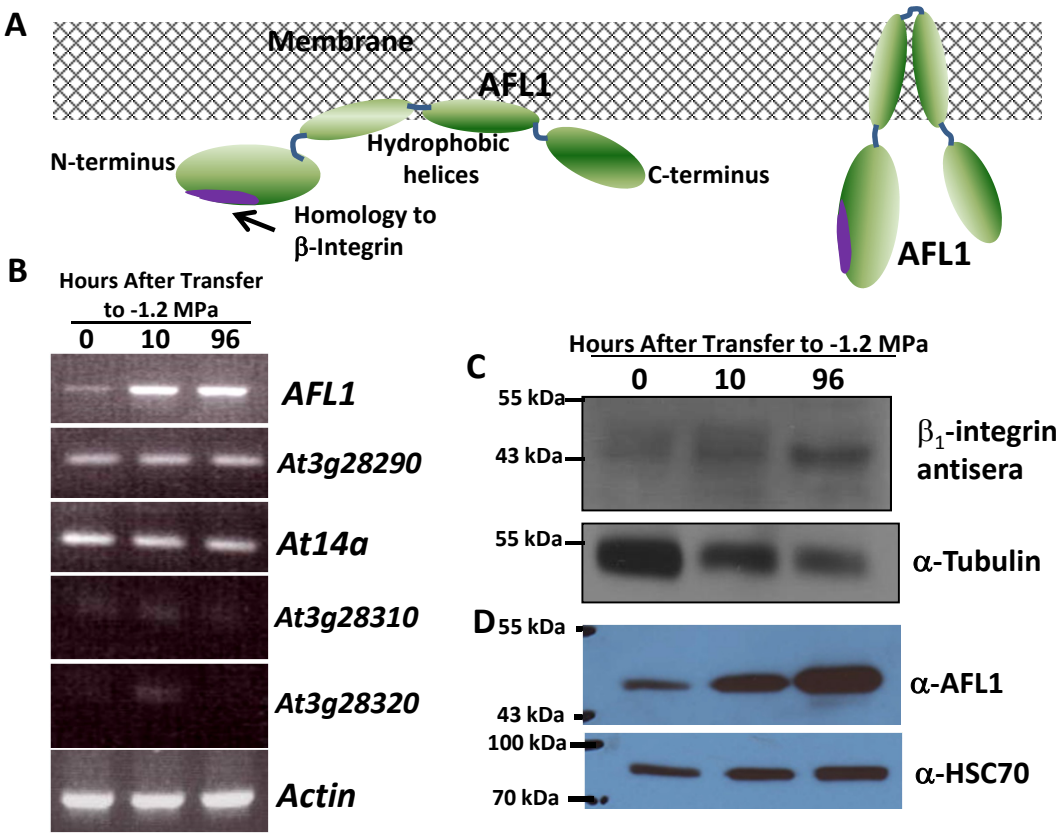

Figure S1: AFL1 structure and expression.

A. Possible structures of AFL1. AFL1 is annotated in TAIR as a membrane protein and contains two predicted hydrophobic helices (based on RHYTHM database) from amino acids 209-231 and amino acids 235-257. Whether or not it was a peripheral membrane protein or transmembrane protein was unclear at the start of this research, thus both possibilities are shown. There are low complexity domains (142-159 and 275-289) that link the N-terminal and C-terminal domains to the helices. The N-terminal domain contains a small region (amino acids 134-144) similar to mammalian β_1-integrin (this is presumably the sequence recognized by β_1-integrin antisera). Despite the presence of this integrin-similarity domain, the overall sequence and domain structure of AFL1 clearly differs from known integrins. Both prediction and our protein interaction data suggest that the N-terminal and C-terminal domains are intracellular. The models shown are not meant to exclude other possibilities for AFL1 structure.

B. RT-PCR shows that among the At14a-like gene cluster in Arabidopsis, only AFL1 was induced by low water potential stress (-1.2 MPa).

C and D. Increased protein abundance of AFL1 at low water potential was also observed using either a commercial β_1-integrin antisera (C) or AFL1-specific antisera (D) generated in our laboratory. Blots were stripped and reprobed with anti-tubulin (C) or HSC70 (D) as loading controls.

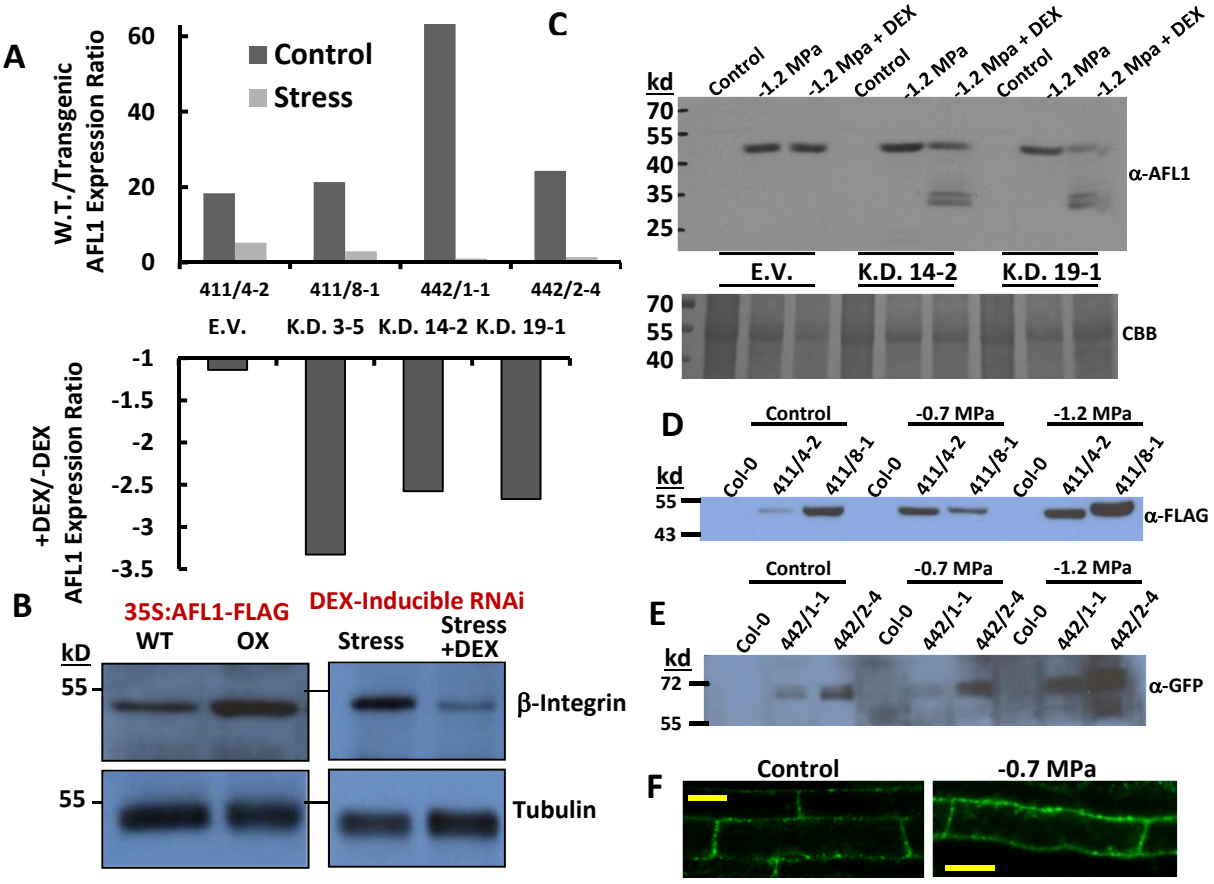

Figure S2: Transgenic plants with increased or decreased AFL1 expression.

A. AFL1 gene expression in transgenic lines expressing either 35S:EYFP-AFL1 or 35S:AFL1-FLAG as well as transgenic lines with Dexamethasone (DEX)-inducible RNAi of AFL1. These are the same transgenic lines used for phenotypic analysis shown in the main figures. O.E. = overexpression, W.T. = Col-0 wild type, 411 = pGWB411 (C-terminal FLAG tag), 442 = pGWB442 (N-terminal EYFP), K.D. = RNAi knock down, E.V. = Empty Vector control for the RNAi knockdown lines. All expression data were normalized using *EFL1α* expression and then the ratio of expression in overexpression transgenics versus wild type or +DEX/-DEX ratio for K.D. lines calculated. Expression in K.D. lines was measured in the stress treatment (-1.2 MPa, 96 h) where AFL1 expression is highly induced.

B. Immunoblots using commercial β_1-integrin antisera show increased AFL1 protein abundance in a representative with 35S:AFL1-FLAG and decreased abundance in a representative AFL1 K.D. RNAi line after DEX application to stress treated seedlings (-1.2 MPa, 96 h).

C. Immunoblots with antisera specifically recognizing AFL1 show decreased AFL1 protein level after DEX application to stress treated (-1.2 MPa, 96 h) AFL1 K.D. seedlings. A limited amount of truncated AFL1 protein is seen, likely because the RNAi targets the 3' UTR of *AFL1* as the most unique part of the gene. A replica commasie stained gel is shown as a loading control. E.V. = empty vector. CBB = Commasie Brillant Blue stained.

D. Transgenic lines with *35S:AFL1-FLAG* show expression of a protein recognized by anti-FLAG antibody at molecular weight consistent with AFL1-FLAG fusion protein.

E. Transgenic lines with *35S:YFP-AFL1* show expression of a protein recognized by anti-GFP antibody at a molecular weight consistent with the YFP-AFL1 fusion protein.

