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Supplemental Information

A Plant-Specific N-terminal Extension Reveals Evolutionary Functional Divergence within Translocator Proteins

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TRANSPARENT METHODS

Plant material

We used *A. thaliana* (ecotype Columbia-0) WT and transgenic plants expressing full-length AtTSPO, point-mutated AtTSPO^{H91A} or Venus-tagged AtTSPO$^{\Delta N}$ (first 41 amino acids removed), all under the control of the p35S promoter (Guillaumot et al., 2009; Vanhee et al., 2011b). An Arabidopsis homozygous T-DNA insertional line (KO) in TSPO (Guillaumot et al., 2009) was also used. Seed origin, sterilisation and growth conditions were as described previously (Guillaumot et al., 2009; Vanhee et al., 2011).

To generate the AtTSPO overexpressing cassette, AtTSPO cDNA (RIKEN, clones RAFL09-68-G14 and RAFL05-18-I12) was PCR-amplified and the PCR product was digested with XbaI/BglII and cloned into pPily (Ferrando et al., 2000) opened with XbaI/BamHI to generate pPily-AtTSPO. The expression cassette driven by a double p35S promoter in pPily was retrieved by digestion with Acc65I and subcloned into pCAMBIA 1300 opened with the same enzyme. To generate the AtTSPO^{H91A} overexpressing cassette, the cDNA of AtTSPO variant H91A was cloned from p426GAL1-AtTSPO^{H91A} (Vanhee et al., 2011b), digested with XbaI/BglII and cloned into pPILY digested with the same restriction enzymes. The expression cassette containing a double p35S promoter was retrieved from pPILY using NotI and subcloned into pAUX-3131 opened with NotI, then into the binary vector pMODUL using I-SCEI (Goderis et al., 2002). The generation of Venus-AtTSPOdeltaN was as for the AtTSPO overexpressing cassette described above except that the first 41 residues of AtTSPO were replaced by the fluorescent protein Venus sequence. The KO plants were offspring of Arabidopsis homozygous T-DNA insertional line in *TSPO* (SALK_066561C). All plasmids were amplified in *Escherichia coli* DH5α and the plasmid was transferred into *Agrobacterium tumefaciens* GV3101 containing the Ti plasmid pMP90 (a gift from Dr C. Koncz, MPI, Cologne, Germany) for Arabidopsis transformation.

Transgenic *A. thaliana* Columbia-0 plants were generated by the standard floral dip method (Clough and Bent, 1998). To this end, plants were grown until flowering and all the fertilized siliques were removed before dipping. *A. tumefaciens* GV3101 bacteria grown in a liquid medium supplemented with appropriate antibiotics were harvested in the mid-log phase. The culture was spun down and resuspended in 5% sucrose solution followed by addition of 0.02% Silwet L-77 surfactant (Lehle Seeds, Round Rock, USA, #VIS-01). The above-ground parts of plants were dipped in bacterial solution for 2-3 seconds with gentle agitation. Then, the pots

with plants were laid on their side, covered by plastic foil to maintain high humidity and put back in a room under dimmed light.

Twenty-four hours later, plants were transferred into the phytotron and further grown under normal watering regime (twice a week) until seeds became mature. Seeds were harvested and screened for homozygous transformants using 20 µg/ml hygromycin B.

Seeds spread on a filter paper were disinfected under laminar flow hood first by pouring 70% ethanol containing 0.02% Silwet L-77 surfactant followed by 3 rounds with 100% ethanol. After each round, the seeds were allowed to dry and after the sterilization process they were brushed off on the agar plate containing appropriate medium. Seeds were stratified for 2–3 days at 4°C in the dark, and transferred into a growth chamber (16h/8h light/dark regime, 22–25°C, 90 µmol photons. $s^{-1}.m^{-2}$). After germination and growth for 10 days on the agar plate, the seedlings were transferred to Jiffy pots (http://www.jiffypot.com) for further growth in soil or subjected to various treatments.

WT Arabidopsis suspension cells (May and Leaver, 1993) were agitated in the dark on a rotary shaker and diluted 10-fold weekly in freshly sterilised LS medium (Duchefa, Haarlem, The Nederlands, #L0230; 4.43 g/L) adjusted to pH 5.7 with KOH and containing 3% sucrose, 50 µg/L kinetin (Duchefa # K0905), and 500 µg/L 1-naphthalene acetic acid (Duchefa #N0903).

Water loss and stomatal conductance assays

To assess dehydration tolerance, WT, TSPO knockout, AtTSPO, $AtTSPO^{H91A}$ and Venus-$AtTSPO^{\Delta N}$ overexpressing plants were grown on half-strength Murashige and Skoog (MS) agar plates (0.2% MS basal salt mixture, MP Biomedicals, Eschewege, Germany, #0926230) containing 1% sucrose, 25 mM 2-(N-morpholine)-ethanesulphonic acid (MES, Sigma-Aldrich, Overijse, Belgium, #69889), pH 5.7, sealed with parafilm in a growth chamber (16 h/8 h light/dark, 22-25°C, 90 µmol photons.s^{-1} .m^{-2}). The 7-day-old seedlings were transferred from agar plates into pots with soil, installed in the phytotron (16 h/8 h light/dark, 20°C, 65% humidity, 120 µmol photons.s^{-1} .m^{-2}) and watered twice weekly. Integral rosette leaves of pre-flowering plants were subjected to abaxial stomatal conductance measurements according to the manufacturer's protocol (automatic mode, SC-1 Leaf Porometer; Decagon Devices, Pullman, USA).

To assess the water loss on detached rossette leaves, the seeds of wild-type, TSPO knockout and AtTSPO, $AtTSPO^{H91A}$ and Venus-$AtTSPO^{\Delta N}$ overexpressing plants were put on soil in a phytotron (average temperature 20°C, approx. 65% humidity, 16 h photoperiod,

~120 µmol.m^{-2}.s^{-1}). Seventeen days after germination, rosettes were isolated using a blade and then were transferred on a weighing paper and weighted on a microbalance at time 0 and after 120 minutes. Water loss was expressed as percentage of the original weight of seedlings. For this experiment, we assayed five rosettes of each genotype harvested from at least two pots (pots were randomly allocated in different areas of phytotron).

Transformation of plant suspension cells and PM preparation

Transgenic Arabidopsis suspension cell lines were co-cultivated with Agrobacterium (Van Leene et al., 2007) (Timentin, Duchefa #T-104-2, replaced vancomycin). The pCambia3300 USER vector cloning system (Nour-Eldin et al., 2006) was used to generate mGFP5-TSPO fusions driven by the p35S promoter. GFP-tagged AtTSPO, AtTSPO$^{\Delta N}$, mTSPO (mouse homolog) and AtTSPO^{Nter}-mTSPO (N-terminus of AtTSPO added to the mouse protein) chimeras were created by PCR using primers listed in Supplemental Table S1. For GFP-AtTSPO$^{\Delta N}$, a seven amino acid linker (GAGAGAG) was introduced to increase flexibility of the chimeric protein.

The vector harboring the PI(4,5)P_2 phosphoinositide sensor (Simon et al., 2014) was a generous gift from Dr Yvon Jaillais (Ecole Normale Supérieure, Lyon, France) and Dr Teun Munnik (University of Amsterdam, The Netherlands).

Plasma membrane-enriched fractions of WT and transgenic Arabidopsis suspension cells were prepared by two-phase partitioning of microsomal fraction (Santoni et al., 2006). All the steps were performed at 4°C. To obtain microsomal membranes, cells were harvested and mechanically disrupted by glass beads in a disruption buffer (2.5 mL buffer/g fresh cells; 50 mM Tris pH 8, 500 mM sucrose, 10% glycerol, 20 mM EDTA, 20 mM EGTA, 50 mM NaF, 10 mM ascorbic acid, 0.6% w/v polyvinylpyrrolidone, 5 mM dithiothreitol and protease inhibitors). The cell homogenate was centrifuged at 10000 g for 10 min and the supernatant was filtered through a 100 µm-diameter nylon cloth. Finally, microsomal pellet was obtained from the supernatant by centrifugation at 50000 g for 35 min.

