Early Response to Dehydration 7 Remodels Cell Membrane Lipid Composition During Cold Stress in Arabidopsis


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Plants adjust to unfavorable conditions by altering physiological activities, such as gene expression. Although previous studies have identified multiple stress-induced genes, the function of many genes during the stress responses remains unclear. Expression of ERD7 (EARLY RESPONSE TO DEHYDRATION 7) is induced in response to dehydration. Here, we show that ERD7 plays essential roles in both plant stress responses and development. In Arabidopsis, ERD7 protein accumulated under various stress conditions, including exposure to low temperature. A triple mutant of Arabidopsis lacking ERD7 and two closely related homologs had an embryonic lethal phenotype, whereas a mutant lacking the two homologs and one ERD7 allele had relatively round leaves, indicating that the ERD7 gene family has essential roles in development. Moreover, the importance of the ERD7 family in stress responses was evidenced by the susceptibility of the mutant lines to cold stress. ERD7 protein was found to bind to several, but not all, negatively charged phospholipids and was associated with membranes.

Keywords: Arabidopsis • Cold stress • ERD7 • Membrane lipid composition.

Introduction

Plants have developed several mechanisms to adapt in response to unfavorable growth conditions. Understanding the mechanisms involved in sensing stress signals and triggering adaptive mechanisms are fundamental biological questions to address to improve plant stress resistance. Cold stress is an environmental factor that has a significant impact on crop growth and limits the geographical distribution of many plants (Liu et al. 2019). Low temperature can arrest plant growth and extended exposure to temperatures below 0°C disrupts cellular membranes, leading to cell death. However, most temperate plants can survive mild freezing after a period of exposure to low and non-lethal temperatures (between 12.5 and 4°C) in a process known as cold acclimation, which involves transcription and metabolic changes that increase the levels of intracellular solutes and metabolites. Cold acclimation also requires rapid and dynamic changes in lipid composition, since membrane stabilization is indispensable for survival in freezing conditions (Webb et al. 1994, Uemura et al. 1995, Zheng et al. 2011, Degenkolbe et al. 2012). During cold acclimation, the total amount of structural phospholipids, such as phosphatidylcholine (PC) and phosphatidyethanolamine (PE), increases in the plasma membrane (PM) (Degenkolbe et al. 2012). Galactolipids, including monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), also play an important role during stress acclimation in plants. These two types of galactolipids are both primarily located in thylakoid membranes and chloroplast inner and outer envelope membranes. These lipids allow the insertion of the cold-regulated protein COR15A into thylakoid membranes during cold stress to stabilize the membranes and maintain the optimal efficiency of photosynthesis (Steponkus et al. 1998, Navarro-Retamal et al. 2018).

In addition, lipids can act as signal transducers to drive biological responses with phosphatidic acid (PA) and polyphosphoinositides (PPIs) being important mediators of stress signals (Munnik and Vermeer 2010, Munnik and Nielsen 2011, Heilmann 2016). PA can trigger a rapid biological response that occurs within seconds–minutes of exposure to a broad variety of stresses, including salinity, cold, drought, heat, wounding and pathogen attack (Arisz et al. 2013, Arisz et al. 2018, Tan et al. 2018). Several PA-binding proteins are directly involved in distinct biotic/abiotic stress-regulating plant responses (Testerink and Munnik 2011, Hou et al. 2016, Yao and Xue 2018). For example, PA-activated MPK6 phosphorylates Na⁺/H⁺ antiporter SOS1 under salt stress (Yu et al. 2010). Subcellular
induced ERD7 protein accumulation, but to a lesser extent under stress conditions. Focused on the role of ERD7 in response to cold, we indicated that ERD7 also accumulated at the protein level in response to abiotic stresses. In subsequent experiments, we examined the relationship between ERD7 and lipid composition of cell membranes, as well as the effects that lipid alterations have on the fluidity of cellular membranes in Arabidopsis exposed to cold temperatures.