F. Localization of YFP-AFL1 in root cells of a representative YFP-AFL1 transgenic line. Scale bars represent 20 μm.

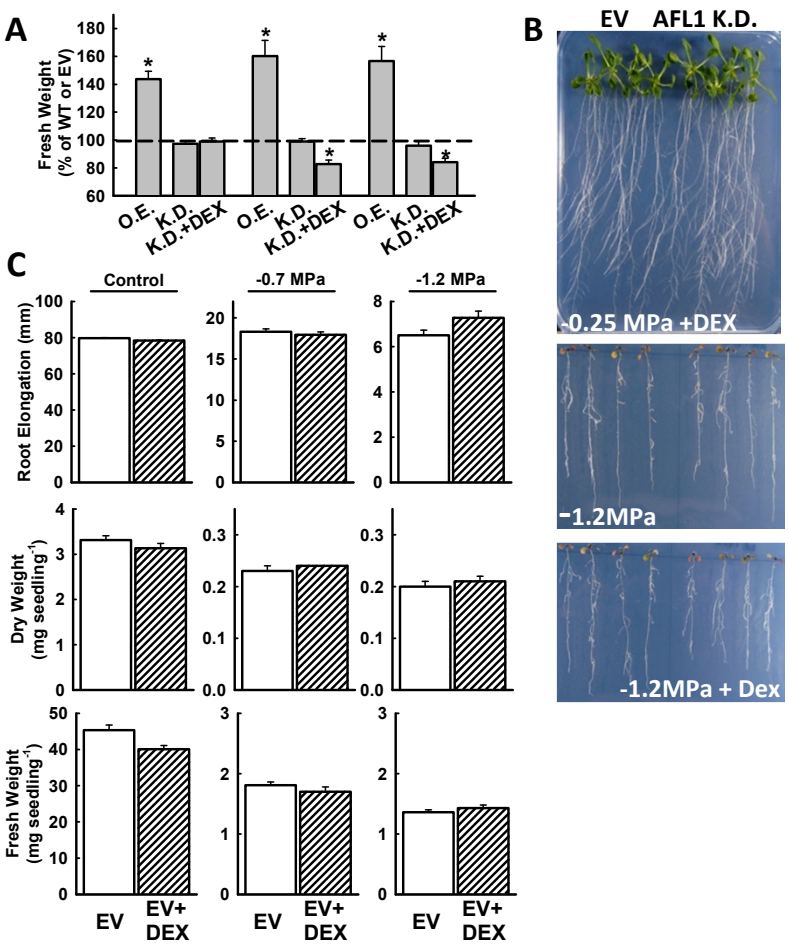

Figure S3: Additional seedling growth data from transgenic lines with increased or decreased AFL1 expression.

A. Seedling fresh weight data from the same experiments as the root elongation and dry weight data shown in Figure 1A.

B. Representative seedlings of empty vector and an AFL1 K.D. RNAi line. DEX application did not affect growth of the empty vector under either control or stress. In contrast, DEX application to the AFL1 RNAi line reduced growth under low water potential stress but had no effect on the control.

C. Root Elongation, Dry Weight and Fresh Weight of the empty vector control lines for the DEX-inducible RNAi. Application of DEX to the empty vector lines had no effect on any of the growth parameters. All the growth parameters for these empty vector control lines were essentially identical to the Col-0 wild type data used to normalize the growth data shown in Figure 1A.

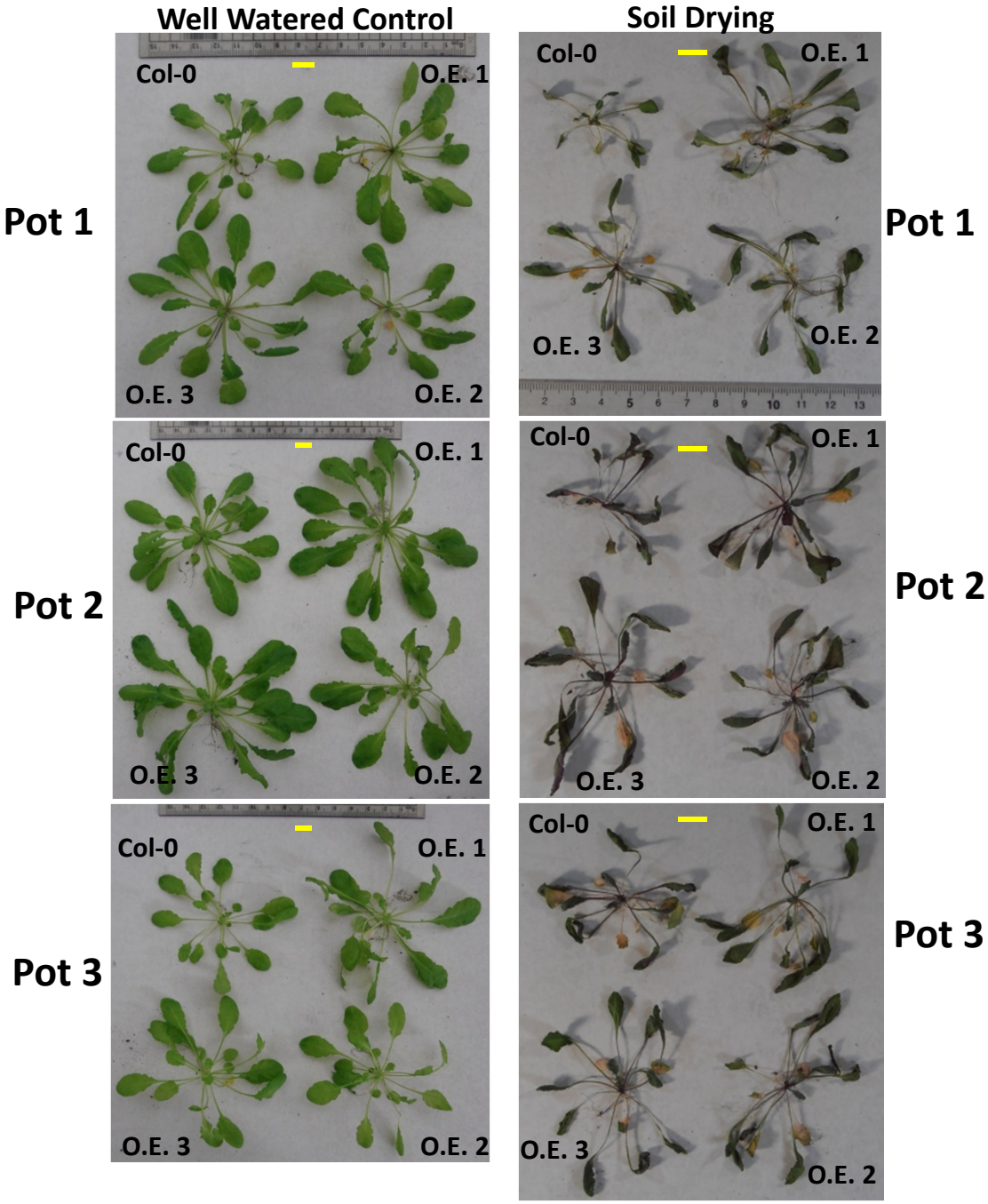

Figure S4: Representative wild type and AFL1 overexpression (O.E.) plants from soil drying experiments reported in Figure 1C.

For soil drying experiments, Col-0 wild type and three *AFL1* overexpression (O.E.) lines were planted in sectors of the same pot. Two plants of each genotype were grown per sector (one representative plant is shown in the photos for clarity). A controlled soil drying was imposed over 18 days with a partial rewatering in the middle of the stress period to equalize water content between pots and maintain a longer period of moderate soil drying. Relative water content did not differ between the genotypes either before or after water with holding, further indicating that all genotypes were exposed to the same severity of stress. At the end of the experiment, three pots were selected arbitrarily for photography while other pots were used for rosette fresh weight and dry weight analysis. Yellow scale bars indicate 1 cm. Note that to allow the soil drying plants to be seen more clearly, they are shown at larger scale than well watered plants.

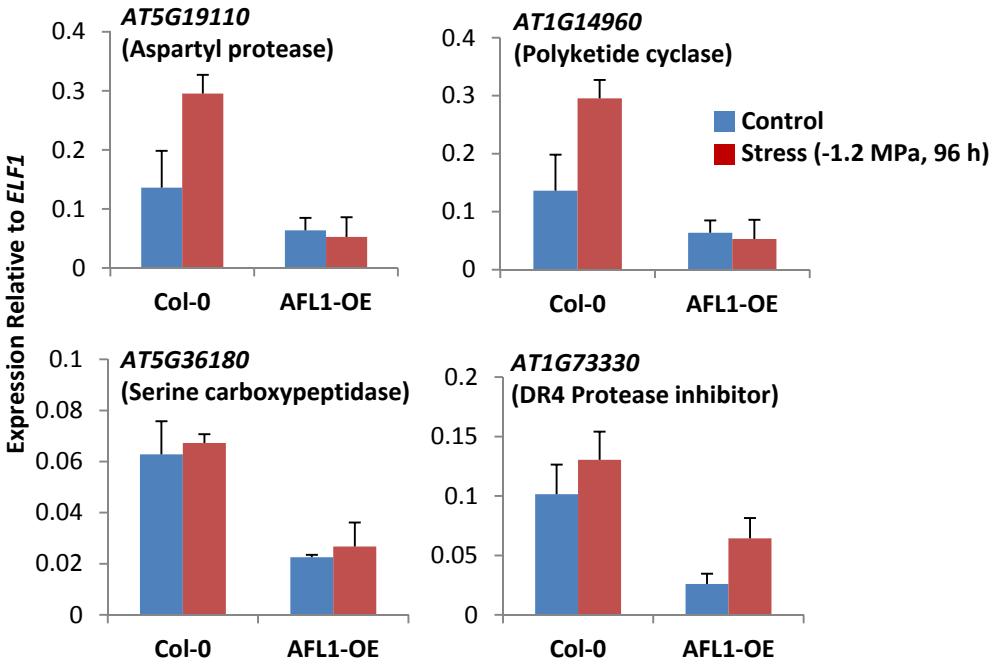

Figure S5: Quantitative RT-PCR validation of AFL1 regulated gene expression. Several genes whose expression was found by microarray analysis to be repressed by AFL1 O.E. (Table S4 and S6) were selected for QPCR validation. In agreement with the microarray data, AFL1 O.E. reduced the expression of these genes in both control and low water potential stress treatments. Data are means ± S.E. (n = 6) combined from two independent experiments.

	Yeast two hybrid library screen (AFL1 N-terminus)	IP/MS YFP-AFL1	mbSUS Interaction assay (Full Length AFL1)	BiFC	Co-IP
Adaptor protein (AP2-2A)	N.D.	✓	✓	✗	✗
Protein Disulfide Isomerase (PDI5)	✓	✓	✓ Weak	✓ Stress More	✓ Stress More
NAI2	✓	✓	✓	✓ Stress More	Non-specific binding
TSA1	✓	✗	✓ Weak	✗	N.D.
AFL1	✓ C-terminal clones of AFL1 and At14a	✓	✓	✓ Stress More	N.D.
HAP6 DRP1A	N.D.	✓	✗	✗(HAP6)	N.D.
Adaptor med subunit, Clathrin, GDI2, ER proteins, proteases, Cytoskeleton proteins (See Table S7)		✓	N.D.	N.D.	N.D.

