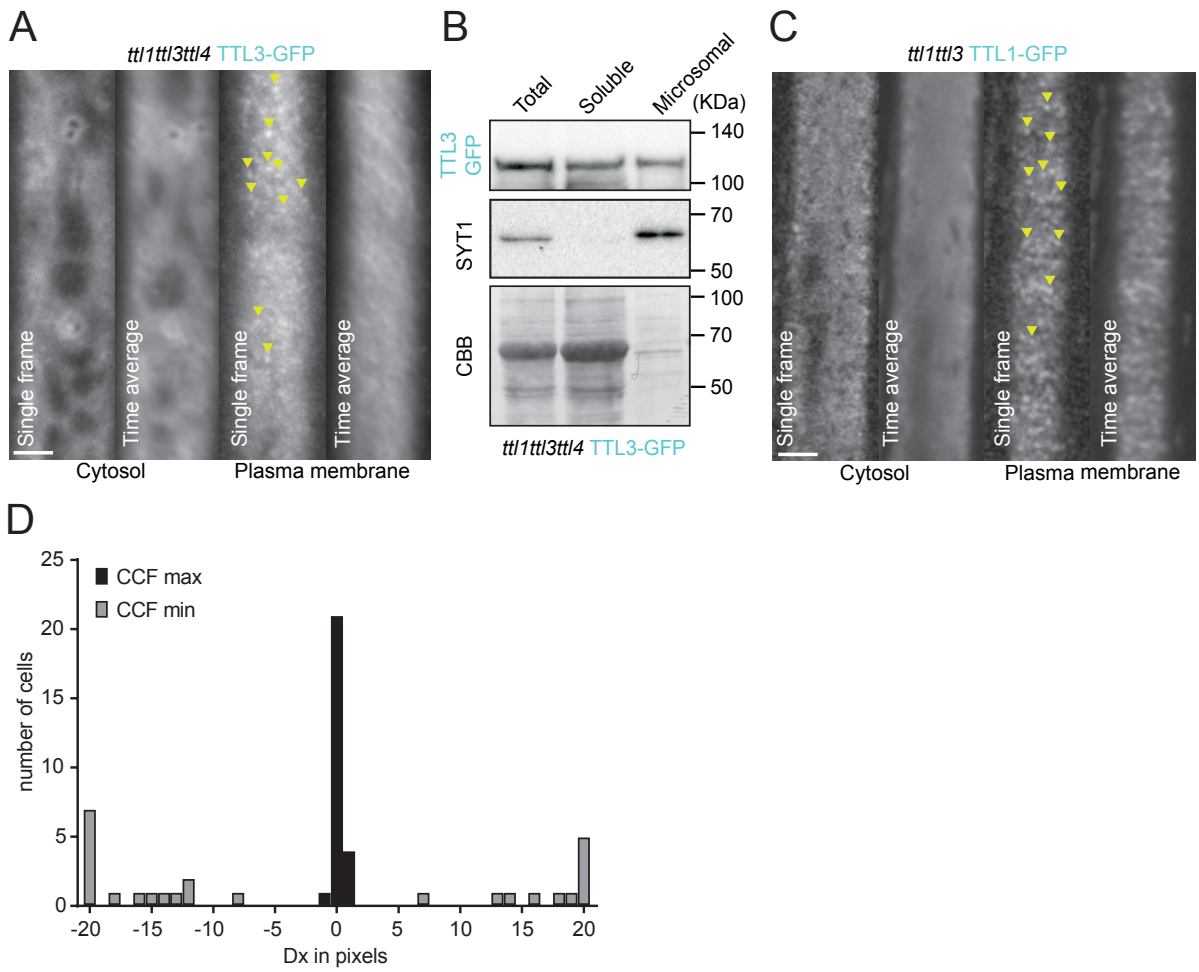

Fig. S5. TTL1 and TTL3 are peripheral membrane proteins that co-migrate with the Cellulose Synthase Complex.