**Results**

**ERD7 protein accumulates under low temperature**

ERD7 mRNA expression is induced by abiotic stresses (Kiyosue et al. 1994, Taji et al. 1999). Here, we evaluated the amount of ERD7 under abiotic stress with an anti-ERD7 antibody produced using a specific ERD7 peptide as an antigen to probe extracts from wild-type Arabidopsis plants and plants having a T-DNA insertion in ERD7. In extracts from plants exposed to low temperature (4°C for 24 h), Western blotting detected a 58-kDa band in wild type but not the erd7 mutant, indicating that ERD7 protein accumulates in response to cold (Fig. 1A). These results indicated that ERD7 also accumulated at the protein level in response to abiotic stresses. In subsequent experiments, we focused on the role of ERD7 in response to cold stress conditions.

**ERD7 binds to phospholipids in vitro**

ERD7 has 452 amino acids and the C-terminal domain (aa69-427) carries a plant Senescence domain, which is homologous to an early response to dehydration protein domain identified in Arabidopsis. ERD7 has 452 amino acids and the C-terminal domain (aa 258–427) carries a plant Senescence domain, which is homologous to the Senescence domain of the human SPARTIN20 (SPG20) protein that mediates interactions between SPG20 and cardiolipin (Joshi and Bakowska 2011). Based on the high sequence similarity between SPG20 and ERD7, we examined whether ERD7 had lipid-binding activity. To test this, we expressed and purified a fusion protein consisting of a maltose-binding protein (MBP) fused to an ERD7 peptide (aa69–440) containing the Senescence domain (MBP-ERD7) from Escherichia coli (Fig. 2A, Supplementary Fig. S1B). The recombinant MBP-ERD7 protein was used for an in vitro protein–lipid overlay assay using nitrilolipase membrane pre-spotted with phospholipids. As shown in Fig. 2B, MBP-ERD7 interacted with cardiolipin, PA and all species of PPIs tested. These data indicated that ERD7 interacts with several types of negatively charged phospholipids.

Arabidopsis has two closely related homologs of ERD7: AT3g51250 (hereafter ERD7-like1: EDN1) and AT4g35985 (hereafter ERD7-like2: EDN2), which have a pairwise identity of >62% based on the Clustal Omega analysis (EMBL-EBI). According to the GENEVESTIGATOR database (Hruz et al. 2008), the ERD7 gene is highly responsive to abiotic stress conditions, such as drought, cold, osmotic and salt stress (Supplementary Fig. S2), whereas only cold and drought conditions triggered the upregulation of EDN2 gene expression. Meanwhile, EDN1 gene expression is not affected by any environmental stress (Supplementary Fig. S2). We cloned both EDN1 and EDN2 for further analysis. GST-fused forms of full-length ERD7 and EDN1 were produced in E. coli (Supplementary Fig. S1B). Unfortunately, the GST-full-length EDN2 could not be purified in our E. coli expression system. The TAIR database indicates that EDN2 has splice variants and our RT-PCR amplified a splice variant of EDN2, in which the third intron was not spliced out, resulting in coding short form (M1-K384) of EDN2 (EDN2-S, EDN2-S, Supplementary Fig. S1C). In addition to the full-length EDN2, we successfully purified the EDN2-S protein from E. coli (Supplementary Fig. S1B). The anti-ERD7 antibody used above did not recognize GST-EDN1 or GST-EDN2-S (Supplementary Fig. S1D), suggesting no cross-reactivity to EDN1 and EDN2.

A liposome flotation assay was performed to exclude the possibility that lipid association is due to the hydrophobicity of...
the ERD7 and to confirm the binding between ERD7 family and
PA under further physiological conditions. GST-ERD7, GST-
EDN1, and GST-EDN2-S, but not GST, were detected in the
top fraction containing PC:PA (9:1) liposome, but not in the
fraction containing PC-only liposome (Fig. 2C). These results
suggest that ERD7 and homologs can bind to the PA head
group. Furthermore, lipids extracted from Arabidopsis leaves
were blotted for overlay assay with an anti-GST antibody. (D) Lipid overlay assay with plant extract or egg yolk PC.

Fig. 2 The ERD7 Senescence domain binds to several phospholipid spe-
cies. (A) Schematic diagram of ERD7 showing the MBP tag and the
senescence domain enclosed by a gray box (258–427). (B) Lipid overlay
assay with MBP-ERD7 or MBP alone. (C) Liposome flotation assay with
GST-ERD7, GST-EDN1, GST-EDN2-S or GST. PC:PA (9:1) or PC only
liposome was floated in the sucrose gradient. Top (containing lipo-
some), middle and bottom fractions were loaded for Western blotting
with anti-GST antibody. (D) Lipid overlay assay with plant extract or
egg yolk PC.