Figure S6: Summary of AFL1 protein interaction experiments

Putative AFL1-interacting proteins were first identified using the N-terminal domain of AFL1 (amino acids 1-208) to screen a yeast two hybrid library prepared in our laboratory using cDNA from low water potential treated seedlings (-1.2 MPa, 96 h). Clones of PDI5, NAI2 and TSA1 as well as clones containing the C-terminal portion of AFL1 or At14a were repeatedly identified in this screen. Concurrently, YFP-AFL1 was immunoprecipitated from transgenic plants and proteins in the immunoprecipitate identified by LC-MS/MS. PDI5 and NAI2 were also found via this method along with other ER proteins, vesicle transport proteins and cytoskeletal proteins (see Table S7 for complete list). Since both AFL1 and many of the putative interactors were membrane associated proteins, selected interactions were further tested using the mating-based split ubiquitin (mbSUS) yeast protein interaction assay which is designed to detect membrane protein interactions. In this case, full length AFL1 was used as bait and full length clones were used for all putative interactors. We also used transient expression in intact seedlings to detect interactions by either bi-molecular fluorescence complementation (BiFC) or co-immunoprecipation (Co-IP). The BiFC and Co-IP assays allowed us to test interactions in vivo and, because intact seedlings were used, to test the interactions under the same stress treatments used for our phenotypic analyses (generally -1.2 MPa for 96 h). N.D. = Not determined

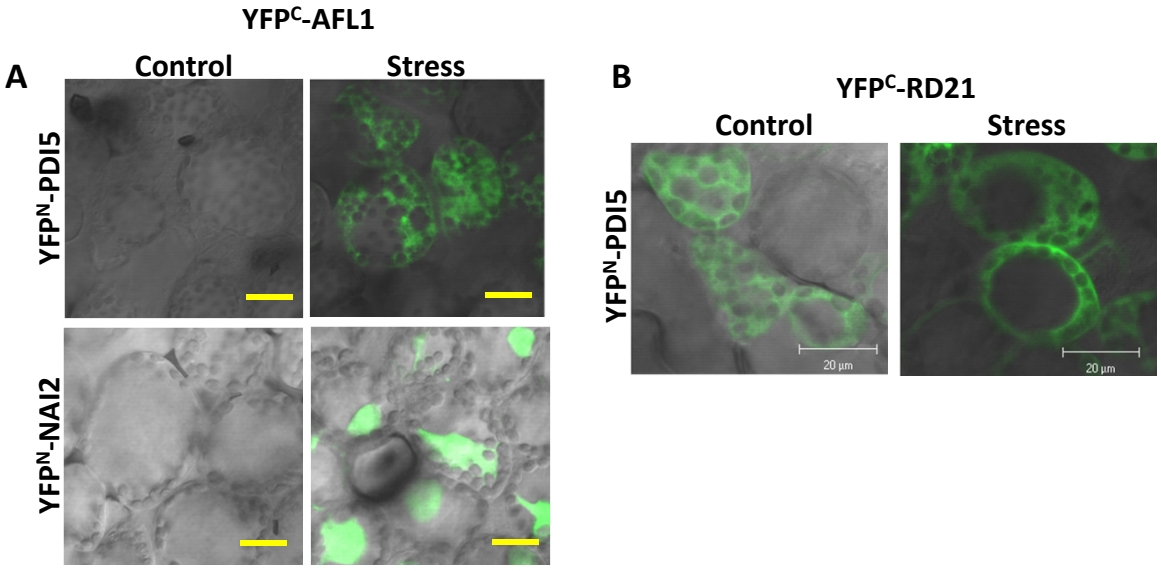

Figure S7: AFL1 interaction with PDI5 and NAI2; interaction of PDI5 and RD21
A. BiFC interaction of PDI5 and NAI2 with AFL1 using the pSite vector system. The results were consistent with the rBiFC analysis in that no interaction could be observed in unstressed seedlings while substantial BiFC signal was observed in stressed plants.
B. BiFC of PDI5 and RD21 (using pSite-NEYFP-C1 and pSite-CEYFP-C1 vectors). In contrast to the AFL1-PDI5 interaction, interaction of PDI5 and RD21 could be readily observed in both control and low water potential stress treatments.

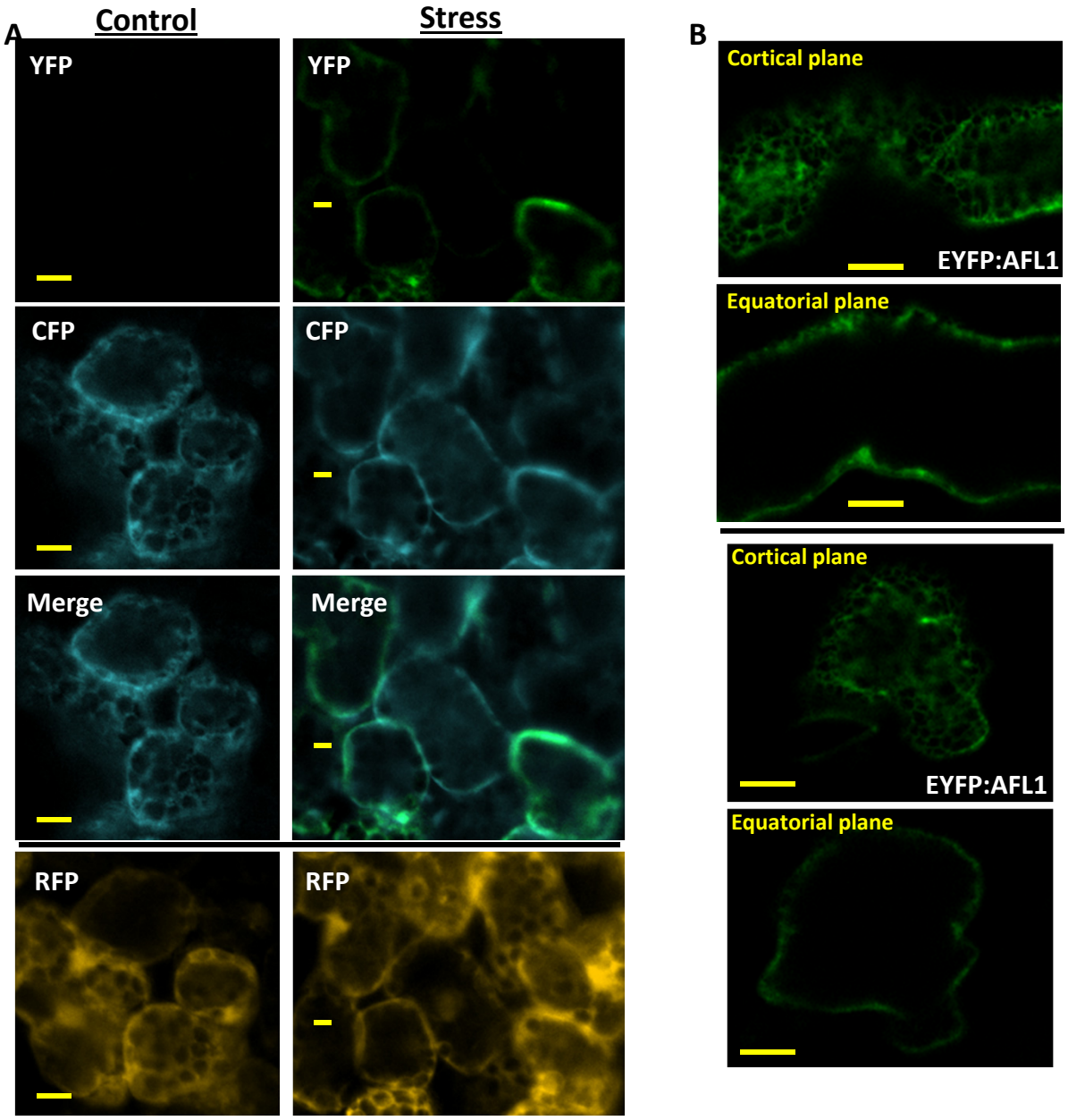

Figure S8: Comparison of ER marker and AFL1-PDI5 rBiFC localization and imaging of leaf epidermal cells indicate partial ER localization of AFL1.

A. rBiFC of AFL1 with PDI5 and NAI2 performed with concurrent expression of CFP labeled ER marker containing the signal peptide of AtWAK2 at its N-terminus and ER retention signal His-Asp-Glu-Leu at the C-terminus (plasmid CD3-953; Nelson et al., Plant J 51: 1126, 2007). Green (top panels) represents the BiFC signal, blue the CFP ER marker, merge is the combined BiFC and ER marker signals. Yellow in the bottom panels is the constitutively expressed RFP reporter of the rBiFC system. The substantial, but not complete, overlap between the rBiFC signal and the ER-marker indicates that the PDI5-AFL1 interaction occurred partially in the ER but also potentially in other endomembrane compartments. The somewhat different localization of the NAI2 interaction (Figure 2 of main text) likely represents clustering of NAI2 in ER bodies (Yamada et al., Plant Cell 20: 2559, 2008). For stress treatment, seedlings were transferred to -1.2 MPa of either 24 or 96 h.

B. Imaging of YFP-AFL1 in leaf epidermal cells (unstressed plants). Images from the cortical plane show the tubule network typical of ER while the equatorial plane shows clustering of the signal along the cell periphery also typical of ER localization

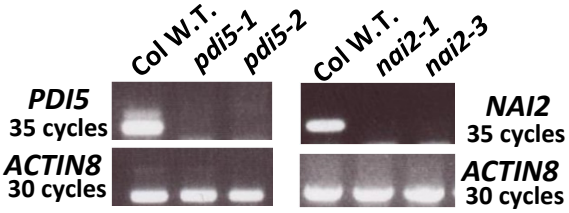

Figure S9: RT-PCR analysis of *pdi5* and *nai2* mutants.
RT-PCR of *pdi5-1* (SALK_015253), *pdi5-2* (SALK_136642), *nai2-1* (SALK_005896) and *nai2-3* (SALK_043149) found lack of PDI5 or NAI2 expression. *pdi5-1* was previously described as a knockout mutant (Ondzighi et al., 2008 Plant Cell 20: 2205-2220) and both *nai2-1* and *nai2-3* were also previously reported as knockout mutants (Yamada et al., 2008 Plant Cell 20:2529-2540).