Nine grams of microsomal fraction was mixed with 27 g of phase system (11.82 g Dextran T-500 20% w/w (Sigma #31392), 5.76 g polyethylene glycol 3350 40% w/w (Sigma #P4338), 5 mM phosphate buffer pH 7.8, 5 mM KCl, 300 mM sucrose, H2O up to 27 g). The mixture was shaken vigorously 15-20 times and allowed to separate out into two phases by centrifugation at 2000 g for 10 min. To purify the PM from the upper phase, this phase was repartitioned against a “fresh” lower phase. “Fresh” lower phase was obtained after centrifugation of a shaken

mixture of 9 g of microsomal buffer and 27 g of phase system. After a series of fractions' repartitioning, the PM-containing phases were diluted in 60 mL of PM washing buffer (10 mM Tris pH 8.3, 10 mM boric acid, 300 mM sucrose, 9 mM KCl, 5 mM EDTA, 5 mM EGTA, 50 mM NaF, 5 mM dithiothreitol, protease inhibitors) before pelleting for 35 min at 85 000g max. Pelleted PM-enriched fraction was resuspended in PM washing buffer, frozen in liquid nitrogen and stored for a short term (days) at -20°C or for a long term (weeks) at -80°C.

BiFC experiments and cell imaging

For BiFC assays, we employed the pDOE12 vector (Gookin and Assmann, 2014) (provided by Dr Sarah Assmann, Pennsylvania State University, USA) encoding a split Venus at the N-terminus of the protein of interest (Venus split into 210 and 29 amino acid N and C parts, respectively). Full-length AtTSPO and AtPIP2;7 cDNAs were PCR-amplified from templates (Vanhee et al., 2011a; Hachez et al., 2014), and N-terminally truncated variants of AtTSPO were generated by PCR. All AtTSPO lysine/arginine point mutants were created by overlapping PCR-mediated site-directed mutagenesis using primers listed in Supplemental Table S1. Transient expression in tobacco (*Nicotiana tabacum*) was performed using *Agrobacterium tumefaciens*-mediated transfection as described previously (Batoko et al. 2000). *N. tabacum* Petit Havana seeds were put on soil and grown at 21°C until the seedling stage (but no flowers) has well established. Each expression vector was introduced by electroporation into *A. tumefaciens* GV3101 (pMP90) strain (Koncz and Schell, 1986). A single colony from the transformants was used to inoculate 2 mL of YEB medium (w/v: 0.5% beef extract, 0.1% yeast extract, 0.5% sucrose, and 2 mM $MgSO_4$), supplemented with 100 mg/mL kanamycin and 20 mg/mL gentamycin. On the next day, 1 ml of culture was transferred into an Eppendorf tube, and the bacteria were pelleted by centrifugation at 2200 g for 5 min at room temperature. The pellet was washed twice with 1 mL of the infiltration buffer (10 mM MES pH 5.6-5.7, 0.5% glucose (w/v), 20 mM $MgSO_4$ and 100 µM acetosyringone (Sigma, Overijse, Belgium)) and then resuspended in 1 mL of the same buffer.

The bacterial suspension was diluted with infiltration buffer to adjust the inoculum concentration to the OD_{600} value of 0.2. The inoculum was delivered to the lamina tissues of tobacco leaves by gentle pressure infiltration through the stomata of the lower epidermis, by using a 1 ml syringe without a needle. The infected area of the leaf was delimited and labeled with an indelible pen, and the plant was incubated under normal growing conditions as above. One tobacco plant was used to infiltrate one genetic construct.

Confocal imaging with a Zeiss LSM710 confocal microscope equipped with a spectral detector was performed as described (Guillaumot et al., 2009; Vanhee et al., 2011a; Hachez et al., 2014). Reconstituted mVenus fluorescence after BiFC was excited with a 514 nm argon multiline laser and the amplified emitted light was recorded between 520 and 610 nm. mTurquoise was excited with a 445 nm laser, and fluorescence used to detect transfected cells and Golgi colocalisation of the BiFC signal. Images were processed using identical parameters. To quantify the BiFC signal, we imaged five randomly chosen independent (non-overlapping) areas of infiltrated or non-infiltrated leaves. Pixel intensities from mTurquoise and mVenus channels were acquired along two diagonals of each image (~700 intensity measurements per diagonal) using ZEN2012 software (Carl Zeiss, Oberkochen, Germany). Average signal intensities were calculated from 10 diagonals, and values for non-infiltrated leaves were subtracted. Ratios between the BiFC/mTurquoise signal intensities were calculated and normalised against full-length AtPIP2;7-AtTSPO positive control.

Human phosphatidylinositol 4-phosphate 5-kinase type-1 (PI4P 5-kinase) alpha fused to mCherry (mCherry-HsPIPK1α) known to be functional in plant cells (Ma et al., 2015) was expressed in tobacco leaves by Agrobacterium-mediated transient expression. The construct was expressed in WT plants and stable transgenic lines overexpressing YFP-AtTSPO. Confocal imaging of the resulting mCherry signal in leaf epidermal cells was performed using a 561 nm 543 excitation laser.

TSPO purification for *in vitro* biochemical assays

Yeast transformation was performed by the LiAc/ss-DNA/PEG method as described (Gietz and Schiestl, 2007). A freshly grown colony of yeast on a solid plate was suspended in 1 ml of sterile miliQ water. Cells were vortexed and then centrifuged at 13000 g for 30 s. The supernatant was discarded and the yeast pellet was covered (the order as given) with 240 µl PEG 3350 (50% w/v), 36 µl lithium acetate (1 M), 50 µl single-stranded DNA (2 mg/ml, to obtain a single-stranded state, the DNA from fish sperm was boiled 5 min followed by a snap cool on ice), and finally 34 µl of sterile water and approximately 25-50 ng of plasmid DNA of interest. The content of the tube was vortexed until resuspended. Then, the tube was placed in a water bath at 42°C and incubated for 20 min. After this period, cells were pelleted at 13000 g for 30 s at room temperature and the transformation mix supernatant was removed. Cells were resuspended gently in 1 ml of sterile water and plated (100 µl and 900 µl) on a selective medium. Colonies of transformants were screened for the presence of gene of interest by colony PCR.

Ten histidine-tagged AtTSPO and AtTSPO$^{\Delta N}$ proteins were expressed and purified from microsomal membranes of *Saccharomyces cerevisiae* (Vanhee et al., 2011b). Microsomes were suspended in solubilization buffer (50 mM Tris pH 8, 500 mM NaCl, 10% glycerol, 20 mM imidazole, protease inhibitors) and solubilized with 2% n-Dodecyl β-D-maltoside (DDM, Enzo #ALX-500-007) for 30 min at room temperature. The mixture was centrifuged for 30 min at 20000 g at room temperature and solubilized proteins (in supernatant) were subsequently mixed with Ni-NTA matrix (Qiagen, Benelux, #30210) pre-equilibrated with solubilisation buffer lacking imidazole. Binding was performed at room temperature for 3 h, after which the flow-through was collected and beads were washed four times with wasing buffer (solubilization buffer containing 0.2% DDM) with increasing imidazole concentrations (20, 20, 40 and 60 mM). Bound proteins were eluted with 300 mM imidazole in washing buffer.

The 6His-mTSPO (pET-15b vector) and 10His-tagged AtTSPO N-terminus fused to mTSPO (AtTSPO^{Nter}-mTSPO; GST fusion in a pGEX-4T-2 vector) were expressed and purified from *Escherichia coli* BL21 (DE3). Protein expression was induced with 1 mM isopropyl-β-D-thiogalactoside (IPTG) at 28°C for 4 h, cells were centrifuged, and the pellet was resuspended in buffer A (50 mM TRIS pH 8.0, 300 mM NaCl, 10% glycerol, 20 mM imidazole) containing protease inhibitor cocktail (Sigma) supplemented with both 1 mg/ml lysozyme and 1000 U benzonase (Sigma-Aldrich, Overijse, Belgium, # E1014) and incubated for 15 min at room temperature with gentle shaking.