ERD7 localizes in the vicinity of the cellular membrane

We next investigated the subcellular localization of ERD7 by
isolating subcellular fractions from tissues taken from
Arabidopsis plants exposed to 4°C for 24 h. The identity of
the fractions was validated with anti-D1, anti-MPK6 and anti-
SOS1 antibodies as markers for the chloroplast, cytosol and
microsomal fractions, respectively. Anti-ERD7 recognized a
~58-kDa band only in the microsomal fraction (Fig. 3A). A
band corresponding to a lower molecular weight was detected
in the soluble fraction of both wild-type and erd7 mutant
plants, indicating that this was the background signal
(Supplementary Fig. S3). To further examine the localization
of the ERD7 family in cellular membranes, we constructed plas-
mids expressing ERD7 family proteins fused to a cyan fluores-
cent protein (CFP) to study its localization in mesophyll
protoplast cells isolated from transgenic lines expressing
YFP fused to the ER retention signal. The ERD7 family protein was fused
to CFP. Co-localization between CFP and YFP is calculated based on
Pearson's correlation showed by Coloc2 Plugin from Fiji. Bars: 10 μm.
The figures are representatives for 10–11 biological replicates.

Fig. 3 ERD7 localization based on subcellular fractionation studies and
confocal microscopy. (A) Cell extracts from plants treated with cold for
24 h were fractionated into the chloroplast, soluble (Sol.100) and micro-
somal fractions (P.100) and examined with anti-ERD7 antibody (arrow-
head). The asterisk indicates background. Anti-D1, anti-MPK6 and anti-
SOS1 antibodies were used as markers for chloroplast, cytosol and
microsomal fractions, respectively, and are indicated by arrowheads.
Twenty micrograms of total protein was loaded per lane. (B) Confocal
microscopy analysis using mesophyll protoplasts from plants expressing
YFP fused to the ER retention signal. The ERD7 family protein was fused
to CFP. Co-localization between CFP and YFP is calculated based on
Pearson's correlation showed by Coloc2 Plugin from Fiji. Bars: 10 μm.
The figures are representatives for 10–11 biological replicates.
places, such as PM, and that the ERD7 family does not distribute uniformly to the whole ER membrane. These results supported that ERD7 localized in the vicinity of cellular membranes (Fig. 3B).

**Mutant plants lacking the ERD7 family show developmental defects**

To analyze the biological role of ERD7 in vivo, erd7 mutant plants were isolated in Arabidopsis. The mutant lines showed no observable phenotype under several stress conditions, although Western blotting showed that the mutants lacked the ERD7 protein (Fig. 1). This result may be due to the presence of the two close ERD7 homologs EDN1 and EDN2. To produce higher-order mutants, we crossed the mutant lines (Fig. 4A) and genotyped the progeny by PCR. We identified semi-triple mutant erd7−/− edn2−/− edn1−/− lines (heterozygous for ERD7 and homozygous for two close homologs: hereafter hHH). RT-PCR showed that there were no EDN1 or EDN2 mRNA in hHH (Supplementary Fig. S4A). In their progenies, no triple mutant was found and there were some empty spots in the silique of hHH (Supplementary Fig. S4B), suggesting that the triple mutant is embryonic lethal. In addition, the ratio of hHH was less (47%) than that expected based on the Mendelian rule (67%), suggesting that some hHH could not survive either. Since we could isolate three single mutants and two double mutants (erd7−/− edn2−/− and edn2−/− edn1−/−), the gene dosage of the ERD7 family may affect developmental success. hHH plants were found to have shorter petioles and rounded rosette leaves (Fig. 4B), while leaf mass per unit area was similar between hHH and wild-type plants (WT: 16.9 ± 2.9, hHH: 16.0 ± 2.6 mg cm−2, n = 6), and hHH plants fully developed and produced seeds. When ERD7 was expressed under 3SS promoter, hHH plants had longer leaves compared to hHH plants without the exogenous expression (Supplementary Fig. S5A), although they were shorter than those of the wild type; this is probably because EDN1 or EDN2 were still lacking. Taken together, these results support an essential function for ERD7 family genes during plant development.