(**A**) TTL3-GFP in 3-day-old etiolated hypocotyl epidermal cells of *ttl1ttl3ttl4* TTL3-GFP seedlings. TTL3-GFP signal is visible as cytosolic signal and as distinctive motile particles (yellow arrows) at the plasma membrane as revealed by single frames and time-average projections. Scale bar=2.5 µm. (**B**) Fractionation analysis (total, soluble, and microsomal fractions) of TTL3-GFP in *ttl1ttl3ttl4* as in (A). TTL3-GFP and the microsomal control SYT1 were detected with an anti-GFP and anti-SYT1 antibodies, respectively. Coomassie brilliant blue (CBB) staining provides a loading control. The experiment was repeated 3 times with similar results. (**C**) TTL1-GFP in 3-day-old root epidermal cells of *ttl1ttl3* TTL1-GFP seedlings. TTL1-GFP signal is visible as cytosolic signal and as distinctive motile particles (yellow arrows) at the plasma membrane as revealed by single frames and time-average projections. Scale bar=2.5 µm. **(D)** Quantification of TTL3-GFP and tdT-C6 co-localization in hypocotyl cells of three-day-old etiolated seedlings. Cross-correlation coefficients (CCFs) were calculated in the original non-shifted images (dx = 0) and after being shifted with dx ± 20 pixels using the van Steensel's algorithm. Note the highest CCFs (CCF max, black bar) were detected at dx = 0 for n = 21 cells. Gray bars represent the shifts that lead to the lowest CCF (CCF min), where the overlap of signals was the lowest.