Cells were lysed by sonication (3 min, amplitude 60%, 10 s pulses; ultrasonic processor Vibra-Cell, Sonics & Materials, Newtown CT, USA). Bacterial membranes containing overexpressed TSPO were obtained after two centrifugation steps at 4°C by centrifuging lysed cells for 30 min at 15,000 g followed by supernatant centrifugation for 1 h at 250,000 g. Sedimented membranes were resuspended in buffer A and solubilised by adding n-dodecyl-β-D-maltoside (DDM; Enzo Life Sciences, Brussels, Belgium, #ALX-500-007) to a final concentration of 2% for 1 h at room temperature on a rotary wheel. Solubilised membranes were centrifuged at 150,000 g for 30 min at 4°C and the supernatant was mixed with Ni-NTA matrix (Qiagen, Benelux, #30210) pre-equilibrated with solubilisation buffer lacking imidazole. Purification was performed as described above. Bound proteins were eluted with 300 mM imidazole in buffer A and dialysed on PD-10 desalting columns (GE Healthcare, Belgium-Luxemburg, #17085101) against imidazole-free buffer A. Eluted fractions of mTSPO were pooled, concentrated and frozen in aliquots.

After dialysis, AtTSPO^{Nter}-mTSPO fusion proteins were subjected to a second purification step by overnight digestion at room temperature with His-tagged tobacco etch virus (TEV) protease (0.1 µg/ml; produced and provided by Marc Boutry's laboratory, UCLouvain, Louvain-la-Neuve, Belgium). The mixture was added to a nickel affinity column and processed as previously described. Washed fractions containing the protein of interest were pooled, dialysed, concentrated and frozen for subsequent biochemical analysis. Primers used for expression and purification of TSPO are listed in Supplemental Table S1.

Expression and purification of the N-terminus of AtTSPO for structural studies

The AtTSPO^{Nter} construct (residues 1-49) was expressed via pGEX-4T-2 vectors in *E. coli* BL21 (DE3) as a GST-fused 10His-tagged protein containing a TEV protease cleavage site. To obtain labelled protein, bacteria were cultured in M9 minimal medium supplemented with [^{15}N]-NH_4Cl and [^{13}C]-glucose at 1 and 4 g/L, respectively. Protein expression in cells cultured in the presence of 0.1 mg/ml ampicillin was induced with IPTG at 37°C for 4 h. Bacteria were collected and protease inhibitor cocktail was added before lysis (by sonication) in 50 mM HEPES-NaOH and 500 mM NaCl (pH 7.8), followed by centrifugation at 14,000 g for 20 min. Protein in the supernatant was purified using Ni-NTA resin and eluted with 250 mM imidazole. Purified proteins were dialysed against 50 mM HEPES-NaOH at pH 7.8 overnight at room temperature and then digested by His-tagged-TEV protease (0.1 µg/ml) overnight (16-20 h) at room temperature. Cleaved AtTSPO^{Nter} was isolated from GST-fusion proteins and proteases using Ni-NTA resin since His-tagged GST and TEV proteins were retained on the column.
Purified proteins were dialysed against 10 mM sodium phosphate and 1 mM NaN_3 at pH 7.8 to perform structural studies. SDS-PAGE analysis was performed on samples collected during all purification steps.
Purified AtTSPO^{Nter} was characterised by (i) dot blots and (ii) mass spectrometry (MS). For dot blots, spots of AtTSPO^{Nter} on PVDF membranes were first incubated with polyclonal rabbit anti-AtTSPO antibody serum followed by anti-rabbit antibody coupled to alkaline phosphatase, and visualisation with specific substrates (BCIP/NBT Sigma-Fast; Sigma-Aldrich, Overijse, Belgium, #B5655). For MS, aliquots of purified AtTSPO^{Nter} were mixed with HCCA matrix and analysed using a 5-17 kDa standard set protein (Proteomics Sigma-Aldrich, Overijse, Belgium) via MALDI-TOF (DE-Pro, ABSciex, Framingham, MA, USA). The concentration of purified AtTSPO^{Nter} was determined by UV spectroscopy in conjunction with the calculated

extinction coefficient (280 nm) of 0.256 for 1 mg/ml. Primers used for expression and purification of AtTSPO^{Nter} are listed in Supplemental Table S1.

Circular dichroism (CD) spectroscopy

CD measurements were carried out on a JASCO spectropolarimeter (JASCO, France) with a 1 mm cuvette at room temperature.

Preparation of bicelles and PI(4,5)P_2 micelles

Bicelles were prepared as previously described (Caillon et al., 2013). Briefly, 25 mM 1,2-dimyristoyl-sn-glycero-3-phosphocholine or 1,2-dimyristoyl-sn-glycero-3-phospho-(1'-*rac*-glycerol), corresponding to DMPC or DMPG, respectively (Avanti polar lipids) and 50 mM 1,2-dihexanoyl-sn-glycero-3-phosphocholine (DHPC; Avanti polar lipids) in 10 mM sodium phosphate at pH 6.1-6.2 were mixed (vortexed for 1 min) and subjected to three freeze-thaw cycles. Next, 1,2-dioleoyl-sn-glycero-3-phospho-(1'-myo-inositol-4',5'-bisphosphate) PI(4,5)P_2 micelles were formed by adding 10 mM sodium phosphate at pH 6.1-6.2 to powder and sonicating to ensure thorough mixing (stock solution 5 mM)

Dynamic light scattering (DLS)

LS measurements were carried out with a DynaPro-99 instrument (Yatt 616 Technology). The sizes (radius) of bicelles (DMPC/DMPG) and PI(4,5)P_2 micelles were determined to be 4.1±0.2 and 65±20 nm, respectively.

NMR spectroscopy

NMR samples were prepared in 5 mm NMR tubes and contained ~100 µM ^{15}N- and ^{13}C-labelled AtTSPO^{Nter} in 10 mM sodium phosphate at pH 6.2 and H2O/D2O (90:10 v/v). A 0.1 mM sample of sodium 2,2-dimethyl-2-silapentane-d6-5-sulfonate (DSS, Sigma) served as an internal chemical shift reference. Protease inhibitor cocktail (Roche) and EDTA (1 mM) were added to prevent degradation. Experiments were recorded on a Bruker 500 MHz Avance III spectrometer equipped with a TCI cryoprobe. Resonances were assigned using 2D ^{1}H-^{15}N HSQC and 3D ^{1}H-^{15}N-^{13}C HNCACB, CBCA(CO)NH, HNCA, HNCO and HN(CA)CO spectra acquired at 20°C. NMR data were processed with Bruker TopSpin 3.2 or NMRPipe and analysed with NMRFAM-SPARKY (Caillon et al., 2013; Delaglio et al., 1995; Lee et al., 2015; Ziarek et al., 2011).

Secondary structure analysis was based on SSP scores (Marsh et al., 2006) calculated from $^{13}C\alpha$, $^{13}C\beta$, ^{13}CO, ^{1}HN and ^{15}N chemical shifts.

Subsequent 2D ^{1}H-^{15}N-HSQC spectra of AtTSPO^{Nter} were recorded in the presence of DMPC/DHPC and DMPG/DHPC bicelles (12.5/25 mM, respectively; q = 0.5), and in the presence of increasing amounts of PI(4,5)P_2 at 1 to 10 equivalents. Both ^{1}H and ^{15}N chemical shift perturbations (CSP) were calculated using the following formula:

$\Delta\delta = \left| \Delta\delta(1H) \right| + 1/10 \left| \Delta\delta(15N) \right|$

where the binding constant (Kd) was estimated from CSP values upon titration with PI(4,5)P_2 using a fast-exchange single-site binding model (Delaglio et al., 1995).

Western blot and antibodies

Extracted proteins were quantified by the Bradford method, mixed with 5× Laemmli buffer and incubated for 30 min at 37°C prior to SDS-PAGE and transfer onto PVDF membranes (Immobilon-P, Merck-Millipore, Darmstadt, Germany, #IPVH00010) using the semi-dry transfer method. Membranes were saturated for 1 h at 25°C in blocking buffer (5% non-fat milk in TBS-Tween-20 supplemented with 0.5% Tween-20 in 1× TBS) and incubated at room temperature for 1 h with primary antibodies diluted in washing buffer (0.5% milk in TBS Tween-20 supplemented with 0.1% Tween-20 in 1× TBS). After washing three times in washing buffer, membranes were incubated with secondary Horseradish peroxidase (HRP)-conjugated antibodies for 1 h at room temperature. After washing three times in washing buffer, membranes were incubated for 3-5 min with ECL (Roche, #11500694001). Emitted light was detected using either a film processor (Optimax X-ray film processor; Protec Medical Systems) or a digital imaging device (Amersham Imager 600, GE Healthcare Life Sciences). All original immunoblots are provided as Supplemental Information.