**hHH mutants have diminished cold acclimation capacity**

Due to the accumulation of ERD7 protein in response to low temperature, we next examined hHH cold tolerance. We transferred 6-week-old WT and hHH plants to a 4°C chamber for 10 d before evaluating anthocyanin accumulation as a sign of stress and ROS production (Xu et al. 2017). Both WT and hHH plants displayed higher anthocyanin content after 10 d at 4°C relative to plants maintained under normal growth conditions. However, hHH plants accumulated more anthocyanin than cold-treated WT plants (Fig. 5A) while the accumulation of anthocyanin was lower in the hHH plants transformed with 3SS-ERD7 (Supplementary Fig. S5B).

We also evaluated the acclimation capacity of hHH plants by measuring the freezing tolerance of leaves taken from WT and hHH plants with or without pre-incubation at 4°C, by determining LT50 value (a parameter showing at which temperature 50% cell disruption occurs). WT plants had an LT50 of −4.8°C, whereas the LT50 for hHH was −3.4°C, indicating that hHH plants are slightly more sensitive to freezing temperatures than WT plants under basal condition. After an acclimation period, the WT LT50 was −9.9°C, but hHH had an LT50 of only −6.1°C (Fig. 5B), indicating that hHH plants also had considerably diminished cold acclimation capacity. The hHH plants transformed with 3SS-ERD7 were more tolerant to freezing conditions after acclimation than hHH plants and the cold acclimation capacity was partly restored (Supplementary Fig. S5C).

Furthermore, we analyzed the cold-induced gene expression. RNA was extracted from rosette leaves before and after cold treatment (3 and 12 h at 4°C). Although an induction in all analyzed genes was detected in hHH plants, the induction of some genes, including COR15A, CBF3 and CBF2, was lower compared to that in the WT (Fig. 5C). These results indicate that cold-induced gene expression is also affected in the hHH plants.

**hHH plants have a distinct membrane lipid composition**

Based on the connection between the lipid composition of cellular membranes and cold acclimation in plants (Steponkus 1984, Degenkolbe et al. 2012), we carried out a comparative analysis of the lipid composition in hHH and WT plants. Total lipids were extracted from plants grown under normal growth conditions or at 4°C for 24 h and analyzed by mass spectrometry. The results showed that several lipid species were altered in hHH plants compared to wild type (Supplementary Table S1, Supplementary Fig. S6). To explore the correlation between the different lipids, and the different conditions and mutants, we performed a principal components analysis (PCA). The PCA showed separation between the four different experimental situations according to plant genotype and treatments (Fig. 6A, Supplementary Table S2). The distribution between control and cold-treated plants could be explained by increases in the level of several PC and PE species that had a high degree of saturation (e.g. PC(36:6), PE(36:5) and PE(36:4)). Although an induction in all analyzed genes was detected in hHH plants, the induction of some genes, including COR15A, CBF3 and CBF2, was lower compared to that in the WT (Fig. 5C). These results indicate that cold-induced gene expression is also affected in the hHH plants.

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ERD7 affects PPI metabolism

We used in vivo radiolabeling (Mishkind et al. 2009, Arisz et al. 2013) to determine whether defects in the ERD7 family affect the metabolism of PPIs or PA, as they have also been implicated in cold signaling (Ruelland et al. 2002, Delage et al. 2012, Arisz et al. 2013). The amount of PIP2 was reduced by almost 50% in hHH plants compared to WT plants after exposure to cold for 30 min (Fig. 7). Overall PIP content was not significantly different while the response in PA was slightly less in hHH plants. These results indicate that ERD7 mediates not only the content of structural lipids but also the metabolism of PIP2.