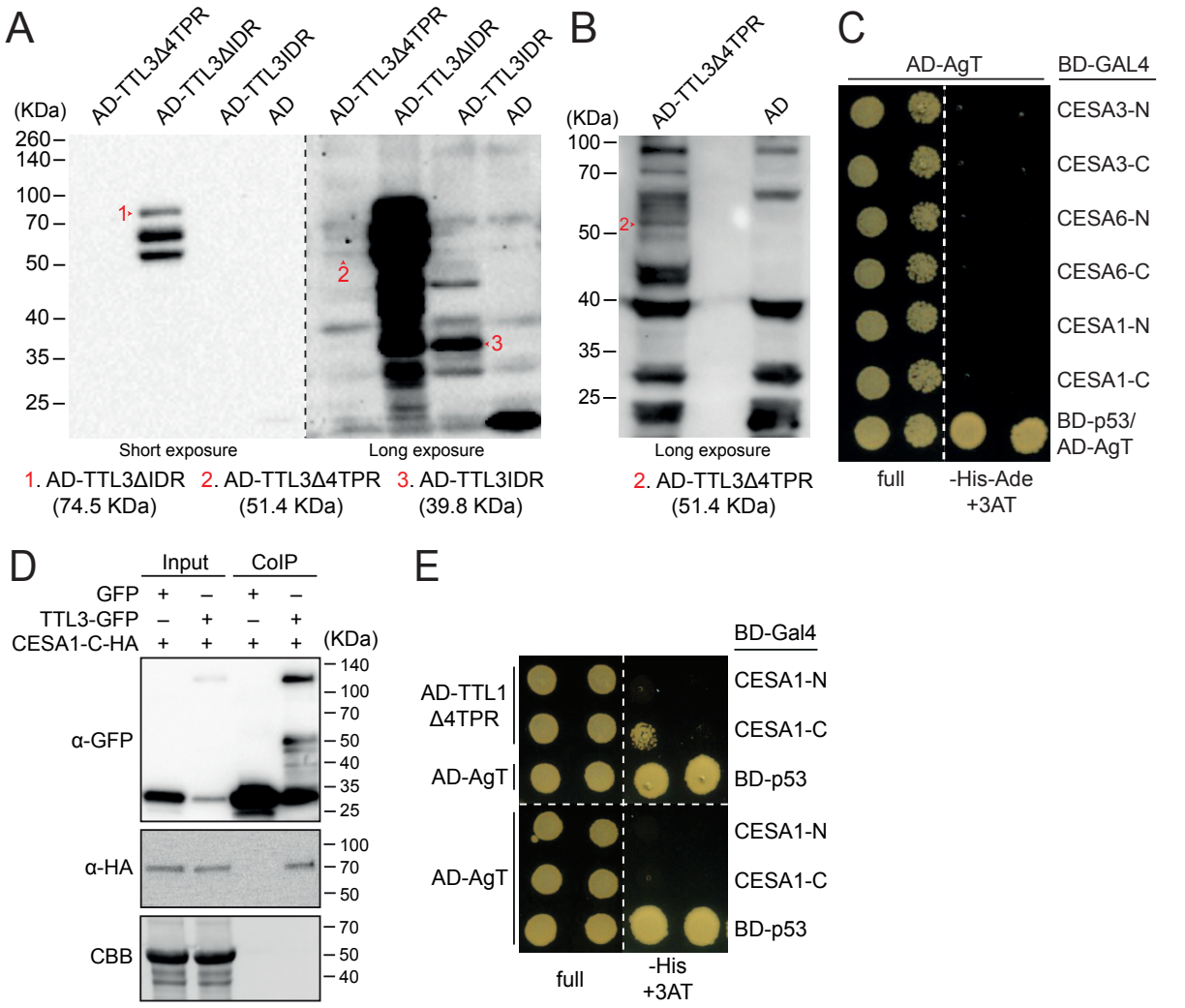

Fig. S6. Yeast-2-hybrid constructs used in the study are expressed and do not auto-activate. TTL1 interacts with CESAs in a similar way as TTL3.

(A) Immunoblot analysis showing the expression of AD-TTL3ΔIDR, AD-TTL3Δ4TPR, and AD-TTL3IDR in protein extracts of yeast transformants used for the yeast-two-hybrid assays. For each of them, yeast transformants were resolved in polyacrylamide/SDS-Page gels and analyzed by immunoblot using an anti-HA Tag monoclonal antibody. The expected molecular size of (1) AD-TTL3ΔIDR, (2) AD-TTL3Δ4TPR, and (3) AD-TTL3IDR is represented in the figure. (**B**) Immunoblot analysis showing the expression of AD-TTL3Δ4TPR and AD in protein extracts of yeast transformants used for the yeast-two-hybrid assays. Representative Western-blot of independent AD-TTL3Δ4TPR and AD yeast transformants of those shown in (A) Western blot was performed as described in Fig.S6A. (**C**) Negative control of yeast-two-hybrid assays of the cytosolic CESA-N and CESA-C BD domains with BD-GAL4. BD-p53/AD-AgT was used as a positive control of the interaction. Growth on non-selective media (full) and interaction-selective media (lacking histidine and adenine [–His–Ade] and supplemented with 3-amino-1,2,4-triazole [+3AT]) are shown. Photographs were taken after 3 days. Three independent experiments yielded similar results. **(D)** TTL3-GFP was transiently co-expressed with CESA1-C-HA or free GFP as a negative control in *N. benthamiana*. Total (Input) and co-immunoprecipitated (CoIP) proteins were analyzed by immunoblotting using anti-GFP and anti-HA antibodies. Equal loading was confirmed

by Coomassie blue staining (CBB) of input samples. Two independent experiments yielded similar results. **(E)** Yeast-two-hybrid assays showing the interaction of TTL1Δ4TPR (amino acids 1 to 307) with CESA1-C. Growth on non-selective media (full) and interaction-selective media (lacking histidine [–His] and supplemented with 3-amino-1,2,4-triazole [+3AT]) are shown. Photographs were taken after 3 days. A positive control (BD-p53/AD-AgT) for the yeast two-hybrid assays is shown. -N=N-terminal region of CESAs; -C=catalytic cytosolic loop of CESAs. The experiment was repeated 3 times with the same results.