Polyclonal anti-AtTSPO antibodies were designed by the host laboratory and raised in a rabbit against the synthetic peptide ^{2}DSQDIRYRGGDDRDA^{16} followed by affinity purification using the same peptide (Eurogentec) (Guillaumot et al., 2009).

HisProbe-HRP conjugates were purchased from ThermoFisher Scientific (#15165). Antibodies against Arabidopsis RbcL and AtPIP2;7 were purchased from Agrisera (#AS03037 and #AS09469, respectively). Anti-GFP was from Abcam (#ab290), and anti-PI(4,5)P_2 was from Enzo Life Sciences (#ADI-915-052-020). The mouse monoclonal anti-actin (β-actin) was purchased from Proteintech (#60008-1-Ig). Anti-FLAG was a generous gift from Dr Michel

Ghislain (UCLouvain, Louvain-la-Neuve, Belgium), and antibodies against plant H^+-ATPases were provided by Dr Marc Boutry (UCLouvain, Louvain-la-Neuve, Belgium).

For western blotting, anti-AtTSPO, anti-AtPIP2;7, anti-GFP and anti-RbcL antibodies were diluted 1:5,000, HisProbe-HRP was diluted 1:1,000, anti-PI(4,5)P_2 and anti-FLAG were diluted 1:2,000, and anti-H^+-ATPase was diluted 1:100,000. HRP-conjugated anti-rabbit and anti-mouse secondary antibodies were from Sigma and diluted 1:10,000.

Overlay and pull-down assays

Protein-lipid overlay assays were performed using commercially available PIP Strips (Echelon Biosciences, Salt Lake City, USA, #P-6001) according to manufacturer's protocols, or using membranes prepared in our laboratory. Briefly, phosphatidic acid (Sigma-Aldrich, Overijse, Belgium, #P9511, dissolved in $CHCl_3$), phosphoinositides PI3P, PI4P and PI(4,5)P_2 (Echelon Biosciences, #P-3016, #P-4016 and #P-4516, respectively; all dissolved in 2% DDM), or SDS-solubilised plasma membrane fractions were spotted on nitrocellulose membranes (Amersham Protran, GE Healthcare, #10600002) and air dried for 1 h. Membranes were then saturated in blocking buffer (3% BSA in TBS-Tween-20, supplemented with 0.1% Tween-20 in TBS). Incubation with proteins in blocking buffer was performed at room temperature for 3 h, followed by incubation with primary and secondary antibodies and ECL exposure as described above.

For lipid-dependent pull-down assays, purified 10His-AtTSPO or 10His-AtTSPO$^{\Delta N}$ were incubated for 3 h at room temperature with phosphatidic acid-coupled agarose beads (Echelon Biosciences, #P-B0PA) followed by flow-through collection, three washes with incubation buffer (50 mM TRIS-HCl pH 8.0, 150 mM NaCl, 10% glycerol, 0.2% DDM, proteases inhibitors) and elution with Laemmli buffer supplemented with 8 M urea. Uncoupled agarose beads were used as a negative control.

Microscale thermophoresis (MST)

MST was used to investigate binding events between purified TSPO and lipid or aquaporin ligands. All experiments were performed on a Monolith NT115 instrument (Nanotemper Technologies, Germany) equipped with red and green filters using standard treated capillaries. Proteins and lipids were stored in the same buffer composed of 50 mM TRIS-HCl pH 8.0, 200 mM NaCl, 10% glycerol, 2% DDM, and proteases inhibitors (Sigma-Aldrich, Overijse, Belgium). Proteins were titrated against a gradient of lipid concentrations, and in the PIP2;7-

AtTSPO interaction assay, PIP2;7 was titrated against different AtTSPO concentrations. In the phosphatidic acid binding experiment, full-length AtTSPO and isolated N-termini were labelled on lysine residues with NT-647-NHS, while PI(4,5)P_2 binding assay proteins were labelled on polyhistidine tags with RED-tris-NTA (Nanotemper, Müchen, Germany). YFP-AtPIP2;7 aquaporin (mVenus variant) was in the form of a DDM-solubilised microsomal fraction of transgenic *A. thaliana* seedlings (Hachez et al., 2014), and YFP fluorescence was probed using a green filter. The laser power was set to 10, 20 or 40%, and the LED power was maintained at 100%.

PLC activity assay

We used the EnzCheck® Direct Phospholipase C Assay kit to measure PLC activity from plant extract according to the manufacturer recommendations. We used the PC-PLC from *Bacillus cereus* present in the kit to normalize the recorded fluorescence emission. Plant extract were obtained from 10-day-old Arabidopsis seedlings growing on MS plate. For ABA treatment, the seedling were incubated for 24 hours in half-strength MS containing 50 µM ABA and in the dark. PLC activity was measured in triplicate (total protein per reaction: 10 µg, 30 µg and 90 µg) and the experiment repeated at least twice.

Statistical analysis

The number of replicates and statistical methods for each experiment are stated within figure legends. GraphPad Prism version 5.03 for Windows (GraphPad Software; San Diego, California, USA; www.graphpad.com) was used to analyse statistical significance. For western blot signal quantification, we used ImageJ version 1.51j8 (NIH, USA; www.imagej.nih.gov/ij/). For quantification, we ensured that exposure conditions provided by the digital imager were below the saturation threshold. For MST data analysis, we used NT Analysis 1.5.37 software (Nanotemper, Müchen, Germany). A non-linear regression model was used (Graphpad Prism) to fit normalised fluorescence data and derive binding constants.

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Supplemental Figures

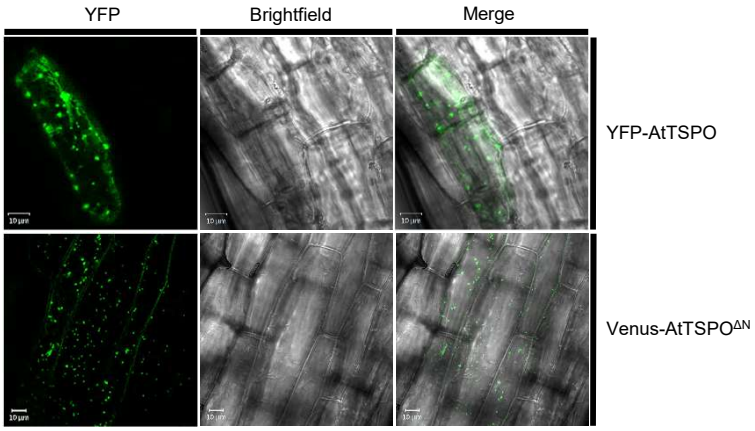


Figure S1. Subcellular localization of AtTSPO and AtTSPO$^{\Delta N}$ expressed in *Arabidopsis thaliana*. Related to Figure 5. Representative confocal images of hypocotyls of transgenic Arabidopsis seedlings overexpressing YFP-AtTSPO or Venus-AtTSPO$^{\Delta N}$. Full-length AtTSPO is detected in the Golgi membranes and in the ER and the N-terminus truncated AtTSPO localizes essentially to the Golgi. Bars = 10 µm.