Since PIP2 can be cleaved by PI-specific phospholipase C (PLC) to generate IP3 and DAG, of which the latter can be phosphorylated to PA under cold conditions (Ruelland et al. 2002, Delage et al. 2012, Arisz et al. 2013), we measured the expression of PLC isoforms. Earlier work had shown that the expression of PLC1, -3, -4, -5 and -7 were upregulated upon cold treatment (Tasma et al. 2008). We found induced expression for PLC3 and PLC5 under our conditions but found no significant difference between WT and hHH plants (Supplementary Fig. S7).

hHH mutants showed lesser membrane flexibility than WT

Cell membrane properties are related to lipid composition, mobility of lipids and molecular dynamics of the membrane due to protein binding to either of immobilized or fluid membrane domain. We used here Electron Paramagnetic Resonance (EPR) spin labeling to explore whether cell membranes in WT and hHH plants behave differently at diverse temperatures. In EPR, a spin label containing a spin sensitive reporter group (nitroxyl group) bound at a specific carbon position in the stearic acid chain can be introduced into biological systems to detect changes in membrane composition and/or biophysical properties. In this case, it was used to study membrane fluidity in the interior of the hydrophobic core of the lipid bilayer in...
mesophyll protoplasts that were stabilized in mannitol solution to avoid disruption of the cellular membrane by 16DS. Under these conditions, we were able to measure the EPR signal from isolate protoplast cells. We have studied here relative change in the fluidity of PM in wild-type and hHH mutants. A change in membrane fluidity affected the rotational dynamics of the nitroxyl group and, thus, caused a change in the shape and intensity of the EPR spectral line (low field to high field) (Fig. 8A). Consequently, the rotational motion of the nitroxyl group calculated as rotational correlation time ($\tau_R$) is changed (Ježek and Freisleben 1994). The rotational motion of the spin label is a resultant of the exchange of resonance frequencies in different orientations of the nitroxyl probe. Any perturbation in motion of either of the orientations, perpendicular or parallel to membrane plane leads to an anisotropy of the EPR spectrum and change in values of $\tau_{2C}$ and $\tau_{2B}$, respectively.

The comparative analysis of EPR spectra of 16DS in mesophyll protoplasts from hHH mutant and WT at 24°C showed slow rotational motion with a higher $\tau_R$ of approximately 129 ps in hHH mutant than in WT of approximately 75 ps (Fig. 8B). Upon further lowering the temperature to 15, 5 and $-10^\circ$C, WT plants showed a gradual decline in fluidity with an increase in $\tau_R$ to approximately 96, 156 and 189 ps, respectively (Fig. 8B). However, hHH mutant showed a relatively higher stable $\tau_R$ up to 15, 5 and $-10^\circ$C, i.e. 127, 165 and 336 ps, respectively. Furthermore, a similar trend of increase in membrane rigidity was observed with lowering the temperature of measurements represented by increased rotational correlation times $\tau_{2C}$ and $\tau_{2B}$ between hHH mutant and WT protoplasts (Fig. 8C, D). However, the difference between the $\tau_{2C}$ and $\tau_{2B}$ at each corresponding temperature was increased upon lowering the temperature in hHH.
mutant with respect to WT, indicating that the ERD7 family in WT has lowered the activation energy for rotational diffusion. By contrast, no significant difference was observed in similar experiments using thylakoids (Supplementary Fig. S8). These results indicate that PMs of hHH plants are more rigid than WT membranes, and this effect is amplified at lower temperatures.

**Discussion**

ERD7 gene expression is induced in response to several abiotic stresses, such as drought, dehydration, cold, salt and light excess (Kiyosue et al. 1994, Taji et al. 1999, Kimura et al. 2003, Bray 2004, Sánchez et al. 2004, Kaplan et al. 2006). However, the function of ERD7 is unclear. In this study, we aimed to assign a functional role for ERD7 during plant stress. We showed that ERD7 protein accumulates under various stress conditions, particularly following exposure to low temperature (Fig. 1). Attempts to generate a true triple mutant that lacked all three members of the ERD7 gene family were unsuccessful, likely due to embryo lethality. However, we did generate a semi-triple mutant, hHH, having the genotype erd7+/− edn2−/− edn1−/−. Under normal growth conditions, hHH plants had a more compact morphology than WT (Fig. 4), indicating that ERD7 family genes are essential for normal growth and development.