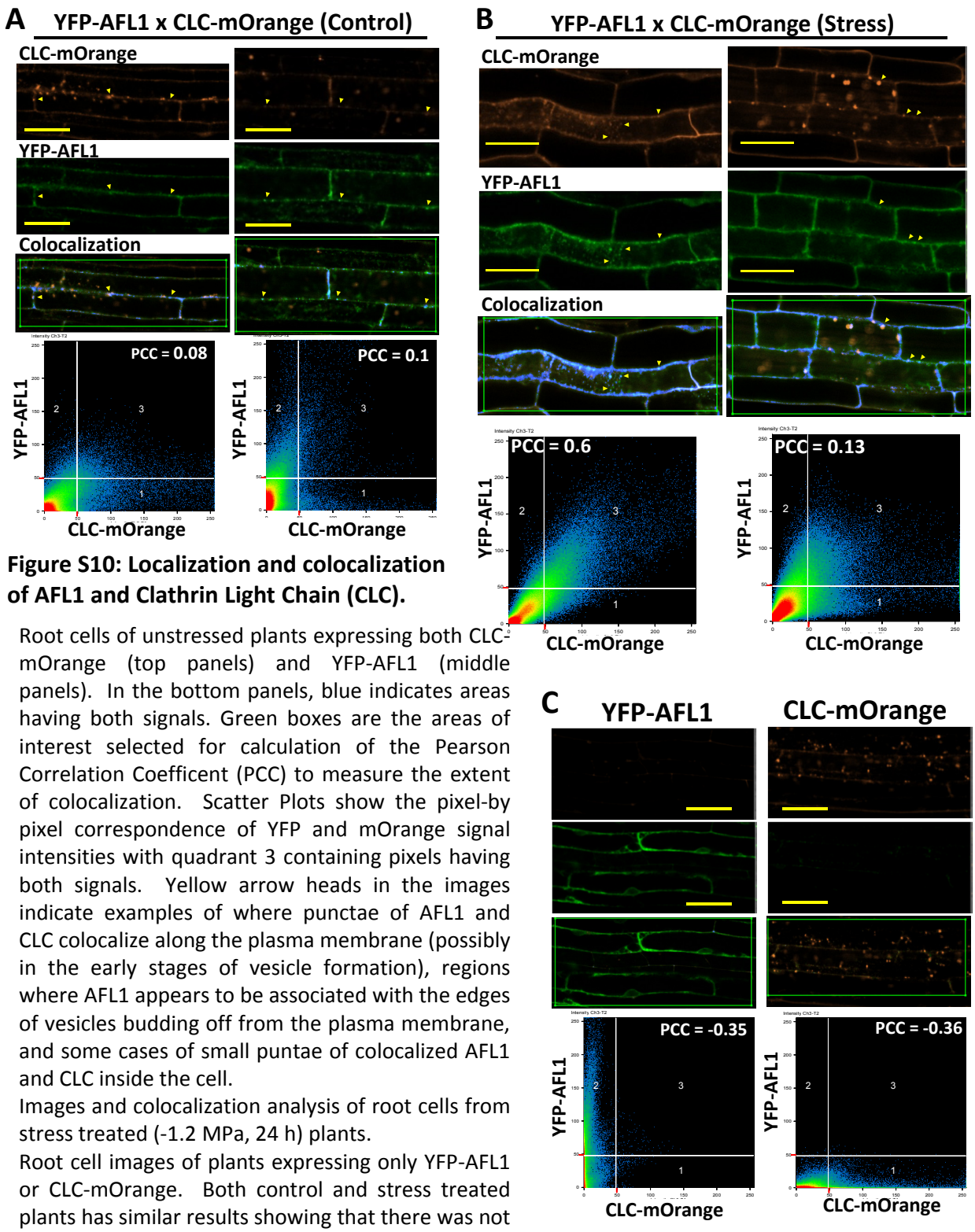

Figure S10: Localization and colocalization of AFL1 and Clathrin Light Chain (CLC).

A. Root cells of unstressed plants expressing both CLC-mOrange (top panels) and YFP-AFL1 (middle panels). In the bottom panels, blue indicates areas having both signals. Green boxes are the areas of interest selected for calculation of the Pearson Correlation Coefficent (PCC) to measure the extent of colocalization. Scatter Plots show the pixel-by pixel correspondence of YFP and mOrange signal intensities with quadrant 3 containing pixels having both signals. Yellow arrow heads in the images indicate examples of where punctae of AFL1 and CLC colocalize along the plasma membrane (possibly in the early stages of vesicle formation), regions where AFL1 appears to be associated with the edges of vesicles budding off from the plasma membrane, and some cases of small puntae of colocalized AFL1 and CLC inside the cell.

B. Images and colocalization analysis of root cells from stress treated (-1.2 MPa, 24 h) plants.

C. Root cell images of plants expressing only YFP-AFL1 or CLC-mOrange. Both control and stress treated plants has similar results showing that there was not substantial bleed through between the YFP and mOrange detection.

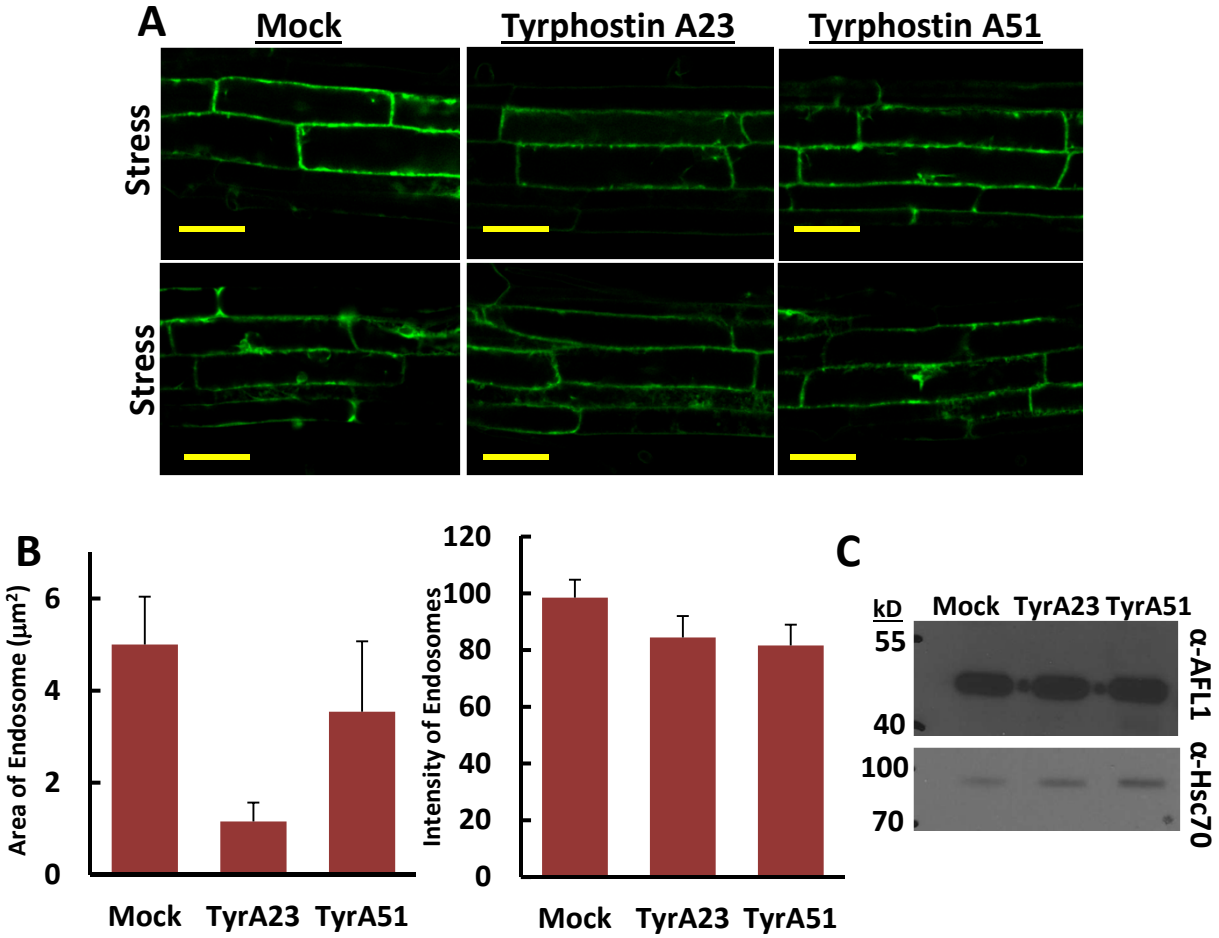

Figure S11: TyrphostinA23 effect on AFL1 localization.

A. Representative images of root cells treated with 15 μM of the endocytosis inhibitor Tyrphostin A23, its negative analog Tyrphostin A51 or a mock treatment. Seven-day-old seedlings were transferred to -0.7 MPa PEG-infused plates containing the indicated treatments and roots imaged 24 h later. AFL1 intensity at the plasma membrane was not substantially affected by any of the treatments. Similar results were observed in unstressed seedlings. Scale bars represent 50 μm.

B. Quantification of area of endosome-like particles decorated with AFL1 and the fluorescence intensity of these particles. TyrA23 treatment decreased the area of AFL1-decorated particles but not their intensity.

C. Western blot showing that TyrA23 treatment had no discernible effect on total amount of AFL1 protein.

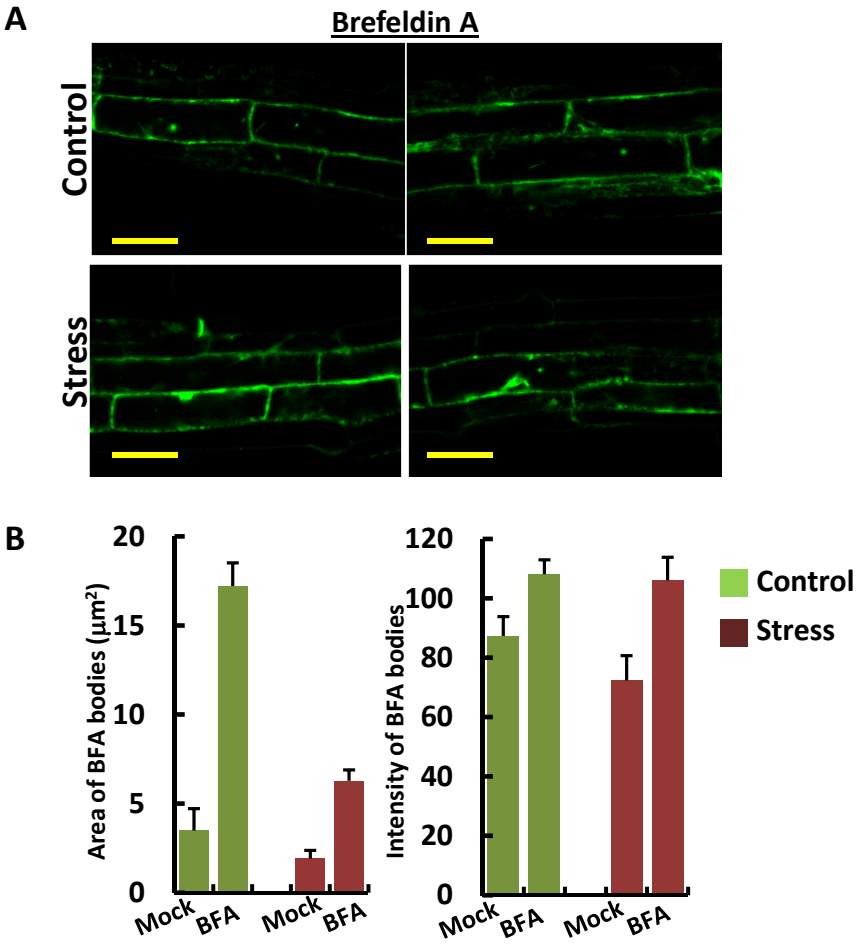

Figure S12: Accumulation of AFL1 in BFA bodies is not increased by stress.

