Phase separation of LEA proteins throws new light on plant stress response

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Final Report

Plants resist abiotic stresses via complex and multi-component molecular mechanisms, typically due to the emerging diverse stress effects, even under a single primary stress condition. For example, under drought stress, plants have to cope with soil hardness, osmotic stress due to dehydration and oxidative stress due to excessive of reactive oxygen species (ROS) formation (Kovtun et al., 2000). In general, abiotic stresses such as drought, salinity or chilling all lead to the disruption of cellular homeostasis and an increase of ROS in plants (Sharma et al., 2012). Therefore, under almost any other type of stress, plants always encounter oxidative stress to some extent.

Besides producing ROS in plants, mitochondria are also considered as the major sensors of environmental stress (Ashrafi and Schwarz, 2013; Barbour and Turner, 2014). They can signal stress via alteration of their membrane potential, Ca^{+} fluxes, ROS production or ATP level (Barbour and Turner, 2014).

Upon mitochondrial dysfunction several components are released into the cytosol, thus, the removal of oxidatively damaged cell components by autophagy and regulating Ca^{2+} homeostasis may prevent cell death and promote cell survival (Kara et al., 2016; Dickman et al., 2017). However, the molecular relationship between autophagy and cell death in plants is far from being fully understood.

The formation of stress granules (SGs) via liquid-liquid phase separation (LLPS) is another important process that helps plants respond to stress conditions and promote cell survival (Mahboubi and Stochaj, 2017). In general, SGs contain mRNA, RNA binding proteins, components of the translation initiation machinery, proteins with predicted prion-like domains (PrLDs) and disordered proteins (Khong et al., 2018), and their exact composition depends on the stress condition (Chen and Liu, 2017; Mahboubi and Stochaj, 2017).

Early Response to Dehydration 14 (ERD14) is a dehydrin - a subgroup of the Late Embryogenesis Abundant (LEA) proteins (Battaglia et al., 2008), which are thought to be involved in dehydration protection (Hughes et al., 2013), and overexpression of several dehydrins enhanced stress tolerance in transgenic plants (Figueras et al., 2004; Shekhawat et al., 2011; Kim et al., 2013). During plant growth and under physiological conditions, the expression level of ERD14 is low, but it is highly upregulated when the plant is exposed to abiotic stresses like cold and salt or abscisic acid (ABA) (Alsheikh et al., 2005; Kiyosue et al., 1994, Nylander et al. 2001). ERD14 is an intrinsically disordered protein (IDP), which possess chaperone-like activity, membrane binding and protection of proteins against aggregation and activity loss (Kovacs et al., 2008).

In the current work our main aim was to characterize the liquid-liquid phase separation (LLPS) capabilities of the plant dehydrin ERD14 and its relevance in the stress tolerance of plants. We also investigated the molecular mechanisms through which ERD14 may exert its protective effect.

To evaluate the physiological function of ERD14 associated with stress response, we created an ERD14 overexpressing (OE) A. thaliana line by expressing ERD14 fused with cyan fluorescent protein (CFP) under the control of the CaMV 35S promoter. Attempts to create transgenic plants overexpressing only ERD14 under the control of 35S promoter have failed.

We compared the expression level of ERD14 of wildtype (WT) and mutant lines (ERD14 OE and KO) at different developmental stages compared on both protein and mRNA level and we could observe a slight decrease in the level of endogenous ERD14 in the mature plants with a high level of transgenic ERD14, while the level of ERD10, an ERD14 homologue, was similar in all 3 lines.

After this, we determined the effect of ERD14 expression on dehydration tolerance. All the plants showed signs of dehydration when the field capacity decreased to 32 ± 2 %. The leaves became thin, curled and lost their turgidity. Older leaves were the most susceptible to drought. The young leaves of the ERD14 KO displayed dehydration signs, which was not the case for the young leaves of WT and ERD14 OE plants. However, all the plants were able to recover from a field capacity of 32

± 2 % when water became available again. If dehydration lasted longer, ERD14 KO plants died first, when the field capacity dropped to 19 ± 1 % (Figure 2), and only the ERD14 OE plants were able to recover upon rehydration. The WT plants still showed some green parts but did not recover after rewatering (Figure 1).