In addition to growth and development, we have observed that ERD7 accumulates under several stress conditions (Fig. 1), suggesting a protective role in response to environmental stresses. Indeed, hHH plants were more susceptible to low temperatures and had reduced their cold acclimation capacity (Fig. 5). These data indicate that the ERD7 family promotes cold and freezing tolerance in plants.
Because ERD7 binds to negatively charged phospholipids, such as PI and PA (Fig. 2), we investigated the connection between ERD7 and lipids. Our protein–lipid assay could not discern specificity between ERD7 and any particular phospholipid species. The Senescence domain of ERD7 has a high pI value (9.79 according to ExPASy Compute pi tool, https://web.expasy.org/compute_pi/) reflecting its positive charge at physiological pH that might facilitate nonspecific electrostatic interactions with negatively charged phospholipids. This characteristic could also explain the localization of ERD7 to the membrane as well as a tight association with the cellular membranes (Fig. 3). The binding of ERD7 to phospholipids could induce structural and mechanical changes in the membrane that affect membrane fluidity. EPR results (Fig. 8) showed that hHH plants have a more rigid PM that could render them vulnerable to mechanical stress and dehydration forces exerted by extracellular ice that forms at freezing temperatures (Steponkus 1984, Thomashow 1999). To obtain these data, we have developed a new protocol that allows the stabilization of mesophyll protoplasts during both EPR-probe incubation and EPR measurements. This approach could be of value for future studies that require analysis of plant cell PMs.

Another possible functional role for the ERD7 family is mediating membrane lipid composition through membrane metabolism and/or trafficking. Some types of changes in lipid composition counteract the loss of membranes’ integrity and reduce the risk of cold or freezing injury (Uemura and Steponkus 1999, Moellering...
The ERD7 gene family plays an important role in both cold expression and development. Since no difference was detected in cold-induced levels of PLC power, ERD7 might mediate the PLC reaction through binding to PIIPs. However, further studies are needed to determine the exact effect of ERD7 on PPI metabolism.

In summary, results in this study show that ERD7 interacts with phospholipids in cellular membranes. This interaction appears to affect lipid trafficking and/or metabolism and cellular membrane fluidity. Taken together, our findings indicated that the ERD7 gene family plays an important role in both cold responses and development.

Materials and Methods

Plant material and phenotypic analyses

The Arabidopsis thaliana lines used in this study were Col-0 wild type, erd7 (AT2G17860) (WISCUFOX452E10), AT3G51250 (Salk_110974) and AT4G35985 (Sail_818_C12). T-DNA insertion lines were obtained from the Arabidopsis Biological Resource Centre (Sessions et al., 2002, Alonso, 2003, Woody et al. 2007). Homozygous insertion lines were selected by PCR following the instructions (http://signal.salk.edu/cgi-bin/tdnaexpress). The following conditions were used: 1 x 95°C for 5 min; 35 x (95°C for 20 s, 55°C for 20 s, 70°C for 1 min) with primers described in Supplementary Table S3. The insertion sites were identified by sequencing of the amplicons. To generate the complementation line, ERD7 cDNA was cloned in pCAMBIA1300 with 35S promoter (pRT105) and transformed with Agrobacterium GV3100. Transformants were screened with hygromycin and PCR for genotyping.

Seeds were frozen for 24 h at −80°C to reduce the likelihood of insect contamination before transfer to soil and stratification at 4°C for 2 d in the dark. Mature plants were grown in soil for 6 weeks under short-day (SD) conditions (120 μmol photons m⁻² s⁻¹, 8 h light/16 h darkness, 22°C). For the evaluation of ERD7 protein content under different stress conditions, Col-0 and erd7 seedlings grew in 1/2 Murashige and Skoog (MS) media plates supplemented with 1% sucrose under long-day conditions (120 μmol photons m⁻² s⁻¹, 16 h light/8 h darkness, 22°C). After 10 d, some of the seedlings were transferred to Petri dishes containing 1/2 MS liquid media + 1% sucrose and 100 mM NaCl or 50 μM ABA for 1 h. For cold treatments, seedlings were incubated at 4°C for 24 h in Petri dishes with 1/2 MS media + 1% sucrose.