A. Seven-day-old seedlings were transferred to either stress or control media for 24 h, then sprayed with 100 μM Brefeldin A and imaged 1 h later. Images of several representative root cells are shown. Scale bars represent 50 μm.

B. Quantification of BFA body area and intensity for representative root cells. Data are ± S.E. (n = 6-20).

BFA bodies decorated with YFP-AFL1 were seen; however, AFL1 was not substantially depleted from the plasma membrane and stress did not increase the prevalence of BFA bodies containing AFL1.

	Template PDB Code	PDB Segment	PDB Comment	CATH coverage or Link	Dataset	Seq Ident	E-value	Model Score	Quality	Modeled Segment
☐	4k1pA	(18-360)		CATH	MW-AFL1	16.00	0	0.49		12-357
☐	1uruA	(40-232)	amphiphysin bar domain from drosophila	CATH	MW-AFL1	11.00	0	0.16		13-208
☐	2i1kA	(338-457)	moesin from spodoptera frugiperda reveals the coiled-coil domain at 3.0 angstrom resolution	CATH	MW-AFL1	18.00	0.39	0.67		261-369

Figure S13: Analysis of AFL1 protein sequence by ModWeb (https://modbase.compbio.ucsf.edu/modweb/). The three top templates used by ModWeb to model the structure of AFL1 are shown. The first entry, 4k1pA, is NheA component of the membrane pore forming Nhe toxin from *Bacillus cereus.* This protein was also found as a structure as a potential structural ortholog of AFL1 by I-Tasser (Fig S14). Amphiphysin is a a protein that associates with the adaptor proteins and dynamin and recruits clathrin to sites where endocytotic vesicles form. It may also have other roles in membrane bending or stabalizing membrane curvature. Moeisins link the cortical actin cytoskeleton to the plasma membrane.

Top 10 Templates used by I-Tasser

Rank	PDB Hit	Iden1	Iden2	Cov.	Norm. Z-score
1	1hciA	0.10	0.17	0.95	1.78
2	4k1pA	0.07	0.20	0.85	1.15
3	3cdvA	0.11	0.16	0.80	1.91
4	4k1pA	0.08	0.19	0.87	1.78
5	4hpqA	0.09	0.21	0.94	1.73
6	3cdvA	0.10	0.16	0.84	1.09
7	1u4qA	0.10	0.18	0.79	1.65
8	4k6jA	0.09	0.18	1.00	1.67
9	1st6A	0.13	0.21	0.98	1.72
10	2wzkA	0.11	0.22	0.94	1.08

1. ROD DOMAIN OF **ALPHA-ACTININ** (spectrin family)
2. NheA component of the Nhe toxin from *Bacillus cereus*
3. Repeats 14-16 of **Beta2-Spectrin**
4. NheA component of the Nhe toxin from *Bacillus cereus*
5. Atg17-Atg31-Atg29 Complex (autophagosome biogenesis)
6. Repeats 14-16 of **Beta2-Spectrin** (ankyrin binding site)
7. Repeats 15, 16 and 17 of Chicken Brain Alpha **Spectrin**
8. Human cohesin inhibitor WapL
9. cytoskeletal protein (**viniculin**)
10. Cul5 N-terminal domain

Top 10 structural analogs PDB

Rank	PDB Hit	TM-score	RMSD[a]	IDEN[a]	Cov.
1	1hciA	0.711	4.51	0.092	0.896
2	1sjjB	0.648	5.22	0.089	0.890
3	3r6nA	0.458	5.18	0.063	0.634
4	1u4qA	0.449	5.25	0.066	0.623
5	1quuA	0.419	4.02	0.083	0.505
6	1e07A	0.402	6.52	0.048	0.647
7	3kbuB	0.398	5.38	0.073	0.564
8	3edvA	0.374	5.98	0.069	0.567
9	2ycuA	0.369	5.86	0.050	0.553
10	3k8pD	0.369	6.12	0.078	0.570

1. Alpha actinin
2. Alpha actinin
3. four spectrin repeat fragment of the human desmoplakin plakin domain
4. Repeats 15, 16 and 17 of Chicken Brain Alpha Spectrin
5. Structure of two central spectrin-like repreats from α-actinin
6. human carcinoembryonic antigen
7. ankyrin binding domain of human erythroid beta spectrin
8. Repeats 14-16 of Beta2-Spectrin
9. Human Non Muscle Myosin 2C in Pre-power stroke state
10. Structural basis for vesicle tethering by the Dsl1 complex

Template proteins with similar binding site

Rank	Cscore[LB]	PDB Hit	TM-score	RMSD[a]	IDEN[a]	Cov.	BS-score	Lig. Name	Download Complex	Predicted binding site residues
1	0.01	1d0zA	0.197	6.69	0.050	0.326	0.15	PNQ	Download	294,295,296,297,298,337
2	0.01	1lvkA	0.217	7.24	0.050	0.382	0.15	Mul.Part	Download	329,333,334,352,353
3	0.01	1h8bA	0.104	4.31	0.088	0.134	0.16	PEPTIDE	Download	69,71,72,75
4	0.01	1c9iB	0.120	7.39	0.041	0.214	0.22	PEPTIDE	Download	288,289,337,350

1. DICTYOSTELIUM MYOSIN S1DC (MOTOR DOMAIN FRAGMENT)
2. NUCLEOTIDE BOUND TO DICTYOSTELIUM DISCOIDEUM MYOSIN MOTOR DOMAIN
3. EF-HANDS 3,4 FROM ALPHA-ACTININ / Z-REPEAT 7 FROM TITIN
4. PEPTIDE-IN-GROOVE INTERACTIONS LINK TARGET PROTEINS TO THE B-PROPELLER OF CLATHRIN

Consensus Prediction of Gene Ontology terms

Molecular Function		Biological Process		Cellular Location	
GO term	GO-Score	GO term	GO-Score	GO term	GO-Score
GO:0008092	0.35	GO:0071844	0.35	GO:0005913	0.35
GO:0043167	0.35	GO:0007015	0.35	GO:0031674	0.35
				GO:0014704	0.35

GO:0008092 cytoskeletal protein binding
GO:0043167 ion binding
GO:0071844 cellular component assembly
GO:0007015 actin filament organization
GO:0005913 cell-cell adherens junction
GO:0031674 I band (A region of a sarcomere that appears as a light band on each side of the Z disc, comprising a region of the sarcomere where thin (actin) filaments are not overlapped by thick (myosin) filaments; contains actin, troponin, and tropomyosin; each sarcomere includes half of an I band at each end)
GO:0014704 intercalated disc (A complex cell-cell junction at which myofibrils terminate in cardiomyocytes; mediates mechanical and electrochemical integration between individual cardiomyocytes. The intercalated disc contains regions of tight mechanical attachment (fasciae adherentes and desmosomes) and electrical coupling (gap junctions) between adjacent cells.)

Figure S14: AFL1 structure modeling and prediction by I-Tasser http://zhanglab.ccmb.med.umich.edu/I-TASSER/ [Yang et al. (2015) Nature Methods 12: 7-8].

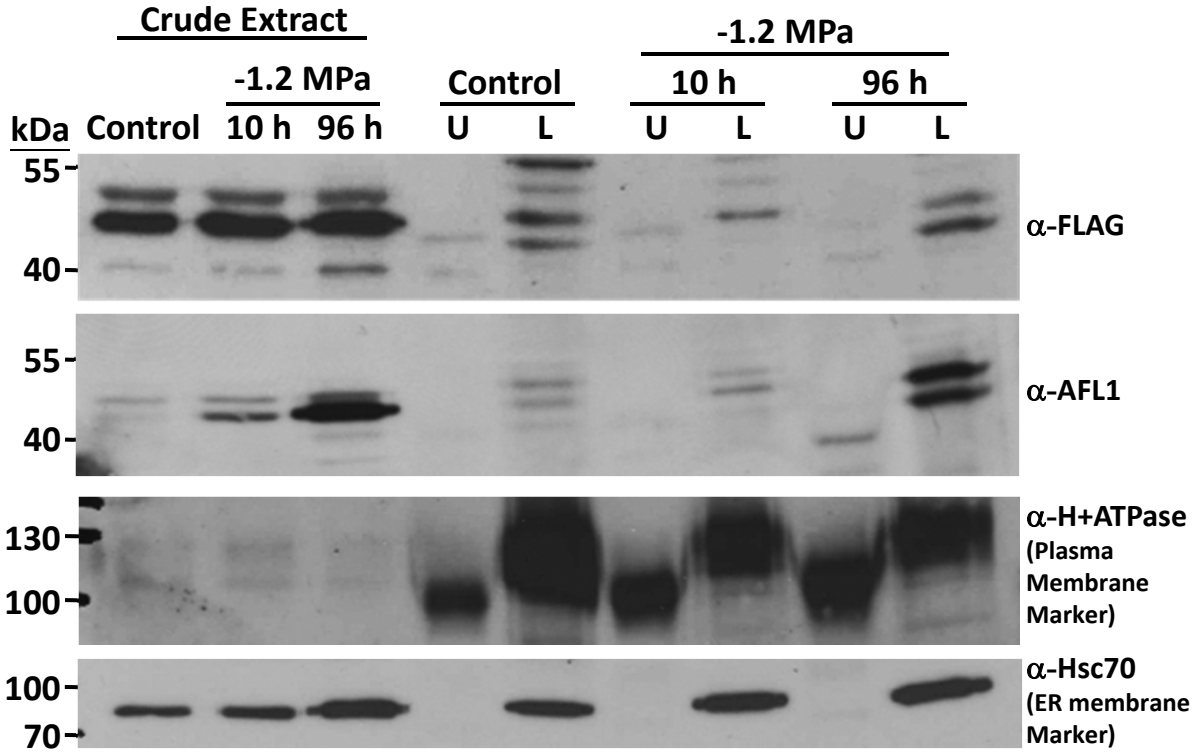

Figure S15: Membrane fractionation of 35S:AFL1-FLAG plants.
35S:AFL1-FLAG plants were grown for seven days, transferred to the indicated treatments and harvested for membrane fractionation. The data show an accumulation of AFL1 in the endomembrane fraction under stress as well a post-translational modification of AFL1. The results are similar to those obtained by membrane fractionation of wild type plants (Figure 3F of main text). AFL1-FLAG was detected in both upper and lower membrane fractions; although at higher abundance in the lower (endomembrane) fraction. Also in agreement with fractionation of wild type, two bands of AFL1 were seen in the lower fraction suggesting the possibility that AFL1 undergoes post-translational modification. The experiment was repeated with consistent results.

