Functional Analysis of Group 3 LEA Proteins

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Abstract

LEA-3 proteins belong to the late embryogenesis abundant (LEA) protein family. Although they have been strongly associated with desiccation tolerance, their physiological and biochemical mechanism remains unknown. To search for their role, we performed a functional analysis of Group 3 LEA proteins. The metal binding properties of LEA3-4 proteins were investigated. Analysis and results reveal that LEA-3 proteins can bind to certain metal ions. This suggests that LEA proteins can reduce the production of reactive oxygen species and reduce oxidative stress by binding to these metal ions.

Keywords: LEA-3 proteins, desiccation tolerance, sequence motifs

Identification and Analysis of LEA-3 Protein Motifs

Late Embryogenesis Abundant (LEA) proteins are proteins that accumulate in the late development stage of plant seeds. They are widely assumed to play a crucial role in cellular dehydration tolerance when plants enter a dry state or desiccation stage (Hinchka and Thalhammer, 2012). They were first characterized in cotton seeds but have also been found to protect other non-plant species such as cyanobacteria, brine shrimp and nematodes from abiotic stress (Hand et al., 2012). To prevent desiccation damage, LEA proteins protect other proteins from aggregation, which happens during dehydration (drought, low temperatures or high levels of salinity) (Goyal et al., 2005). Despite many studies associating this correlation, the mechanism behind LEA protein's biochemical function remains very unclear.

In terms of structure, LEA proteins are highly hydrophilic and belong to the structural group called intrinsically disordered proteins (IDP) (Hinchka and Thalhammer, 2012). IDPs are proteins that have no stable or fixed tertiary structure. Despite not having this defined structure, IDPs tend to be dynamic when bounded to different ligands, which could imply various important functions (Xue and Uversky, 2016).

LEA proteins have been grouped into various subgroups or families based on their sequence similarity. In *Arabidopsis thaliana*, Hundertmark and Hincha classified LEA proteins into nine families which includes dehydrins. Another subgroup, LEA-3 proteins, are more hydrophobic than the average LEA protein and are predicted to be targeted in mitochondria or chloroplast (Hundertmark and Hincha, 2012). Salleh et. al hypothesized that these proteins interact with other proteins that are involved in mitochondrial reactive oxygen species (ROS) signalling, which induces biotic responses in root development and other pathogenic reactions.

There is still no novel function defined for this protein and remains under-researched compared to other LEA proteins. We therefore set out to perform a functional analysis to determine the mechanism behind the function of LEA-3 proteins and to find its role in dehydration tolerance.

Materials and Methods

Expression of LEA-3 Protein

Our gene of interest, LEA3-4 gene, was inserted into a plasmid. Another gene, pET-SUMO, was also inserted for solubility purposes. Two versions of recombinant plasmid were created: one with full-length LEA3-4 gene and one with truncated LEA3-4 gene. Full-length LEA3-4 comprises the whole sequence of the protein without its N-terminus or C-terminus removed. On the other hand, truncated LEA3-4 gene has its N-terminus eliminated. Recombinant plasmids were transformed into the bacteria, *E.coli* BL21 (DE3). These bacterial cultures were grown in LB (Luria–Bertani) medium with kanamycin antibiotic at 37°C. Next, these samples containing the bacterial culture were treated with isopropyl-B-D-thiogalactopyranoside (IPTG) to induce the expression of SUMO-tagged LEA3-4 proteins.

Sumo-LEA Protein Purification

After the expression of both full-length and truncated SUMO-tagged LEA3-4 protein, our samples were lysed using a sonication equipment to solubilize proteins from cell contents. Next, fast protein liquid chromatography (FPLC) was used to purify SUMO-LEA3-4 proteins. In order to cleave SUMO from LEA3-4, the protease, ULP1, was added to the sample. Then, FPLC and High Performance Liquid Chromatography (HPLC) were performed to fully isolate LEA3-4 from SUMO and other unwanted contents.

Immobilized metal ion affinity chromatography (IMAC)

As expected, the isolated LEA3-4 protein was used as the main protein of interest while Bovine Serum Albumin (BSA) protein acted as the positive control for this experiment. 8 physiologically important metal ions were used as metal ions of interest: calcium (Ca^{2+}) , cobalt (Co^{2+}), copper (Cu^{2+}), iron (Fe^{2+}, Fe^{3+}), magnesium (Mg^{2+}), nickel (Ni^{2+}) and zinc (Zn^{2+}) ions

Firstly, a metal ion of interest, followed by the protein of interest, was loaded to the column. Next, equilibiration buffer (EQ buffer) was run to elute proteins that did not bind to the column and therefore, did not directly bind to the metal ion of interest. Lastly, EDTA buffer was run to elute proteins that did bind to the metal ion of interest. This cycle was repeated for each metal ion.