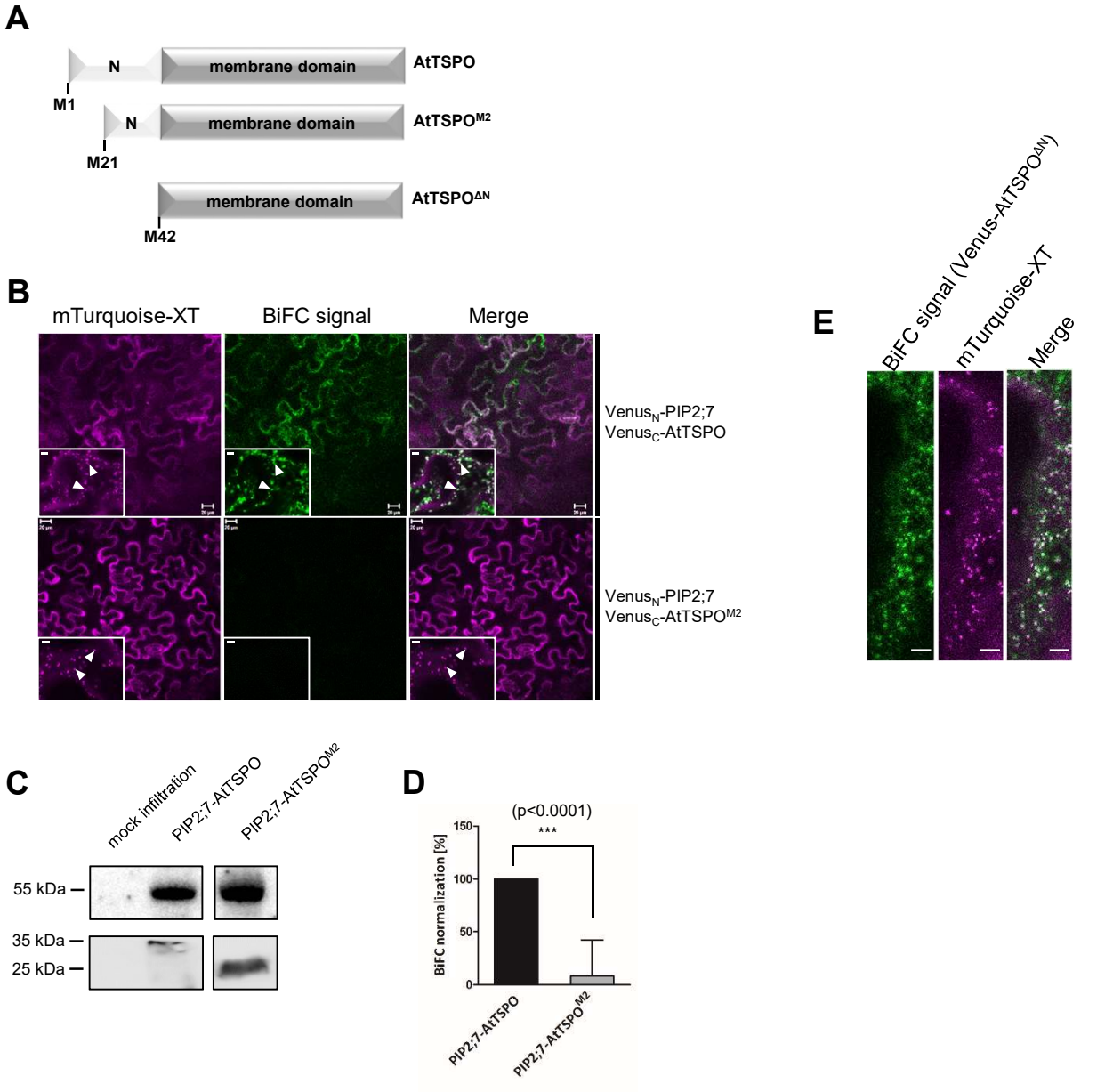
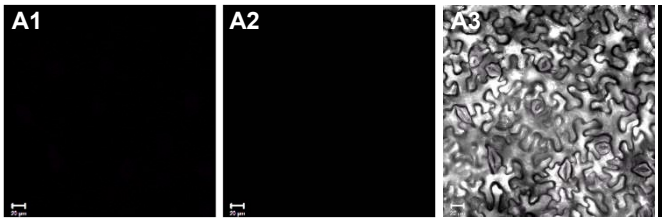
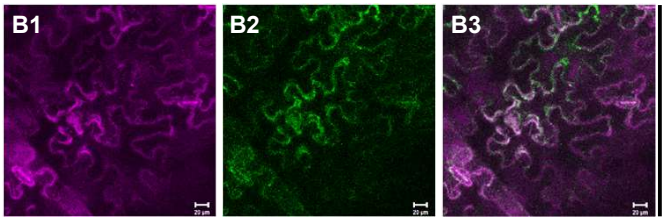


Figure S2. The first 20 amino acids of AtTSPO are also required for AtTSPO interaction *in vivo* with PIP2;7. Related to Figure 2. (A) Schematic representation of AtTSPO genetic constructs used in BiFC approach. As a positive control in this assay we used both full-length PIP2;7 and AtTSPO. N-terminus truncated variant (M2) starts with the methionine at the position 21, and the variant AtTSPO$^{\Delta N}$ starts with the methionine at the position 42. (B) BiFC assay on Agrobacterium-infiltrated *Nicotiana benthamiana* leaves. Xylosyltransferase-mTurquoise fluorescent chimera was imaged as cell transfection control (in magenta). Low magnification images qualitatively demonstrate occurrence or lack of BiFC (in green) and the insets with high magnification images show Golgi stacks (arrowheads). Bars = 20 µm and 5 µm for low and high magnification, respectively. Experiment was repeated at least twice. (C) Western blot of infiltrated leaves areas used for BiFC microscopy analysis (B). PIP2;7 expression was detected by anti-GFP. AtTSPO full-length and truncated form were detected by anti-FLAG (the tag was originally cloned in between Venus$_C$ and cloning site for AtTSPO). Non-infiltrated leaf area was used as a negative control. (D) Quantitative data of the signal show a drastic reduction of BIFC between PIP2;7 and truncated AtTSPO mutant as compared to full-length AtTSPO. For statistical significance evaluation an independent samples t-test was performed using Graphpad Prism 5 (San Diego, USA). Bars represent means +/- SD. See methods section for quantification procedure. (E) BiFC as in (B) but both AtTSPO and PIP2;7 cDNA were replaced by AtTSPO$^{\Delta N}$ coding sequence. The merge image shows colocalization between the BiFC signal (green) generated by interacting AtTSPO$^{\Delta N}$ and the fluorescence of the Golgi marker (magenta). Bars = 5 µm.



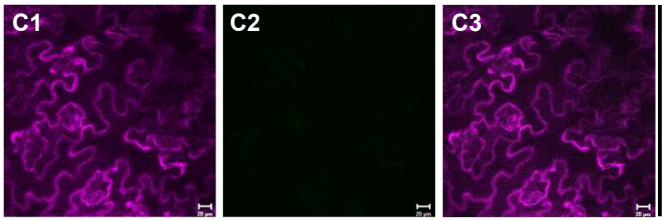
45.42 ± 13.57 | 15.00 ± 4.99 | ratio= BiFC/mTurquoise

Mock infiltration



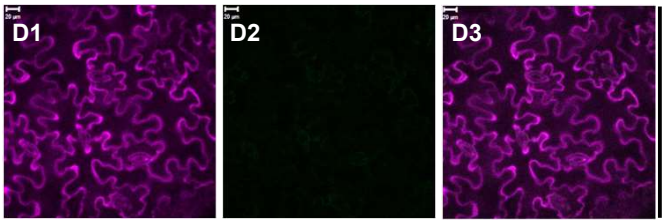
35.48 ± 30.45 | 9.11 ± 16.71 | ratio= 0.257 ± 0.660, norm.= 100%

$Venus_N$-PIP2;7
$Venus_C$-AtTSPO



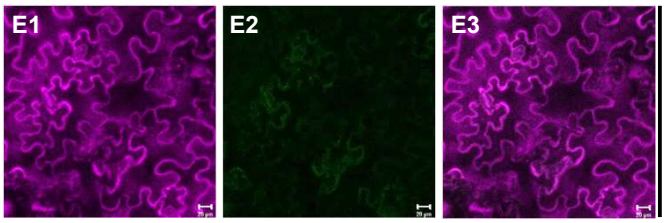
50.60 ± 43.48 | 1.48 ± 8.90 | ratio= 0.029 ± 0.198, norm.= 11.4%

$Venus_N$-PIP2;7
$Venus_C$-AtTSPO$^{\Delta N}$



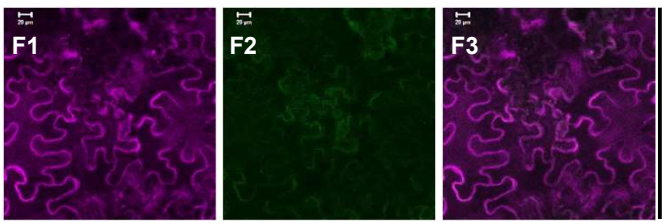
49.64 ± 42.56 | 1.04 ± 7.97 | ratio= 0.021 ± 0.176, norm.= 8.2%