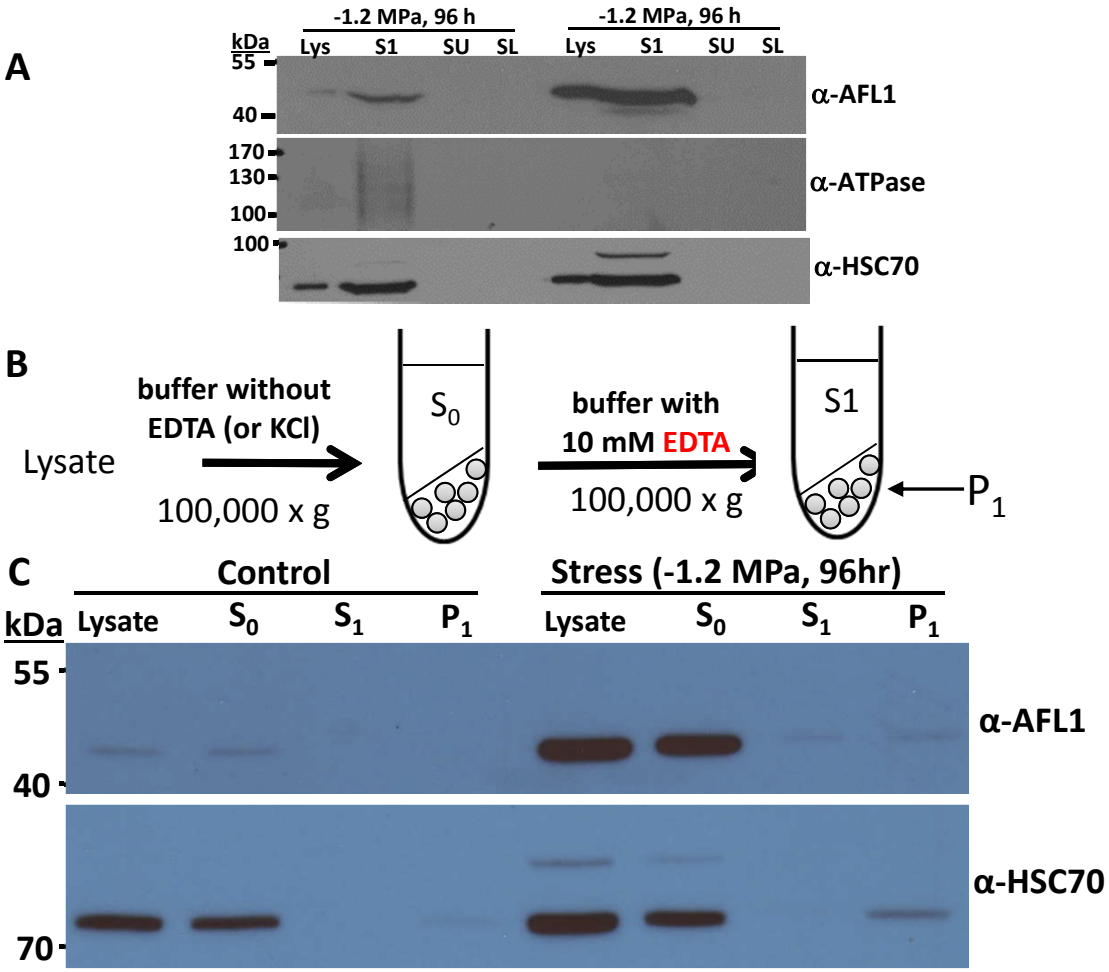

Figure S16: Analysis of membrane fractionation supernatants as well as low salt or EDTA washes indicate that AFL1 is a peripheral membrane associated protein.

A. Detection of AFL1 in the supernatant after high speed centrifugation to pellet membrane fractions (Figure 3F of main text). Lys, total lysate; S1, supernatant after initial pelleting of membranes; SU and SL, supernatants after spin down of plasma membrane enriched (U) and endomembrane enriched (L) fractions. 10 mg of protein (lysate) or 40 µl of unconcentrated supernatant was loaded. This loading is **1%** of total supernatant (S1), **0.2%** of total upper phase supernatant (SU), and **0.1%** of total lower phase supernatant (SL). In comparison **20%** of the total membrane pellet was loaded for both the U and L fractions shown in Figure 3E. The large amount of AFL1 detected in the lysate and S_1 fractions indicates that most of the AFL1 was dissociated from the membrane during fractionation. This results also shows that the the higher molecular weight AFL1 is the predominant form of AFL1.

B. Diagram of low salt or EDTA membrane washes to analyze the membrane association of AFL1. Lysates were suspended in EDTA buffer (or without KCl, shown in Figure 3G) and the supernatant collected. Membranes were then washed with buffer containing KCl and both supernatant and pellet collected.

C. Immunoblot showing that most AFL1 is removed from the membrane by EDTA wash (S_0) and little remained in the membrane pellet (P_1). A similar pattern was observed for HSC70 which is also a peripheral membrane protein. Loading of the gel was **0.1%** of total first supernatant (S_0), **0.1%** of total S1 supernatant and **1%** of total pellet resuspension (P_1). Thus, AFL1 content of the membrane pellet was low despite higher loading of this fraction.

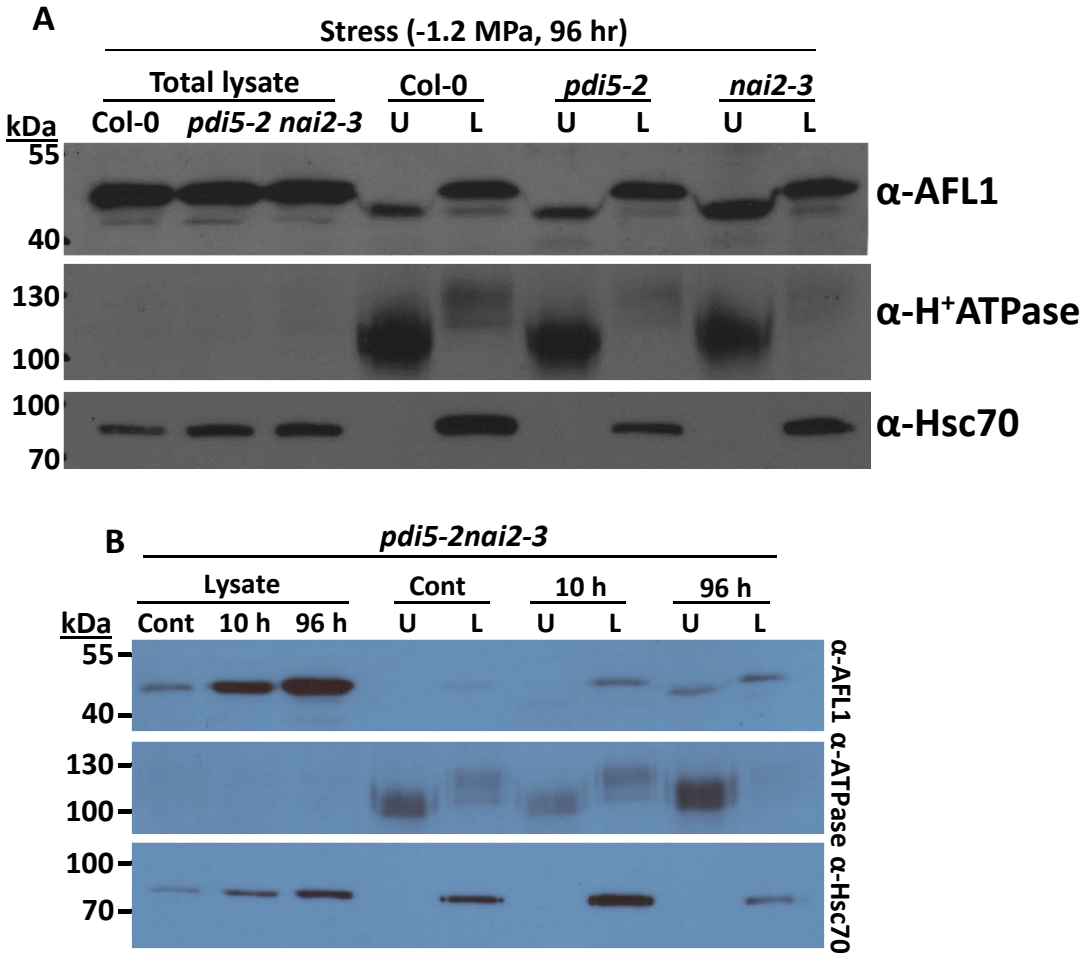

Figure S17: Membrane fractionation of *pdi5-2*, *nai2-3* and *pdi5-2nai2-3* indicate a lack of effect on AFL1 protein abundance or localization.

A. Plants were grown for seven days, transferred to -1.2 MPa for 96 h and harvested for membrane fractionation. The data show an accumulation of AFL1 in the endomembrane enriched fraction as well as plasma membrane enriched fraction which was unaffected by either the *pdi5-2* or *nai2-3* mutants. The proportion of AFL1 in the high molecular weight form versus lower molecular weight form was also unaffected by either mutant

B. Fractionation of *pdi5-2nai2-3* double mutant under both control and stress conditions also shows similar AFL1 abundance and molecular weight distribution as wild type.

Supplemental Materials and Methods

Transgenic Plants and T-DNA mutants

Total RNA was isolated using RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions and AFL1 amplified using Phusion DNA polymerase (New England BioLabs) and gene specific primers containing part of the Gateway cloning sequences (see primer sequences in Table S15). A second nested PCR was performed to add the remaining Gateway cloning sequence and the PCR product integrated into pDONOR 207 by BP reaction (Invitrogen). After sequencing, the AFL1 clone was transferred by LR reaction to pGWB411 to generate a C-terminal fusion of AFL1 to the FLAG epitope with expression driven by the 35S promoter. Likewise, 35S:YFP-AFL1 constructs were generated using the pGWB442 vector. pGWB vectors are described in Nakagawa et al. *(1).* These constructs were transferred to *Agrobacterium tumefacians* GV3101 and transformed into *Arabidopsis thaliana* Col-0 (hereafter referred to as wild type or WT) by floral dip transformation *(2).* Transgenic plants were selected by Kanamycin (50 µg ml^{-1}). Gene and protein expression of transgenic lines were assayed using quantitative RT-PCR (QPCR) and immunoblotting using commercial antisera recognizing mammalian β_1-integrin or AFL1 specific antisera (see below) or commercial anti-FLAG or anti-GFP antisera. Homozygous T_3 lines were used for all further analyses.