Stress causes earlier flowering of the plant and can affect the quality and viability of the seeds produced during the stress, therefore the time before flower development can be a measure of the sensitivity of plants to stress. Based on this, we determined the flowering time of WT, ERD14 OE and ERD14 KO lines during drought treatment in the greenhouse as well as under osmotic stress in vitro. In the greenhouse, more ERD14 KO plants produced flowers than the WT and ERD14 OE lines after a seven-day drought period. The percentage of flowering plants were similar in ERD14 OE and WT. All lines displayed flowers after 12 days of drought. The same response was also observed in plants subjected to osmotic stress upon which ERD14 KO showed significantly early flowering time. The postponed flowering during osmotic stress of the ERD14 OE plants compared to the ERD14 KO plants in two completely independent experiments suggested that ERD14 OE plants are less sensitive to osmotic stress, suggesting a protection by ERD14 during drought stress.

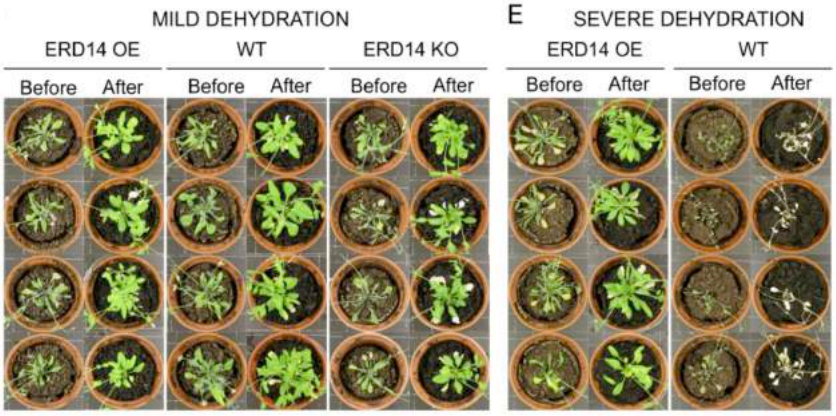

Figure 1. ERD14 overexpression improves plant tolerance to water loss condition.
Effects of ERD14 on mild dehydration tolerance. Mature plants were subjected to drought until the field capacity dropped to 32 ± 2 % then the plants were watered and left for four days to recover.

Oxidative stress is unavoidable, due to an increase of reactive oxygen species (ROS) as one major consequence of drought. Besides, H_2O_2 elevation is also a signal for ABA-dependent stomatal closure, an important response to water loss. For these reasons, when studying the molecular functions of ERD14 in stress response, first we focused on its role in tackling ROS, specifically H_2O_2, or oxidative response in general. ERD14 OE plants have the lowest H_2O_2 content under osmotic stress, but ED14 showed no ROS scavenging ability.

We could show a lower level of H_2O_2 accumulation in ERD14 OE plants than in either WT or ERD14 KO, under osmotic stress for 3 weeks (Figure 2).

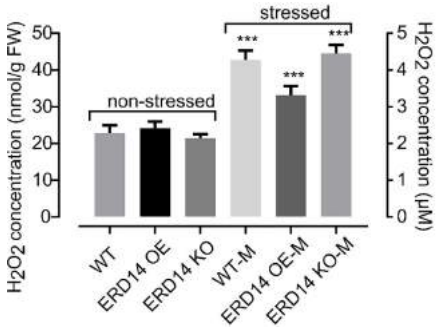

Figure 2. Hydrogen peroxide accumulates in plants under osmotic stress. The bar chart shows the H_2O_2 concentration (nmol/g FW or µM) in extracts obtained from WT, ERD14 KO and OE plants kept without stress or

exposed to mannitol-induced stress. FW refers to fresh weight of plants. The concentrations obtained spectrophotometrically at 390 nm with potassium-iodide show that plants subjected to 180 mM mannitol for 3 weeks accumulate higher levels of H_2O_2 compared to non-stressed plants. All data represent the mean +/− standard deviation of three replicates. Statistical analysis was performed by one-way ANOVA with multiple comparisons, which revealed significant differences between different group of samples (stressed and non-stressed plants; stressed WT and stressed ERD14 OE; stressed WT and stressed ERD14 KO, stressed ERD14 KO and stressed ERD14 OE), indicated in the figure by asterisk *** $p < 0.0001$.

We could also show that as a chaperone, ERD14 can activate redox enzymes such as GSTF9 and catalase, and protect them from oxidation by H_2O_2 (Nguyen et al., 2020). We performed an *in vitro* ferrous oxidation-xylenol orange (FOX) assay to check whether ERD14 can have a direct effect on H_2O_2 removal. The result showed no change in H_2O_2 levels when H_2O_2 was incubated with ERD14 for different times. In accord, direct ROS scavenging is not a function of ERD14.