Anthocyanin levels were measured according to Loreti et al. (Loreti et al., 2008). For the measurement of leaf mass per area, similar-sized rosette leaves were compared between WT and the mutant. Plant area, length of petiole per leaf, leaf roundness (plant area/total leaf area) and plant diameter were quantified using LeafTools plugin for FIJI software (Malloof et al. 2013).
**ERD7 protein localization**

To determine the subcellular localization of ERD7, full-length ERD7 and homolog cDNAs were amplified from Col-0 cDNA or cDNA in pGEX plasmids using the primer pairs in **Supplementary Table S3**. The ampiclon was cloned into the binary plasmid pm-cyk CD3-1001 (NASC) containing CFP following digestion with XbaI/BamHI. The resulting construct ERD7-CFP was transferred into mesophyll protoplasts isolated from different plants expressing different organellar markers as described in Wu et al. (2009). Fluorescence emission from YFP, CFP and chlorophyll was monitored using an SP2 confocal laser scanning system equipped with an inverted microscope. Confocal images were generated with Zeiss Zen 2012 software version 8.0.0.273 (http://www.zeiss.com). For the co-localization analysis, we made use of the Coloc2 plugin after the removal of images’ background.

**Western blotting**

To detect ERD7 protein, an anti-ERD7 antibody was generated by immunizing rabbits with the synthetic peptide CRTPKKEISHDSSDEEGD that includes amino acids 141–157 of ERD7 as an antigen. The antibody was produced by AgriSera (product number: AS 413457, SE, SE-91121, Västra, Sweden).

Total protein was extracted from 5-week-old plants grown in the SD condition. Tissue was collected and ground in liquid nitrogen. Protein extracts were generated using protein extraction buffer (Tris-HCl 50 mM, pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% Na-Deoxycholate, 2 mM phenylmethylsulfonyl fluoride, 2 mM dithiothreitol).

Isolation of microsomes and chloroplasts was performed according to Abas and Luzschig (2010) and Koskela et al. (2018). Protein content was quantified using the Bradford method (Bradford 1976), and protein analysis was performed as described previously (Barajas-Lopez et al. 2018) using anti-ERD7, anti-MPK6 (Agrisera), anti-D1 (Agrisera) and anti-SOS1 (Quintero et al. 2002) antibodies.

**Freezing damage measured by electrolyte leakage**

WT and hhh plants were grown in the SD condition for 6 weeks. Fully developed leaves from cold-acclimated (4°C during 10 d) or non-acclimated plants were excised at the base of the petiole and placed in 15-mL Falcon tubes containing 1 mL of deionized water. The tubes were placed in a circulating water bath at 2°C and incubated for 30 min to allow temperature equilibration. The temperature was then decreased from 0 to −15°C at a rate of 2°C per hour. At the indicated temperatures, the tubes were removed from the water bath and immediately placed on ice to allow gradual thawing. The contents of each tube were transferred to new tubes containing 25 mL of deionized water, and the conductivity of the combined extract was measured by a sample-centric approach and log10-transformed. Centered and scaled z-scores (z-scores) were subjected to PCA. PCA was performed in the R environment v.3.1.1 (R Development Core Team 2011) using mixOmics v.4.0.2 (Rohart et al. 2017).

**Lipid-binding assay**

A truncated peptide from ERD7 (aa 69–440) that contained the Senescence domain (aa 258–427) was fused to the C-terminus of the MBP epitope. The recombinant protein was purified with amylose resin and used in a Lipid-Binding Assay with Membrane Lipid strip (Echelon Bioscience, 75 Arapeen Dr Ste 302, Salt Lake City, Utah 84108 US) as described previously (Joshi and Bakowska 2011). Liposome flotation assay was performed according to Tronchere and Boal (2017) with PC (Sigma-Aldrich, Feldbergstrasse 80, Darmstadt, 64293, Germany) and PA (Sigma-Aldrich). Centrifuged sucrose gradient (500 µL) was separated into five fractions (100 µL each), followed by Western blotting with anti-GST antibody (Upstate, 3 Trask Lane, Danvers, MA, 01923, US). To produce GST-fused recombinant proteins, cDNAs were amplified by PCR with primers (Supplementary Table S3) and cloned into pGEX-4T1. The proteins were purified as described previously (Barajas-Lopez et al. 2018). For the overlay assay with plant extract, 10 µg of lipids extracted as described above or PC from egg yolk (Sigma-Aldrich) were resolved in chloroform and blotted onto methanol-activated polyvinylidene fluoride membrane. After dried and soaked in methanol, the membrane was blocked with fat-free bovine serum albumin (BSA, Sigma-Aldrich) for the binding assay.