$Venus_N$-PIP2;7
$Venus_C$-AtTSPO^{M2}



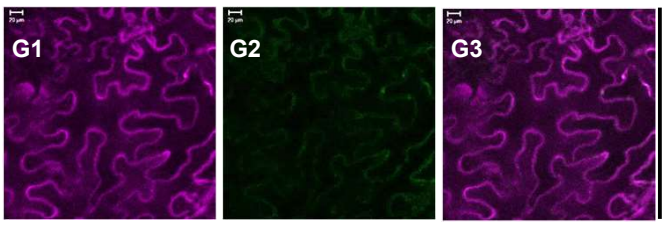
88.43 ± 59.65 | 2.91 ± 10.15 | ratio= 0.033 ± 0.130, norm.= 12.8%

$Venus_N$-PIP2;7
$Venus_C$-AtTSPO$^{K35A/R36A}$



56.36 ± 45.92 | 2.78 ± 9.44 | ratio= 0.049 ± 0.200, norm.= 19.2%

$Venus_N$-PIP2;7
$Venus_C$-AtTSPO$^{K39A/R40A}$



33.72 ± 33.96 | 1.53 ± 9.29 | ratio= 0.045 ± 0.321, norm.= 17.7%

$Venus_N$-PIP2;7
$Venus_C$-AtTSPO$^{K39A/R40A/K44A/R45A}$

Figure S3. Methodology of BiFC signal quantification. Related to Figure 2. Five independent randomly chosen (but not overlapping) areas of the infiltrated or non-infiltrated leaf were imaged. Pixel intensities from mTurquoise (magenta) and mVenus (green) channels were acquired along two diagonals of each image (around 700 intensity measurements per one diagonal) using ZEN2012 software (Zeiss). An average signal intensity was calculated from 10 diagonals (around 7000 intensities measurements). An average signal intensity from non-infiltrated leaves was subtracted. Ratio between an average BiFC/mTurquoise signal intensity was calculated and normalized against the control (combination PIP2;7-AtTSPO full-length).

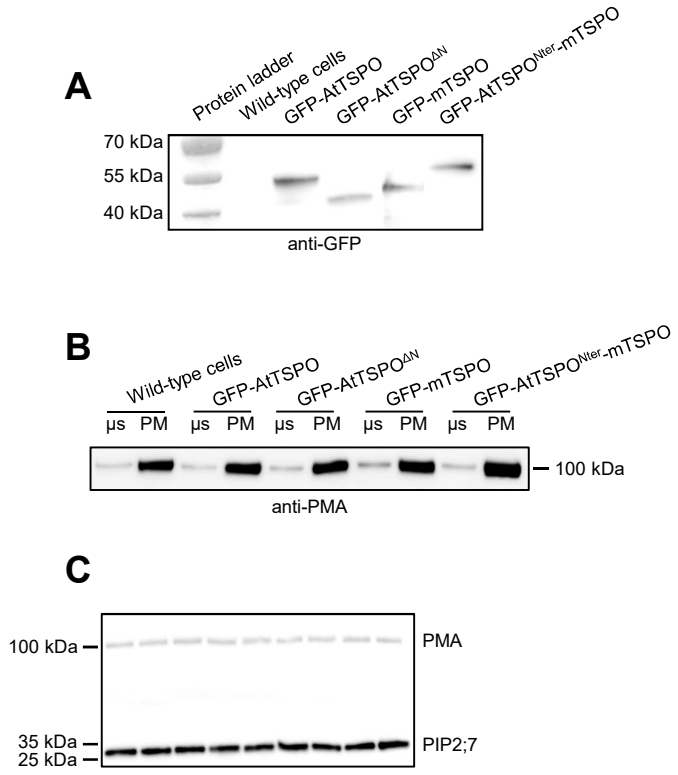


Figure S4. Representative Western blots for PIP2;7 aquaporin signal quantification in the plasma membrane-enriched fractions. Related to Figure 2. (A) Expression of GFP-TSPOs fusions in Arabidopsis PSB-D suspension cells. Each signal was exposed separately followed by superimposition on the protein ladder. (B) Preparation of plasma membrane-enriched fractions (PM) from Arabidopsis suspension cells. The plant plasma membrane H(+)-ATPase (PMA) was probed to check for relative enrichment (5 µg protein in each well); µs, microsomal fraction. (C) Relative amount of PIP2;7 aquaporin was checked by Western blotting. PMA was used as reference protein for the signal quantification. Shown is a representative membrane exposition. Both PMA and PIP2;7 were detected by specific antibodies.

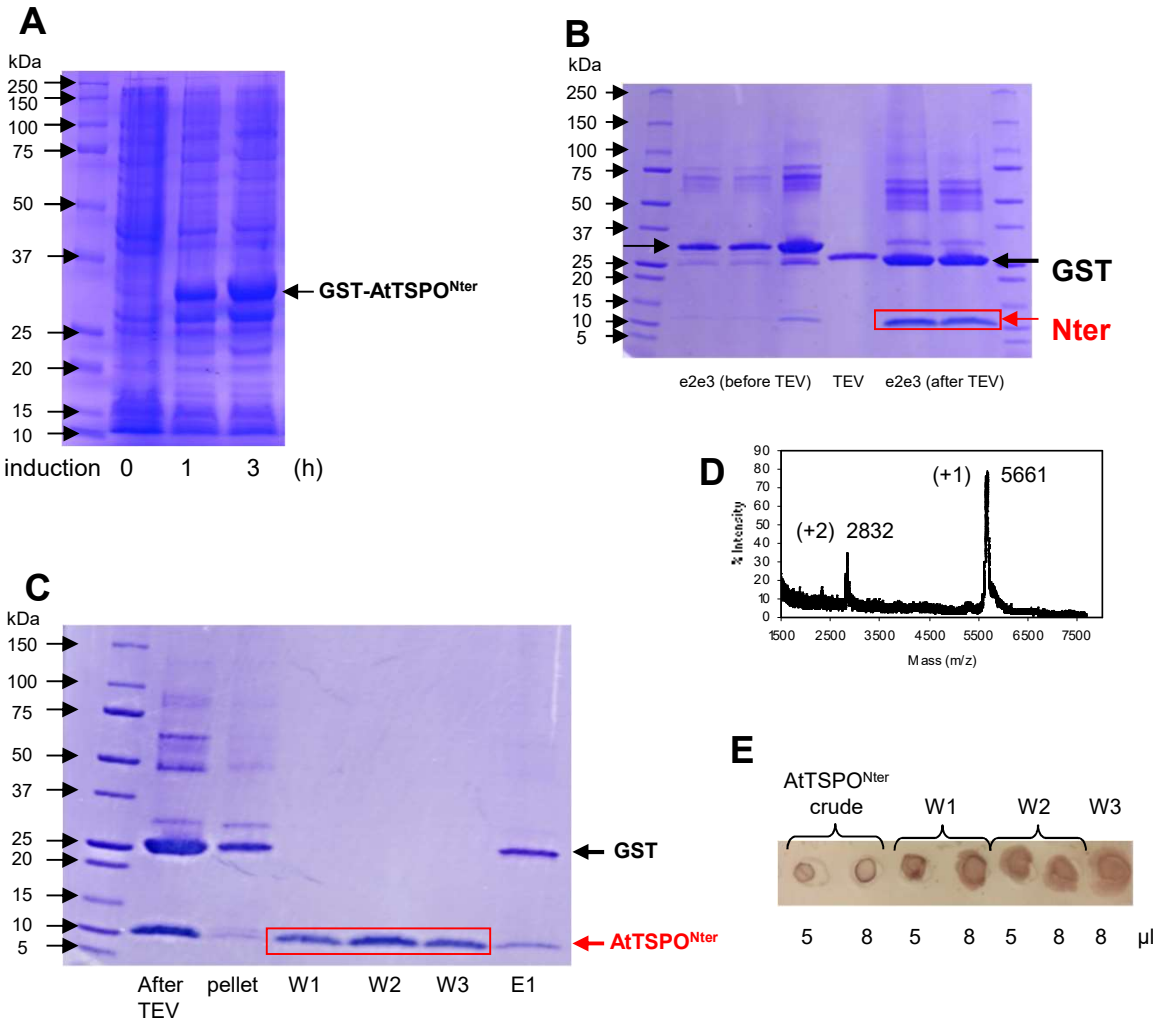


Figure S5. Expression, purification and characterization of the recombinant AtTSPO N-terminal peptide. **Related to Figure 3 and Figure 6.** (A) SDS-PAGE of bacterial culture samples taken along the expression time. (B) SDS-PAGE of elution fraction (e2 and e3) from the first step of bacterial cytosol purification containing fused protein GST-AtTSPO^{Nter} on Ni-NTA affinity column followed by dialysis to remove imidazole and further proteolytic digestion (TEV) to remove fusion protein (GST). (C) SDS-PAGE of the second step of purification on Ni-NTA column to remove His-tagged GST and His-tagged TEV proteins. Digested sample was centrifuged to remove GST precipitated as observed in the pellet. AtTSPO^{Nter} peptide was not retained on the column and collected by washing (W1-W3). GST was removed from the column by imidazole elution. (D) MALDI-TOF mass spectrum of purified AtTSPO Nter showing the expected molecular weight for [^{15}N] labelled peptide. (E) Dot blot of "crude AtTSPO^{Nter}" after proteolytic digestion, before final purification, and samples collected (W1-W3) after Ni-NTA purifcation.