SDS-Page Gel analysis

The SDS-PAGE gel used for analysis was run in gel electrophoresis at 75 V for 15 minutes and 155 V for 1 hour. After gel electrophoresis, the gel was stained by Coomassie Brilliant R-250 for 30 minutes followed by destaining overnight.

Results

After the expression and purification of protein, the truncated LEA-3 protein was successfully isolated, which is shown in the SDS-page gel in Figure 2. This proves that LEA3-4 is soluble after bacterial cell lysis and purification. Successful purification of the protein led to the execution of metal affinity chromatography. The results of metal affinity chromatography, displayed in Figure 3 and Figure, indicates that truncated LEA3-4 binds to certain metal ions such as Fe^{2+}, Fe^{3+} and Cu^{2+}ions. However, LEA3-4 did not bind to Ca^{2+}, Co^{2+}, Mg^{2+}, Ni^{2+} and

Zn^{2+} ions. Compared to the positive control, BSA, truncated LEA3-4 also binds to iron and copper ions but does not bind to nickel and zinc ions.

On the other hand, full-length LEA3-4 protein was insoluble after numerous attempts of purification. Attempts made differed by lengths of incubation period (overnight and 3 hours) and by different temperatures during incubation (16°C and 37°C). However, LEA-3 protein was not detected in SDS-page gel (not shown) after running bacterial cell lysis and FPLC. Another attempt to solubilize LEA3-protein was to denature the cell contents by urea. However, this attempt was also unsuccessful. Therefore, full length LEA-3 proteins are insoluble *in vitro*. As a consequence, we are unable to purify protein and unable to perform IMAC chromatography for full-length LEA3-4 protein. A summary of results is shown in Table 1.

Discussion

Metal-binding properties of LEA3-4 proteins

Abiotic stresses such as drought, low temperatures and high levels of salinity affect the metabolic and physiological activities of plant cells. Ion toxicity is one of the significant consequences that can occur under such extreme conditions (Hara et al., 2004). Transition metals, such as iron and copper ions, can participate in the generation of reactive oxygen species such as hydroxyl radicals (Hara et al., 2004). The increase of concentration in such metals can cause oxidative stress in plants (Hara et al., 2004). For example, the Fe^{3+} ion is involved in various physiological functions such as nitrogen fixation, photosynthesis and respiration in plants. However, during desiccation, Fe^{3+} metal ions can be involved in Fenton reactions and catalyze the production of hydroxyl radicals and other reactive oxygen species, which could result to metabolic dysfunction and eventually, cell death (Becana et al. 1998).

Other subgroups such as dehydrins and LEA4 are also reported to have metal binding properties (Tunnacliffe and Wise 2007). For instance, Group 2 LEA proteins (dehydrins) in Arabidopsis (RAB18) could bind to nickel and copper ions. Additionally, LEA4 proteins in soybean (*Glycine max* L.) such as GmPM1 and GmPM9 could bind to iron, nickel, copper and zinc ions (Liu et al., 2011). Becana et al. reported that metal-binding proteins could reduce the generation of reaction oxygen species by binding to such metal ions. Therefore, LEA3-4 proteins binding to specific metal ions suggests that they have an antioxidant role in desiccation tolerance.

Moreover, histidine residues can be involved in the metal binding of LEA proteins. For example, dehydrins in castor bean and soybean could be eluted with a histidine analog (imidazole) in copper-charged columns (Kruger et al. 2002, Herzer et al. 2003), which suggests that histidine residues are involved in the metal binding properties of LEA2 proteins (Tunnacliffe and Wise 2007). Additionally, Liu et al. speculates that LEA4 proteins has metal binding properties due to its higher proportion of histidine residues. Therefore, it can be also be suggested that the histidine residue in LEA3-4, shown in Figure 1, could be involved in metal binding.

Insolubility of full-Length 3-4 protein

Another important finding to discuss is the insolubility of full-length LEA3-4 *in vitro*. Prior to this functional analysis, a structural analysis of LEA3 proteins has also been performed, which identified and analyzed the sequence motifs of this protein. In this study, four LEA3 protein motifs was proposed, shown in Figure 6. One of the motifs, MARS-motif is predicted to be located at the N-terminus, which could act as a localization signal peptide. As previously

mentioned, Hundertmark and Hincha suggested that LEA3 proteins are localized in the mitochondria or chloroplast. This suggests that full-length LEA3-4 proteins could be imported in these subcompartments making it insoluble during bacterial cell lysis.

Future Direction

A possible further step to pursue for this study is the use of isothermal titration calorimetry (ITC). ITC can be used to quantitatively estimate the binding affinity between LEA3 protein and metal ions. An analysis of circular dichroism can also be used to investigate the effect of metal ions on the secondary structure of LEA3-4 protein and if an interaction with membranes is possible using SDS-micelles, which mimics *in vivo* conditions. Lactate dehydrogenase (LDH) assay could also be used to detect if LEA-3 proteins can protect LDH from aggregation.