Due to the basic effect of ERD14 on plant survival and water retention, but lack of a direct scavenging effect on H_2O_2, we turned to elucidating the cellular localization and possible function of ERD14 under dehydration conditions. Previously, we have shown that ERD14 interacts with catalase (KD = 126 nM) and also observed that in ERD14 OE there is less accumulation of H_2O_2 than in WT and KO plants under osmotic stress (Nguyen et al., 2020). On the other hand, our first observation on ERD14 OE leaves under cold, mannitol and H_2O_2 treatments was that similar ERD14 punctate structures form. Although these structures display similar behaviour, their numbers increase differently depending on the stress condition. Due to the influence of ERD14 on H_2O_2 level and ROS production, we turned to check the relation of ERD14 and other organelles as sources of ROS or sites of ROS detoxification. There are several organelles in the cell that can generate ROS such as mitochondria, chloroplasts, peroxisomes, etc. For this reason, we looked for the subcellular localization of ERD14 in the plant cells to find the potential target site of ERD14 activity with regards to H_2O_2-producing or detoxifying organelles.

Co-expressing ERD14-CFP with several specific organellar markers under oxidative conditions of 10 mM H_2O_2 were performed in Arabidopsis hybrid seedlings. Peroxisomes were our first choice to investigate as they generate more ROS, especially H_2O_2 than chloroplasts and mitochondria. They are also a detoxification site of cytosolic H_2O_2 due to containing the majority of cellular catalase. Two further organelles that take part in ROS production, mitochondria and chloroplasts were also investigated.

Live-cell imaging of hybrid plants under oxidative conditions did not show any colocalization of ERD14-CFP with several organellar markers, such as RBCS-YFP (chloroplast), PTS1-YFP (peroxisomes), ARA7-YFP (late endosomes), and VTI12-YFP (Trans-Golgi). However, we found that ERD14-CFP co-localizes with COX4-YFP, a mitochondrial marker during 10mM H_2O_2 treatment. Microscopy showed more co-localized structures of ERD14-CFP and COX4-YFP under oxidative stress conditions than under non-stress conditions.

To provide further evidence for the co-localization of ERD14 with mitochondria under stress conditions, we isolated mitochondria from WT plants under both mannitol stress and non-stress conditions. Western-blotting with an anti-ERD14 antibody showed the presence of ERD14 in the mitochondrial extract in the stressed sample but its absence in non-stressed plants. Therefore, we concluded that ERD14 only translocates to mitochondria under stress conditions (Figure 3).

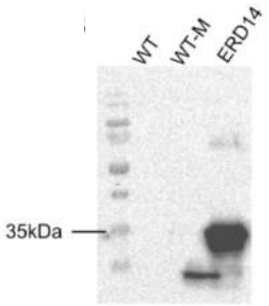

Figure 3. ERD14 is present in mitochondria during stress. ERD14 in a mitochondrial extract of WT plants subjected to mannitol stress. Mitochondria were isolated from WT plants subjected to no stress (WT) and mannitol stress (WT-M). ERD14 was visualized by WB with a specific anti-ERD14 antibody.

Interestingly, the MW of ERD14 in the WT-M sample shifts to lower MW (21 kDa) instead of 35 kDa in the native ERD14 sample, suggesting a proteolysis which may result from the ubiquitination of ERD14, due to its interaction with mitochondria upon stress.

To investigate the targets of ERD14 in direct interaction studies, pull-down experiments were performed to collect the protein interactome of ERD14 in WT plants subjected to an overnight, 180 mM mannitol treatment. All possible binding partners were crosslinked by dithiobis-(succinimidyl propionate) (DSP) crosslinker, and pulled down by a specific anti-ERD14 antibody. The interactome was then sent for MS analysis.

Gene ontology analysis showed most of the interacting proteins associated with chloroplasts, vacuole and mitochondria. Interestingly, mitochondrial outer-membrane proteins were enriched in the interactome more than 40 times compared to their abundance in the non-stressed *Arabidopsis* proteome. These results, in combination with previous observations that ERD14 is able to bind to membranes through peripheral electrostatic interactions with phospholipid head groups (Kovacs et al., 2008), adds significantly to our hypothesis that ERD14 colocalizes with mitochondria upon stress in the cell. Although the interactome also contains many chloroplast proteins, and chloroplasts are also one of main sources of ROS production, there was no colocalization of ERD14 and chloroplast observed under quick oxidative stress (10 min.), indicating no influence of ERD14 on ROS production in chloroplast during short-term stress treatment. Therefore, mitochondria are the most reasonable targets of ERD14 in oxidative stress response.

Under stress, the decrease of mitochondrial membrane potential and the release of cyt c strongly affect cellular homeostasis and are important signals for apoptosis. As SG formation is an essential regulatory activity in cells during stress, which minimizes the stress damage and promotes cell survival, we first investigated the influence of ERD14 on liquid-liquid phase separation (LLPS), a main process driving SG formation. Since transcriptional arrest due to stress can induce SG formation, and mRNA being the core component of SGs, we also investigated whether the presence of RNA is critical for ERD14 phase separation.