Fig. S7. *ttl1ttl3* exacerbates the *prc1-1* developmental defects.
Morphological phenotypes of 5-week-old plants of WT (Col-0), *ttl1ttl3*, *prc1-1*, and *ttl1ttl3prc1-1* grown in long-day conditions. Scale bar=1 cm.

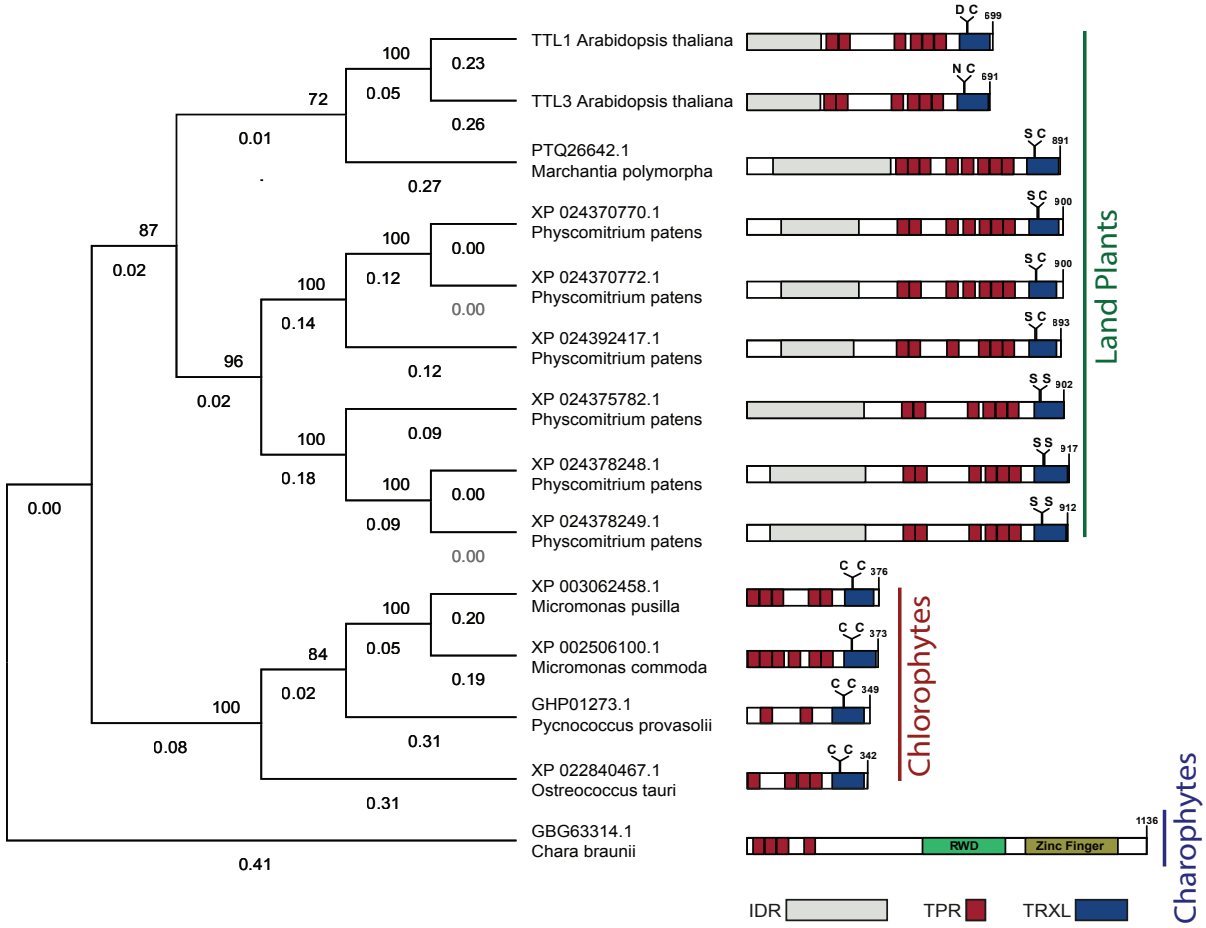

Fig. S8. *TTL* genes have emerged in land plants.
Identification of Arabidopsis TTL1 and TTL3 orthologs in Embryophytes (earliest land plants), Chlorophyta, and Charophytes. The earliest TTL orthologs can only be identified in Embryophytes. IDR = intrinsically disordered region; TPR = tetratricopeptide repeat; TRX = C-terminal sequence with homology to thioredoxins and conserving the essential Cys residues required for thioredoxin activity; TRXL = TRX domain lacking essential Cys residues conserved for thioredoxin activity. Bootstrap test (0-100): 500 replicates; evolutionary distances: *p*-distance (0-1).

Table S1. Primers used in this study

Description	Forward	Reverse
TTL1 dPCR	TGGACTCACCACCACCACTA (LP)	ACCGAGTCTGCGAACAAGAT (RP)
TTL3 dPCR	AGAGAGCTGCGATGCTTGAT (LP)	ATGCTCTCCTCCACATCCAC (RP)
TTL4 dPCR	AATGAACCATTAAATTGGGGC (RP)	ATGTGAAGAATGTGGCGAAAG (LP)
CESA1-C in pDONR/Zeo	AAAAAGCAGGCTCCATGGATCAGTTTCCCAA ATGGTAC	AGAAAGCTGGGTCGGTGTTGATATAAGCG ATCCTCTC
pTTL1::TTL1g in pENTR	CACCACAAATTTCGTTTCGTACACGGT	ACCGCTATAGTGTCTCACCG