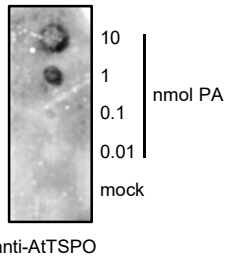


Figure S6. Purified AtTSPO N-terminal peptide binds phosphatidic acid (PA) *in vitro*. Related to Figure 3. Bacterially expressed and purified N-terminal peptide of AtTSPO was incubated with a spotted gradient of PA concentrations and detected with anti-AtTSPO antibodies. Experiment was repeated at least twice.

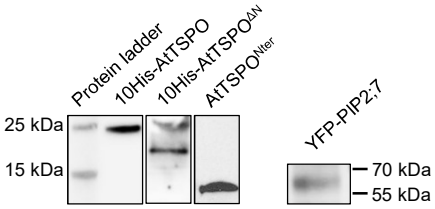


Figure S7. Representative western blot of protein samples used in thermophoresis experiments. Related to Figure 3, Figure 4 and Figure 6. AtTSPO and the truncated mutant were detected by HisProbe (Thermo Fisher Scientific) while the purified AtTSPO N-terminus peptide was detected using anti-AtTSPO antibodies. YFP-PIP2;7 fusion was detected using anti-GFP antibodies.

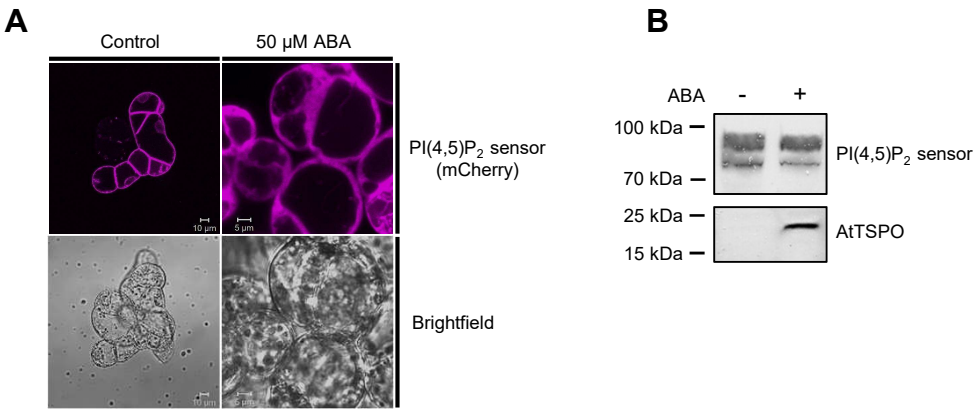


Figure S8. The PI(4,5)P_2 biosensor was depleted from the plasma membrane upon ABA treatment. Related to Figure 5. (A) Arabidopsis PSB-D suspension cells stably expressing the mCherry-tagged PI(4,5)P_2 sensor (double pleckstrin homology domain from phospholipase C) were treated with 50 µM ABA for 24h for endogenous AtTSPO induction. Control cells showed mCherry fluorescence mainly outlining the cell, most likely in the plasma membrane, and some intracellular signals in punctate structures around the nucleus. After ABA treatment, the mCherry fluorescence was essentially detected in the cytosol. Bars = 10 µm and 5 µm for control and treated cells, respectively. (B) Western blotting of ABA-dependent AtTSPO induction in cells imaged in (A). The lipid sensor was detected using anti-mCherry (Abcam ab167453) and served as a loading control. AtTSPO was detected using anti-AtTSPO antibodies.

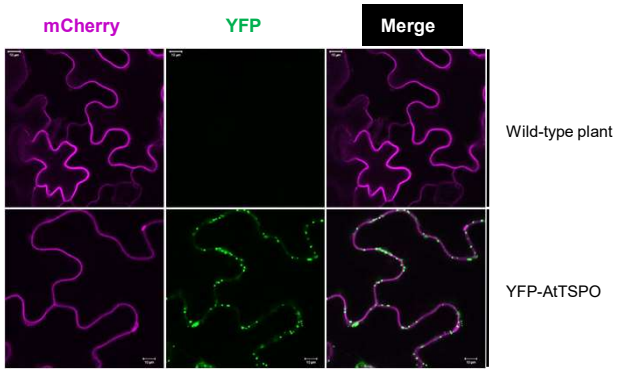


Figure S9. Expression of AtTSPO does not affect the targeting of PI4P 5-kinase to the plasma membrane. **Related to Figure 5.** The phosphatidylinositol 4-phosphate 5-kinase type-1 alpha fused to mcherry (mCherry-HsPIPK1a; in magenta) was transiently expressed in tobacco wild type or stably expressing YFP-AtTSPO (in green). Bars = 10 µm.

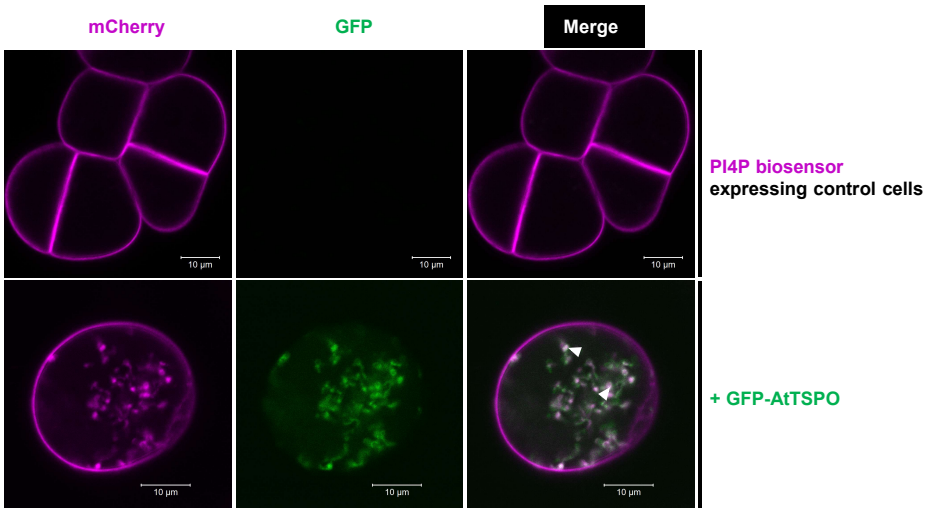


Figure S10. In presence of AtTSPO, the PI4P biosensor was partially depleted from the plasma membrane and partially colocalized with AtTSPO. Related to Figure 5. Representative confocal images of Arabidopsis suspension cells stably coexpressing the PI4P biosensor (mCherry-tagged double pleckstrin homology domain from Fapp1 protein; in magenta) and the full-length AtTSPO (GFP-tagged; in green). The arrowheads indicate mCherry and GFP signals colocalizing likely at Golgi membranes. Bars = 10 µm.