Binding assays by biolayer interferometry (BLI) showed an interaction between cyt c and ERD14 with a dissociation constant KD = 47μM. In a similar experiment, ERD14 and the mRNA mimic poly(U), also showed a strong interaction with a KD = 20nM, in the presence of 10mM $CaCl_2$. In a turbidity test at 600nm, when we slightly decreased pH of ERD14 in the presence of poly(U) and the molecular crowder 8% PEG, ERD14 showed ability to phase separate. As the pH dropped, ERD14 quickly nucleated into liquid droplets which remained in gel-likestructure after 60 min. Cyt c promotes LLPS, causing more droplet formation. In both cases, 5mM $CaCl_2$ speeded up the LLPS of ERD14. Evaluating the size of droplets by dynamic light scattering (DLS) showed results consistent with the turbidity test and microscopy imaging; for cyt c alone, there were no droplets observed in the same condition. ERD14 droplets are highly dynamic, as demonstrated by fluorescence recovery after photobleaching (FRAP), with a fluorescence recovery value of about 60% in about 100s. These results indicated that ERD14 can phase separate, and pH decrease, poly(U), cyt c, and crowded conditions all promote its LLPS. Moreover, Ca^{+} could also be an important factor, the importance of which may derive from the fact that a change in cytosolic $Ca2^{+}$ also leads to the cytosolic acidification upon stress.

All these observations suggest that mitochondrial disfunction may give multiple signals for ERD14 phase separation, which then potentially drives SG formation. The colocalization of ERD14-CFP in the hybrid plants with PABP8-RFP, a SG marker, confirmed this possibility (Figure 4).

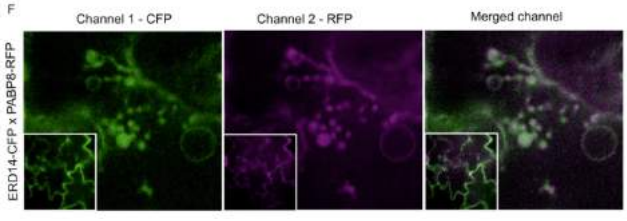

Figure 4. Phase separation of ERD14
ERD14 colocalizes with stress granules under oxidative stress. Leaves of hybrid plants were visualized by Spinning-disc microscopy. Channel 1: CFP-ERD14, Channel 2: RFP-PABP8 (stress-granule marker).

Under stress conditions, high level of ROS can damage the mitochondria, which are then eliminated via autophagy. To find out if there is any relation between ERD14 and autophagy, live cell imaging was performed with the hybrid line containing ERD14-CFP and ATG8-RFP, an autophagosome marker. Similar colocalization of ERD14 with autophagosomes was observed as with mitochondria and SGs under the same conditions. These results indicate a possible signaling connection between stressed mitochondria, SGs and autophagosomes under oxidative stress.

To gain a better insight into this connection, the effects of ERD14 on autophagic components were investigated. We observed a higher level of ATG8 in WT and ERD14 OE plants treated with 180 mM mannitol. By a BLI assay, ERD14 was showed to interact with ATG8 weakly, with a dissociation constant of about 1.1 mM; this is in the range observed in previous studies with other ATG8/LC3 partners containing LC3 interacting region (LIR). This motif plays a crucial role in targeting autophagic receptors to LC3/ATG8 on the phagophores. *In silico* identification of a potential functional LIR motif on ERD14 sequence showed such interacting region between residues 29 and 34. When deleting the Ka segment which contains this motif, the binding affinity of ERD14 mutant (ΔKa) largely decreased. As this LIR motif is quite conserved in other dehydrins, it indicates a possible role of dehydrins in autophagy. ERD14 also contains a short linear motif (SLiM) – WXXL motif – between residues 31 and 34. Both these motifs are crucial for selective autophagy.

SGs can be targeted to the autophagy machinery which help improve cell survival; however, over-activity of autophagy due to increased Ca^{2+} levels can lead to vacuolar disruption and subsequent cell death in plants. In our study, the presence of ERD14 in plants increases cell survival under stress via high autophagic activity but without induced cell death. We have also observed that the expression of ERD14 on an ERD14 KO background (knockout rescued line – ERD14 KOR) can even recover plant resistance upon drought (Figure 5). Besides, staining roots of plants under oxidative conditions by fluorescein diacetate, which reacts with only viable cells, also showed more viable cells in the root tips of stressed ERD14 OE plants than other lines. These results indicate role of ERD14 in regulating homeostasis, especially cyt c and Ca^{2+} during autophagy process.