**3P_l-phospholipid labeling, extraction and analysis**

Phospholipid levels were measured as described earlier (Munnik and Zarza 2013). Briefly, leaf discs (5 mm) were excised from 4-week-old Arabidopsis plants grown at SD conditions (16/8 13 light/dark). Leaves were metabolically labeled overnight by flotation on 200 µL of incubation buffer (2.5 mM MES-KOH, pH 5.7, 1 mM KCl) containing 2.5–10 µCi 32P-OH (32P carrier free, 2.5–10 µCi µL−1) in 2-mL safe-lock Eppendorf tubes in continuous light. Treatments were performed by placing tubes on ice water and stopped after 30 min by adding perchloric acid (Munnik and Zarza 2013). Lipids were extracted and analyzed by thin-layer chromatography (TLC) using alkaline and ethyl acetate solvent systems (Munnik and Laxalt 2013, Munnik and Zarza 2013). Radioactivity was visualized by autoradiography, and individual lipids were quantified by phosphomaging (Typhoon FLA 7000, GE Healthcare, 3350 North Ridge Avenue Arlington Heights, IL 60004 US).

**EPR analysis**

To assess cell membrane fluidity, chloroplasts were isolated following a similar protocol described by Koskela et al. (2018). Briefly, fresh Arabidopsis leaves were gently blended in grinding buffer (330 mM sorbitol, 50 mM HEPES-KOH, pH 7.6, 1 mM MgCl2 and 5 mM Na-EDTA, 0.1% BSA, 5 mM ascorbate). The suspension was filtered through two layers of Miracloth that had been presoaked with the grinding buffer. Filters were then centrifuged at 1,000 × g for 5 min and the resulting pellets were carefully resuspended in a small volume of grinding buffer. The resuspended pellet in grinding buffer was gently loaded on the top of 40:70% Percoll gradient and subsequently tubes were centrifuged at 4,000 × g for 10 min. The intact chloroplasts were collected from the interface and washed twice with washing buffer (330 mM sorbitol, 50 mM HEPES-KOH, pH 7.6, 2 mM Na-EDTA). The number of chloroplasts was quantified based on the chlorophyll content measured by spectrophotometry (Porra et al. 1989). Chloroplast thylakoids were obtained after resuspension of the chloroplasts in shock buffer (50 mM HEPES-KOH, pH 7.6, 5 mM sorbitol, 5 mM MgCl2) and two freeze–thaw cycles. Mesophyll protoplast cells were isolated as described above except that the cells were stabilized in stab-buffer (154 mM NaCl, 125 mM, CaCl2, 5 mM KCl, 5 mM glucose, 2 mM MES, pH 5.7, and 400 mM mannitol). Radioactivity was visualized by autoradiography, and individual lipids were quantified by phosphomaging (Typhoon FLA 7000, GE Healthcare, 3350 North Ridge Avenue Arlington Heights, IL 60004 US).
performed at 22, 15, 5 and −10°C on Miniscope (MS5000) EPR spectrometer using a variable temperature accessory (TC-HO4) in a 50-μl capillary. The EPR settings used were a center magnetic field of 336.95 mT with a sweep width of ± 5 mT, a modulation width of 0.1 mT and a microwave power of 3 mW. The final concentration of spin-label used in chloroplasts was 150 μM 16DS for each 50 μg of chlorophyll.

The rotational motion of the nitroxyl group inside the membrane was calculated as rotational correlation time (τr) from EPR spectra using the formula explained earlier (Jezek and Freisleben 1994). Any disorder in spin probe’s motion either in parallel or perpendicular to membrane plane generates an anisotropy of the EPR spectrum, which are manifested in the calculated values of rotational correlation time along the axis (τ28) and perpendicular to the axis (τ2C), respectively. The values of τ28 and τ2C were calculated as explained previously (Strzałka et al. 1995).

quantitative PCR (qPCR)

qPCR was performed following Tasma et al. (2008) using TIP4 like (AT4g32370) as a reference gene. Primers that are not described in Tasma et al. (2008) are shown in Supplementary Table S3.

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Disclosures

The authors declare that there is no conflict of interest.

References


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