To generate AFL1 knock down (K.D.) lines, a 301 bp Gene specific Tag (GST) sequence targeting the C-terminal region of AFL1 was selected using the CATMA database (www.catma.org) and an entry clone was made with pDONOR 207 as described above. The C-terminal region of AFL1 was targeted for RNAi to maximize the specificity of the RNAi knockdown. This entry clone was initially transferred to the pAgrikola vector (www.agrikola.org) for constitutive RNAi suppression of AFL1; however, we failed to recover transgenic lines with substantial suppression of AFL1 expression using this method. The same entry clone was then transferred to the pOpOff2 vector *(3)* for Dexamethasone (DEX)-inducible RNAi suppression of AFL1. Simultaneously, an empty vector was also made without the AFL1 sequence as a negative control. After sequencing, the vectors were transferred to Agrobacterium and used to generate transgenic plants as described above. The effectiveness of the RNAi was confirmed by application of 10 µM DEX, transfer of seedlings to low water potential stress (see below) followed by quantitative RT-PCR and immunoblot analysis.

T-DNA mutants of *PDI5* and *NAI2* were obtained from the Arabidopsis Biological Resource Center and homozygous lines confirmed by PCR genotyping using primers from the Signal (www.signal.salk.edu) data base. RT-PCR was used to confirm absence of gene expression. All the physiological data presented are the combined data of two T-DNA alleles for each gene. A list of T-DNA mutants used is given in Supplemental Table S8. A list of primers used for cloning and genotyping is given in Supplemental Table S9.

Plant Growth Conditions, Stress and Inhibitor Treatments, Growth and Proline assays

Plants were routinely propagated in a growth room at 23 °C and 16 h light period. For seedling experiments, seed was sterilized, plated on agar plates, stratified for 4 d and the plates incubated vertically in a growth chamber at 23 °C and continuous light (70-90 µmol photons m^{-2} sec^{-1}) as previously described for experiments in our laboratory *(4-6).* The growth media consisted

of half-strength MS media with 2 mM MES buffer (pH 5.7). No sugar was added to any of the plant growth media. Low water potential stress was imposed by transferring either 7-day-old (for proline and gene expression assays) or 4-day-old (for root elongation, fresh weight and dry weight measurements) to agar plates infused with PEG-8000 as previously described *(7)*. For root growth and fresh weight measurements, position of the root apices was marked and root elongation measured over the subsequent 8 days after transfer. Seedling fresh weight and dry weight were quantified at the end of each experiment. Root elongation data were normalized to Col-0 wild type which was grown on the same plate as mutant or transgenic seedlings. Growth data (Fig 1) are the combined data (means ± S.E., n=12 from 2 independent experiments) of four independent AFL1 O.E. lines (expressing either YFP-AFL1 or AFL-FLAG) or three independent AFL1 K.D. RNAi lines. Fresh weight and dry weight data (Fig 1A and S3) are from the same experiments as the root elongation data.

Proline was assayed by ninhydrin assay *(8)* adapted to 96-well plate format *(9)*. Proline data in Figure 1D are means ± S.E., n = 12-36, combined from two independent experiments. Data are combined from four lines expressing YFP-AFL1 or AFL1-FLAG or three independent RNAi transgenic lines. Proline data of *pdi5* and *nai2* (Fig 2F) are combined data of two T-DNA mutant lines for each (means ± S.E., n =12-24).

For experiments with AFL1 K.D. lines, DEX pretreatment was performed by transferring 5 or 6 day-old plants to control plates with 10 μM DEX added. Seven day old seedlings were then transferred to either fresh DEX-containing control media or PEG-infused plates with DEX and samples collected 96 h after transfer. For experiments measuring root elongation and plant weight, 4 day-old plants were transferred directly to 10 μM DEX media and were sprayed with 10 μM DEX on alternate days to maintain repression of AFL1. For inhibitor experiments, stocks of Tyrphostin A23 and its negative analog Tyrphostin A51 (Sigma) were made in DMSO, stored at -20^0C and added to either control or PEG-infused agar plates to a final concentration of 15 μM. Brefeldin A (BFA, Sigma) was applied by spraying 100 μM solution onto seedlings that had been transferred to either control or low water potential PEG plates.

For soil drying experiments, potting mix was supplemented with 25% Turface (Turface Athletics, USA) to increase porosity, autoclaved, and distributed to 8 cm x 8 cm x 10 cm (LxWxH) plastic pots (180 g per pot). The soil-Turface mix was watered to saturation and four genotypes (wild type and three transgenic lines) planted in different sectors of the each pot. The pots were incubated in a short day growth chamber (8 h photoperiod; 100 μmol m^{-2} sec^{-1} light; 23 ° C) and the plants thinned to two plants per sector. The pots were watered with Hyponex nutrient solution diluted to 0.3 g l^{-1}. On the fifteenth day of growth, the pots were watered to saturation and allowed to drain completely over several hours. The weight of each pot was recorded. The pots were allowed to dry over the subsequent eight days. After eight days of drying, the pots were reweighed and water added (by injecting into the middle of the pot with a syringe) to bring each pot back to 75% of its initial weight. The pots were then allowed to dry for another 10 days. Control pots were maintained at fully saturated water content during this period. At the end of the drying period, rosette fresh weight (F.W.), hydrated weight (H.W.; measured after floating rosettes on cold water for ~ 6 h) and dry weight (D.W.) were measured and relative water content calculated as (F.W.-D.W.)/(H.W.-D.W.) x 100. Soil water potential was checked using a Psypro system with c52 sample chambers (Wescor) after collecting and well-mixing of soil samples from several pots. Water potentials at the end of the drying period range were approximately -1.4 MPa with most of

the decrease in water potential occurring over the last few days of soil drying. Rosette F.W. and D.W. were normalized using the W.T. plants growing in the same pot. Rosette F.W. and D.W data shown in Fig 1C represents means ± S.E., n = 10-12, combined from three independent experiments. Data are combined from three overexpression lines (expressing either YFP-AFL1 or AFL1-FLAG).

Recombinant Protein Production and Generation of AFL1 Antisera

AFL1-N terminal fragment (amino acids 1-208) was cloned into pDONR207 and transferred to pET300 (Invitrogen). His-tagged fusion proteins were produced in *Escherichia coli*, Rosetta strain (Novagen). Recombinant protein production was inducted by addition of 1 mM IPTG to late log phase cultures and incubation for 3 h at 37 ^{0}C. Cells were harvested by centrifugation and disrupted using Constant Cell disruptor (Constant Systems TS Cell Disruptor, UK.). Recombinant protein was present in the insoluble fraction of the extract and was resolubilized using 8 M urea before being applied to HisPur Cobalt Spin columns (Thermo Scientific, USA) and purified protein eluted following the manufacturer's instructions. Protein purity was checked by SDS-PAGE and Commassie staining before being used to generate polyclonal antisera. AFL1 polyclonal antisera was generated in rabbit by LTK Biolaboratories (Taiwan). Antibody titer and specificity was checked by immunoblotting using the purified recombinant N-terminal AFL1 fragment as well as total protein extracts from control and stress treated seedlings.

Immunoblot Detection of AFL1

Samples (50-100 mg of seedlings) were ground in liquid N_2 and 100 µL extraction buffer (125 mM Tris-Cl pH 8.8, 1% SDS, 10% glycerol and 1 mM PMSF, Complete Protease Inhibitor [Roche]) added. Samples were centrifuged at 7000 g for 10 min and supernatant collected. Protein concentration was checked using Pierce BCA protein assay kit (Thermo Scientific, USA) and typically 50 µg protein was loaded onto 10% SDS-PAGE gels. Proteins were blotted onto PVDF membranes and probed with AFL1 antisera (1/5000) or an antisera recognizing mammalian β_1 integrin (GTX112971, GeneTex) used at 1/3000 dilution. Tagged AFL1 from transgenic lines was detected with anti-FLAG (Sigma) or GFP antibodies (AB290 ABCAM). HRP- conjugated anti-rabbit secondary antibody was used and blots developed with chemiluminescent substrate (Thermo Scientific) and exposed to film.

Yeast Two Hybrid Screening

A yeast two hybrid library was prepared using mRNA from seedlings exposed to -1.2 MPa low water potential stress for 96 h. The library was prepared using the Cloneminer II cDNA construction kit (Invitrogen) according to the manufacturer's instructions. Yeast two hybrid screening was performed using the ProQuest Two-hybrid system (Invitrogen) following manufacturer's instructions with an N-terminal fragment of AFL1 (amino acids 1 to 208) as bait. The bait fragment was cloned into destination vector pDEST32 and co-transformed into MaV203 along with clones from the cDNA library using LiAc transformation. Transformed yeast cells were plated on SC-Leu-Trp-His with 55 mM 3-AT as preliminary test found that this 3-AT concentration was sufficient to suppress auto activation by the bait construct. Colonies that grew on the selective media were re-streaked and subjected to β-galactosidase filter assay to confirm interaction. Out of approximately $8x10^5$ colony forming units screened, clones of PDI5, NAI2, interactions were detected and interaction with TSA1 and C-terminal fragments of AFL1 were detected repeatedly based on both growth on selective media and β-galactosidase filter assays. These clones were C-

terminal truncations. Full length cDNA clones of PDI5, NAI2 and TSA1 (obtained using the cloning procedures described above) were also tested but did not show interaction with AFL1 in the ProQuest yeast two hybrid system. This was likely caused by retention of these proteins in the ER or by their membrane association preventing translocation to the nucleus and activation of the nuclear reporter genes.

Split-Ubiquitin Protein Interaction Assays

Mating-based Split-Ubiquitin System (mbSUS) assays were performed as previously described *(10)* using vectors and yeast strains obtained from the Arabidopsis Biological Resource Center. Full length AFL1 as well as putative interactors were expressed in yeast strains THY.AP4 and THY.AP5, respectively, by recombinational *in vivo* cloning and plated on SC-Leu-Met or SC-Trp-Ura-Met plates for selection. Protein interaction was tested by X-gal agarose overlay assay *(10).*

YFP-AFL1 Immunoprecipitation and mass spectrometry protein identification

Seven-day-old seedlings from transgenic lines with stable expression of 35S:YFP-AFL1 were transferred to control or low water potential stress treatments (as described above) and samples collected at 10 or 96 h after transfer. Samples consisting of approximately 5 g of tissue were homogenized in liquid nitrogen and extracted in lysis buffer consisting of 50 mM Tris (pH = 7.5), 150 mM NaCl, 0.5% TritonX100, 0.5 M EDTA and Complete Protease Inhibitor (Roche). The cell lysate was collected by centrifuging the homogenate at 20,000g for 10 min. GFP-trap beads (GFP-Trap-A kit, Chromotek) were equilibrated with dilution buffer (same as the extraction buffer except for the omission of TritonX100). Equilibrated GFP-trap beads (20-30 μl) were added to the cell lysate and kept under constant mixing at 4 ^{0}C for 2 h. Beads were collected by centrifugation at 2500g, washed one additional time with lysis buffer and resuspended in 2x SDS-PAGE loading buffer. Proteins were separated on 10% SDS-PAGE gels, stained with colloidal Coomassie stain and gel regions containing visible staining excised for in-gel trypsin digestion. Tryptic peptides were separated by reverse phase chromatography, analyzed by MS/MS on a Q-Exactive mass spectrometer. MS data were processed by Proteome discoverer and Mascot analysis (Mass spectrometry and peptide identification were conducted by the proteomics core facility of the Institute of Plant and Microbial Biology). Three independent immunoprecipitation experiments were conducted for both control and stress treated seedlings and Table S1 shows the combined list of proteins identified in these experiments.