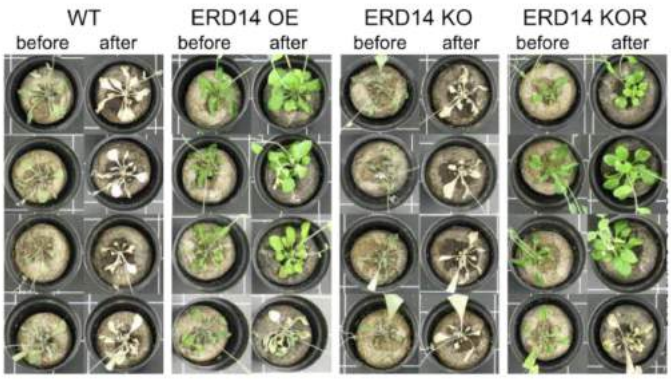

Figure 6. Survival of plants under drought conditions. The experiment was stopped when the field capacity reduced below 15%.

Drought-triggered dehydration causes a dynamically changing physiological behaviour of plants. One of the first reactions is to prevent fast water loss via regulating their stomatal transpiration. The fastest water loss observed in the in vitro leaves that lack ERD14 suggests that ERD14 can prevent fast water loss and help plants to overcome a stress. Importantly, we found ERD14 localization in guard cells, which suggests a possible role of ERD14 in controlling stoma movement.

Besides, the enhancement of ERD14 phase separation in the presence of Ca^{2+} and cyt c, which appear as the consequence of high ROS levels, propose a regulatory role of ERD14 in autophagy. Although autophagy in plants is critical for cell death, balancing homeostasis by autophagy contributes to cell survival. Our study showed a high autophagy activity in the presence of ERD14 but also a higher cell survival in ERD14 OE plants, which propose that ERD14 influences stress tolerance via autophagy regulation. To adapt to unfavourable conditions, plants develop a stress-response network controlling physiological behaviour and expression levels of implicated genes. The expression pattern of these stress-inducible genes is not uniform and is not always overlapping. Our

research provides evidence that ERD14 plays an essential role in plant response to dehydration and an increase in ERD14 levels can lead to increased tolerance of plants. The protective function of ERD14 is multy-layered and specific. In general, ERD14 appears to assist the removal of dysfunctional mitochondria that would increase the toxic accumulation of H_2O_2, cyt c and excess Ca^{2+}, and thus helps to avoid cell death, as outlined in Figure 6.

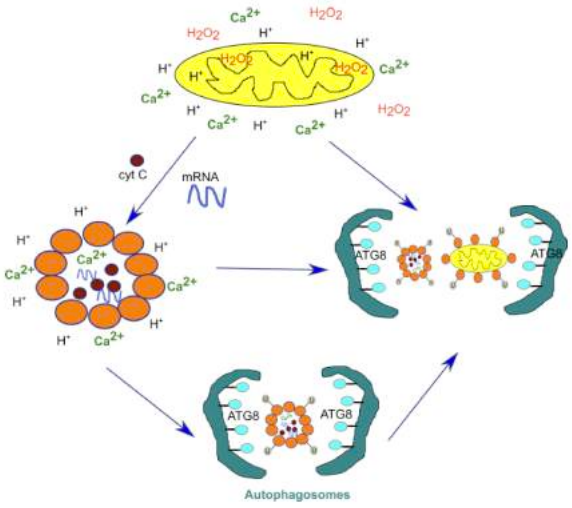

Figure 6. Proposed mechanism of ERD14 in mitigating stress effect by regulating cell homeostasis via autophagy machinery. Possible processes by which ERD14 eliminates toxic components and dysfunctional mitochondria under oxidative stress are shown. LLPS of ERD14 recruits cyt c and mRNA under low pH conditions. This process is induced by the presence of calcium ions which level increases in the cytosol as signalling of stress. Moreover, ERD14 can bind to the membrane of dysfunctional mitochondria, be ubiquitinated and trigger autophagy machinery. In both cases, ERD14 plays as a receptor for autophagosome recognition.

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Publications directly related to the grant

Murvai, N.; Kalmar, L.; Szabo, B.; Schad, E.; Micsonai, A.; Kardos, J.; Buday, L.; Han, K.-H.; Tompa, P.; Tantos, A. (2021) **Cellular chaperone function of intrinsically disordered dehydrin erd14.** INTERNATIONAL JOURNAL OF MOLECULAR SCIENCES 22: 12 Paper: 6190 (2021)
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