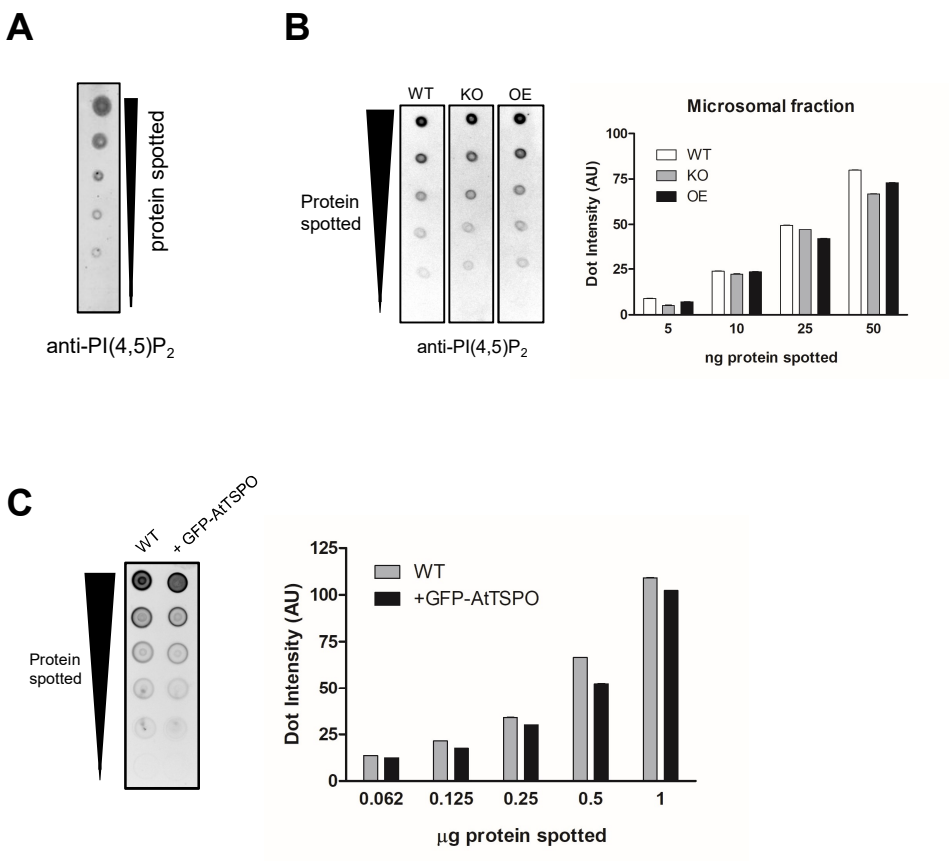


Figure S11. Overexpression of AtTSPO does not impact the overall content of PI(4,5)P_2 lipid in microsomal fraction. Related to Figure 5. (A) SDS-solubilized total extract of Arabidopsis plants was probed for PI(4,5)P_2 to validate the assay and antibodies. (B) SDS-solubilized microsomal fractions from the Arabidopsis wild-type (WT), knock-out for AtTSPO (KO) or AtTSPO overexpressing (OE) plant were spotted on the nitrocellulose membrane and probed for PI(4,5)P_2. (C) SDS-solubilized microsomal fractions from the Arabidopsis wild-type (WT) and GFP-AtTSPO overexpressing suspension cell lines were spotted on the nitrocellulose membrane and probed for PI(4,5)P_2. Average dot intensities were quantified using the ImageJ software and the plots represent a mean of three technical repetitions.

Supplemental Table S1. Primers used in this study. Related to figures 1-7.

Primers used to generate genetic constructs for BiFC analyses

Primer name	Sequence
SpeI-PIP2;7 Fw	5-AAAAAAAACTAGTATGTCGAAAGAAGTGAGCGAAGAAG-3
PIP2;7-XbaI Rv	5-AAAAAAATCTAGATTAATTGGTTGCGTTGCTTCGGAAC-3
AatII-AtTSPO Fw	5-AAAAAGACGTCCATGGATTCTCAGGACATCAG-3
AatII-AtTSPO^{M2} Fw	5-AAAAAGACGTCCATGGCCGAGACAGAGAGGAAAAG-3
AatII-AtTSPO$^{\Delta N}$ Fw	5-AAAAAGACGTCCATGGCGAAACGTGGTCTCAAG-3
AtTSPO-AsiSI Rv	5-AAAAAGCGATCGCTCACGCGACTGCAAGCTTTAC-3
AtTSPO^{K35AR36A} Int Fw	5-GACGACAACAAAGGAGCTGCTGATCAAAAGAGGGCGATG-3
AtTSPO^{K35AR36A} Int Rv	5-CATCGCCCTCTTTTGATCAGCAGCTCCTTTGTTGTCGTC-3
AtTSPO^{K39AR40A} Int Fw	5-GGAAAACGCGATCAAGCTGCTGCGATGGCGAAACGTG-3
AtTSPO^{K39AR40A} Int Rv	5-CACGTTTCGCCATCGCAGCAGCTTGATCGCGTTTTCC-3
AtTSPO$^{K39A/R40A/K44A/R45A}$ Int Fw	5-GAAAACGCGATCAAGCTGCTGCGATGGCGGCTGCTGGTCTCAAGTCTCTG-3
AtTSPO$^{K39A/R40A/K44A/R45A}$ Int Rv	5-CAGAGACTTGAGACCAGCAGCCGCCATCGCAGCAGCTTGATCGCGTTTTC-3

Primers used to generate genetic constructs expressed in Arabidopsis transgenic suspension cells

Primer name	Sequence
USER-kozak-GFP Fw	5-GGCTTAAUCCACCATGAGTAAAGGAGAAGAAC-3
GFP-AtTSPO Int Rv	5-CTGATGTCCTGAGAATCCATTTTGTATAGTTCATCCATG-3
GFP-AtTSPO Int Fw	5-CATGGATGAACTATACAAAATGGATTCTCAGGACATCAG-3
USER-AtTSPO Rv	5-GGTTTAAUTCACGCGACTGCAAGCTTTAC-3
L-GFP Rv	5- ACCAGCTCCAGCACCAGCTCCTTTGTATAGTTCATCCATG-3
L-AtTSPO$^{\Delta N}$ Fw	5-GGAGCTGGTGCTGGAGCTGGTCTGACGGTAGCGGTTGCG-3
GFP-mTSPO Int Rv	5-CACCCAGGATTCAGGCATTTTGTATAGTTCATCCATG-3
GFP-mTSPO Int Fw	5-CATGGATGAACTATACAAAATGCCTGAATCCTGGGTG-3
USER-mTSPO Rv	5-GGTTTAAUTCACTCTGGGAGCCGGGAG-3
USER-kozak-AtTSPO Fw	5-GGCTTAAUCCACCATGGATTCTCAGGACATC -3
AtTSPO^{Nter}-GFP Int Rv	5- GTTCTTCTCCTTTACTCATAGACTTGAGACCACGTTTC -3
AtTSPO^{Nter}-GFP Int Fw	5-GAAACGTGGTCTCAAGTCTATGAGTAAAGGAGAAGAAC-3

Primers used to generate TSPOs variants expressed and purified for biochemical analyses

Primer name	Sequence
XbaI-10His Fw	5-GGGGTCTAGAATGCATCACCATCACCATCACCATCACCATCAC-3
$10His^{C}$-AtTSPO Fw	5-CATCACCATCACCATCACATGGATTCTCAGGACATCAG-3
AtTSPO-XhoI Rv	5-AAAACTCGAGTCACGCGACTGCAAGC-3
XbaI-$10His^{N}$ Fw	5-GGGGTCTAGAATGCATCACCATCACCATC-3
10His-TEV^{N} Fw	5-CATCACCATCACCATCACCATCACCATCACGGTGAAAATTTATAC-3
TEV-$AtTSPO^{\Delta N}$ Fw	5-GGTGAAAATTTATACTTCCAAGGTATGGCGAAACGTGGTCTC-3
$AtTSPO^{\Delta N}$-XhoI Rv	5-GGGGCTCGAGTCACGCGACTGCAAGC-3
BamHI-10His-TEV^{N} Fw	5-GGGGGGATCCCATCACCATCACCATCACCATCACCATCACGAAAATTTATAC-3
TEV-$AtTSPO^{Nter}$ Fw	5-GAAAATTTATACTTCCAAGGTATGGATTCTCAGGACATCAGATAC-3
$AtTSPO^{Nter}$-XhoI Rv	5-GGGGCTCGAGTCAAGACTTGAGACCACGTTTC-3
NdeI-mTSPO Fw	5-AAAAACATATGCCTGAATCCTGGGTG-3
mTSPO-BamHI Rv	5-AAAAGGATCCTCACTCTGGGAGCCG-3
$AtTSPO^{Nter}$-mTSPO Int Fw	5-GAAACGTGGTCTCAAGTCTATGCCTGAATCCTGGGTG-3
$AtTSPO^{Nter}$-mTSPO Int Rv	5-CACCCAGGATTCAGGCATAGACTTGAGACCACGTTTC-3