TTL3IDR in pDONR/Zeo	TAGAAGGGTGGGCGCGCCGAC	TCCTCCGCCGCTGCGTAC
TTL3IDR ColonyPCR	CTGGTAAACCGTCGGTGAGT	GGGATATCAGCTGGATGGCAAA
SALK dPCR (LBb1.3)	ATTTTGCCGATTTCGGAAC	
SAIL dPCR (LB3)	TAGCATCTGAATTTCATAACCAATCTCGATACAC	
Cesa1-N-Y2H	GACCTGCATATGATGGAGGCCAGTGCC	ATTCGGCCTCCATGGCCTTAGCGAGAAGATG GGATAGG
Cesa1-C-Y2H	GACCTGCATATGATGGATCAGTTTCCCAAAT GGTACC	TCGGCCTCCATGGCCTTAGGTGTTGATATAA GCGATCCTC
Cesa3-N-Y2H	GACCTGCATATGATGGAATCCGAAGGAGAA ACC	ATCGGCCTCCATGGCCTTACATTCTGTAAGG ATTGATCCG
Cesa3-C-Y2H	GACCTGCATATGATGGATCAGTTTCCCAAGT GGTTTC	ATTCGGCCTCCATGGCCTTAGGTGGTGTTCAC ATACGC
Cesa6-N-Y2H	GACCTGCATATGATGAACACCGGTGGTCG	TCGGCCTCCATGGCCTTACATCCGGTAAGGA TTTATCTTG
Cesa6-C-Y2H	GACCTGCATATGATGGATCAGTTCCCTAAAT GGTACC	ATTCGGCCTCCATGGCCTTAAGAGGTCCACG GGTAAAC
TTL3IDR in pPROEX HTb	AGGGCGCCATGGGATCCGGAATGTCGCATTC CCGTCGC	CCTCGAGACTGCAGGCTCTAGATCACGCTTT CCCAGAGGTACCG
TTL3 in petM11SUMO3GFP	GTTCCAGCAACAGACCGGTGGATCCATGTCT CATTCTAGAAGACTTTCGTTGGA	CTCGCCCTTGCTCACGGATCCTCATAAGAGG AAATGCTTTATAGAGTCCTCTAG

Caption for Movie S1

TTL3-GFP particles co-localize with tdT-CesA6 at the plasma membrane (see merged image series). TTL3-GFP image series are shown non-modified (non-mod) and after extracting particles at the plasma membrane with an image modification pipeline (mod).