Transient Expression, Bi-molecular Fluorescence Complementation and Co-Immunoprecipitation

Two vector systems were used for BiFC analysis. Initially, the full-length sequence of AFL1 was cloned into pSite-NEYFP-C1 and candidate genes (PDI5, NAI2, TSA1) cloned into pSite-CEYFP-C1 *(11).* To implement the ratiometric BiFC (rBiFC) system *(12),* AFL1 and candidate genes were cloned into pDONR221 P3-P2 and pDONR221 P1-P4 (Invitrogen), respectively (primers used are listed in Table S9). The resulting constructs were transferred to destination vector pBiFCt-2in1-NN with LR clonase (Invitrogen). For both BiFC systems, plasmids were transformed into Agrobacterium strain GV3101. Transient expression was performed in seedlings with DEX-inducible AvrPto expression *(13).* AvrPto seedlings were grown on agar plates as described above and four-day-old seedlings sprayed with 10 μM DEX to induce AvrPto expression. Concurrently, 24 h *A. tumefacians* cultures (150 ml) were grown for BiFC constructs. *A. tumefacians* cells were

collected by centrifugation, resuspended in 10 ml of infiltration media (5% sucrose, 5 mM MES, 200 μM Acetosyringone) and the two cultures containing pSite-NEYFP-C1 and pSite-CEYFP-C1 mixed together. Five-day-old AvrPto seedlings (approximately 24 h after DEX application) were overlaid with the mixed *A. tumefacians* cells in infiltration solution and vacuum infiltrated using two applications of 10 mm Hg for 1 minute each time *(14).* Infiltration solution was then removed and the plate with seedlings returned to the growth chamber. The next day the seedlings were rinsed with sterile water to remove excess infiltration solution and transferred either to a fresh control plates or PEG-infused agar plates (-1.2 MPa) for low water potential treatment. At 24-96 h after transfer, seedlings were analyzed by confocal microscopy (Zeiss LSM 510 Meta 510-2) to detect the BiFC signal. For rBiFC, the same procedure was followed except that only one Agrobacterium strain containing the rBiFC plasmid was used. For co-localization with ER marker, Agrobacterium containing rBiFC plasmid were mixed with Agrobacterium contain the ER marker construct (see below) and co-infiltrated. For rBiFC, the YFP and RFP fluorescence intensities of individual cells were quantified using ImageJ and used to calculate YFP/RFP ratios.

For co-immunoprecipitation, infiltration and transient expression using mixed Agrobacterium cultures containing the two tagged protein constructs was performed in the same manner as for BiFC assays. Samples for protein extraction were collected 96 h after transfer of infiltrated seedlings to either control or low water potential stress plates. Tissues were extracted in 50 mM Tris (pH=7.5), 150 mM NaCl, 0.5% Triton X-100, 0.5 mM EDTA and protease inhibitor (Roche). GFP-trap A beads (Chromotek) were used for immunoprecipitation following the manufacturer's instructions. For each sample, 20 μl of bead slurry was washed three times, incubated with a sample volume containing 3 mg of total protein (protein content assayed by Pierce BCA assay kit) for 2 hours at 4^0 C under constant mixing. Beads were collected by centrifugation or a magnetic stand and protein eluted by incubation in SDS-PAGE loading buffer at 95^0 C for 10 minutes. Immunoblotting was performed as described above.

Aqueous Two-Phase Partitioning

Aqueous two-phase partitioning was performed as previously described *(15).* Seedling tissue (1 g) was collected under control conditions or 10 and 96 h after transfer to low water potential (-1.2 MPa). Samples were ground and dissolved in 330 mM sucrose, 50 mM Tris (pH 7.5), 10 mM KCl, 5 mM EDTA, 5 mM DTT, 5 mM ascorbic acid and protease inhibitor (Roche). The homogenate was centrifuged at 10,000g for 15 min to remove debris. The supernatant was centrifuged at 100,000g for 1 h to pellet the microsomal membranes. The pellet was resuspended and added to phase mixture of 6.2% (w/w) PEG/Dextran. The resulting upper and lower phases were diluted and centrifuged at 100,000g for 1 hour. The pellets were resuspended and analyzed by SDS-PAGE and immunoblotting. For some experiments, the supernatant collected after the first or second high speed centrifugation was retained and the presence of AFL1 analyzed by immunoblotting. The fractionation of wild type was repeated three times with similar results.

For low salt membrane extraction, tissues were ground and dissolved in buffer containing 330 mM Sucrose, 50 mM Tris (pH 7.5), 5 mM DTT, 5 mM ascorbic acid and protease inhibitor and centrifuged at 10,000g for 15 min to remove debris. The supernatant was centrifuged at 100,000g for 1 hour. The following pellets were resuspended in the buffer of the same composition but with addition of 0.5 M KCl and centrifuged at 100,000g for 1 hour. The resulting pellets were resuspended and collected along with the supernatant for Western blot (400 μg total protein was

loaded for each lane). Membrane extraction in buffer with EDTA was conducted in the same manner.

AFL1 subcellular localization and co-localization of AFL1 with FM4-64 and CLC-mOrange

Seven day old seedlings were observed for co-localization analysis using a confocal microscope (LSM 510-Meta, Carl Zeiss.) with 63X or 40X water immersion lenses. Observations were made on seedlings under either control conditions or 2-96 h after transfer to -0.7 MPa. The -0.7 MPa treatment was used for these experiments as the more severe -1.2 MPa stress occasionally caused membrane damage which interfered with microscopy. For AFL1 subcellular localization, T_3 homozygous transgenic lines with expression of 35S:YFP-AFL1 were observed at excitation/emission wavelengths of 514/530-590 nm. For FM4-64 (Merck) treatment, roots of intact seedlings were immersed in 2 µM FM4-64 on a glass slide for 3 minutes before observation. FM4-64 was detected with excitation/emission wavelengths of 488/575-610nm. The same section of the root just behind the cell expansion zone was imaged in all experiments. Images were analyzed using ImageJ software. Colocalization of YFP-AFL1 with mOrange-tagged clathrin light chain (CLC) was observed in similar manner using F2 seedlings obtained from a cross of YFP-AFL1 O.E. line with a CLC-mOrange line (obtained from the laboratory of Sebastian Bednarek, University of Wisconsin-Madison). Colocalization was quantified using the Pearson correlation co-efficient PCC). Areas of interest were selected and PCC calculated using LSM510 expert mode analysis software. PCC ranges from 1 to -1 with Positive PCC values indicating similar location and intensity of the signals while negative values indicate a lack of correspondence in signal location and intensity *(16, 17).*

The number of endosome like structures and BFA bodies were counted and their area and intensity were analyzed by using tools in ImageJ.

Microarray and Gene Expression Analysis

Microarray analysis using Agilent one color arrays was performed by the microarray core facility of the Institute of Plant and Microbial Biology. Seven day old seedlings were transferred to either fresh control media or -1.2 MPa low water potential stress media as described above. Samples were collected 10 h after transfer and total RNA extracted using RNeasy Plant Mini Kits (Qiagen). Quality of RNA was checked using an Agilent 2100 Bioanalyzer. For labeling, 15 µg of total RNA was annealed to Oligo dTV DNA primer, and cDNA was synthesized in a reverse transcription reaction with an amino allyl modified dUTP. The amino allyl labeled cDNA was then coupled to Alexa 555 dye (Invitrogen) containing a NHS-ester leaving group. Unreacted NHS-ester Alexa dyes were quenched with addition of 4.5 µl of 4 M hydroxylamine and removed by PCR clean up kit (QIAGEN). For further details, see http://ipmb.sinica.edu.tw/microarray/protocol.htm.

For array hybridization, a volume of 44 µl of Alexa555-labeled cDNA was denatured at 98℃ for 3 min and cooled to room temperature. The cDNA solution was mixed with 11µl of 10x Agilent blocking agent followed by 55 µl of 2x Agilent hybridization buffer. The 100 µl of reaction mix was hybridized to Agilent Arabidopsis (V4) Gene Expression Microarrays (G2519F) for 17 hours at 65°C in a rotating Agilent hybridization oven. After hybridization, microarrays were washed 1 minute at room temperature with GE Wash Buffer 1 (Agilent) and 1 minute with 37°C GE Wash buffer 2 (Agilent), then dried immediately by brief centrifugation.

Slides were scanned immediately after washing on the Agilent DNA Microarray Scanner (G2505C) using one color scan setting for 4x44k array slides. Scan resolution 5μm, Dye channel is set to Green and Green PMT is set to 100%. The scanned images were analyzed with Feature Extraction Software 10.7.1.1 (Agilent) using default parameters (protocol GE1_107_Sep09 and Grid: 021169_D_F_20100217) to obtain background subtracted and spatially detrended Processed Signal intensities. Features flagged in Feature Extraction as Feature Non-uniform outliers were excluded.

The scanned images were analyzed with Feature Extraction Software 10.7.1.1 (Agilent) to obtain Processed Signal intensities. Signal intensities were analyzed with Genespring 11.1 software. A 1.5-fold change in expression and corrected P value of 0.05 (false discovery rate of 0.05) were used as cutoffs to determine differentially expressed probe sets.

Gene Ontolology (GO) enrichment of genes up or down regulated in the AFL1 overexpression line relative to wild type was computed using TopGO elim method *(18)* using the Gene Ontology Browsing Utility (GOBU) with its MultiView plugin *(19)*.

For quantitative RT-PCR, RNA was extracted in the same manner and quantified by Nanodrop spectrophotometer. cDNA was synthesized using 1μg of total RNA and Superscript III reverse transcriptase (Invitrogen). PCR was performed with gene specific primers (Table S9) and a SYBR green master mix (Kappa Biosystems). The PCR program used was 95 ºC for 3 min followed by 40 cycles of 95 ºC for 15 seconds and 60 ºC for 1 minute. Melt curve analysis was used to verify specific amplification. Gene expression difference was quantified by the $\Delta\Delta C_t$ method with *ELF1α,* whose expression is unaffected by abiotic stress *(20),* used as the reference gene for normalization. Three technical replicates were performed for each sample. Data represent presented are means ± S.E. (n = 6) for samples combined from two independent biological experiments.

Statistical analysis

Data typically represent the combined results of 2-3 independent biological experiments. Significant differences were determined by either t- test or two-factor ANOVA (for experiments involving multiple treatments or genotypes) performed using SigmaPlot 11.

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