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Table 1

Results of immobilized metal binding chromatography (IMAC)

Metal ion	**BSA (+)**	**Truncated**	**Full Length**
Fe^{2+}	+	+	Insoluble
Ni^{2+}	+	-	Insoluble
Cu^{2+}	+	+	Insoluble
Zn^{2+}	+	-	Insoluble
Ca^{2+}	-	-	Insoluble
Mg^{2+}	-	-	Insoluble
Co^{2+}	-	-	Insoluble
Fe^{3+}	+	+	Insoluble

Full-Length LEA3-4:

MSQSLFNLKSLSRSINNTIRMRRYIVITKASQRAYTIGSSQEKPSWASDPDTGYFRPET
AAKELDPYIAKTSQVQGKMMRGEELWWMPDPQTGYYRPDNFARELDAVELRSLHF
NKNQKTYVVS

Truncated LEA3-4:
TIGSSQEKPSWASDPDTGYFRPETAAKELDPYIAKTSQVQGKMMRGEELWWMPDPQ
TGYYRPDNFARELDAVELRSLHFNKNQKTYVVS

Figure 1. Full length LEA3-4 sequence and truncated LEA3-4 sequence

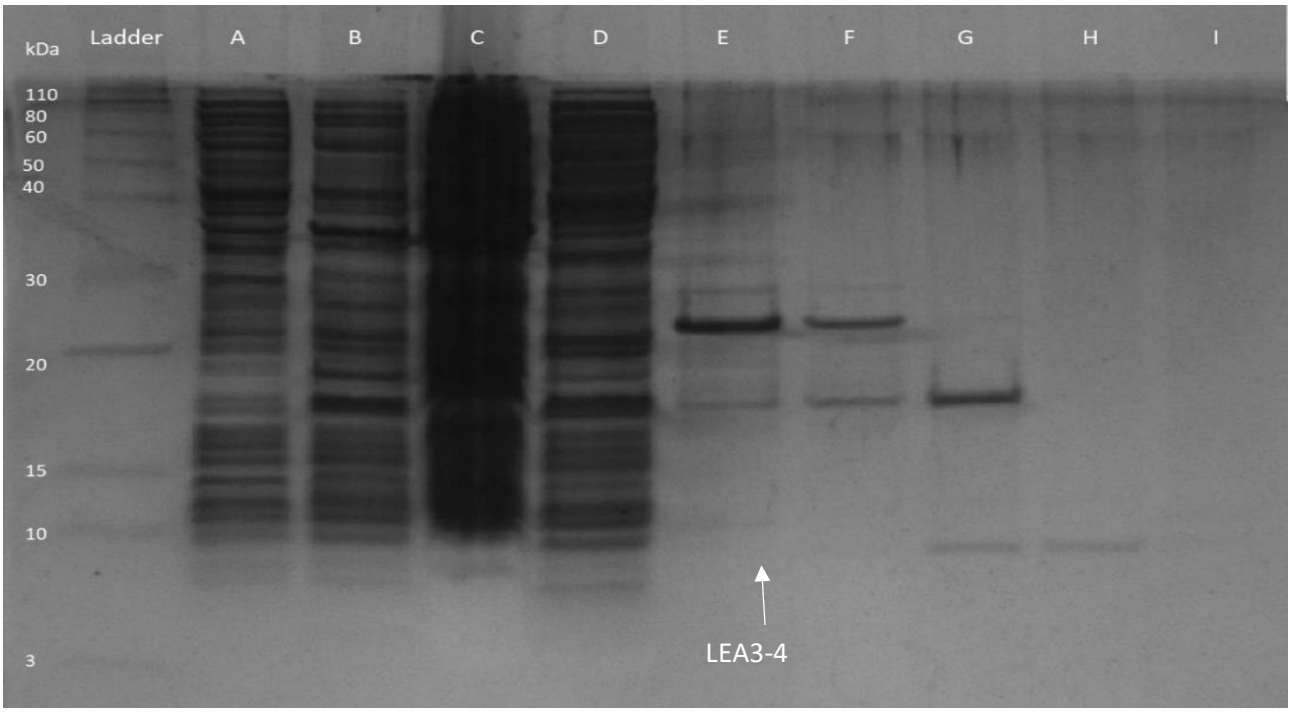

Figure 2. SDS-PAGE gel showing the purification of LEA3-4. Pre-induction (A), Post-induction (B), Insoluble fraction after bacterial pellet lysis (C), Soluble fraction after bacterial lysis (D), Eluted FPLC #1 fraction (E), Pre-digestion with ULP1 (F), Post-digestion with ULP1 (G), Flow through from FPLC #2 (H), Eluted HPLC fraction (I).

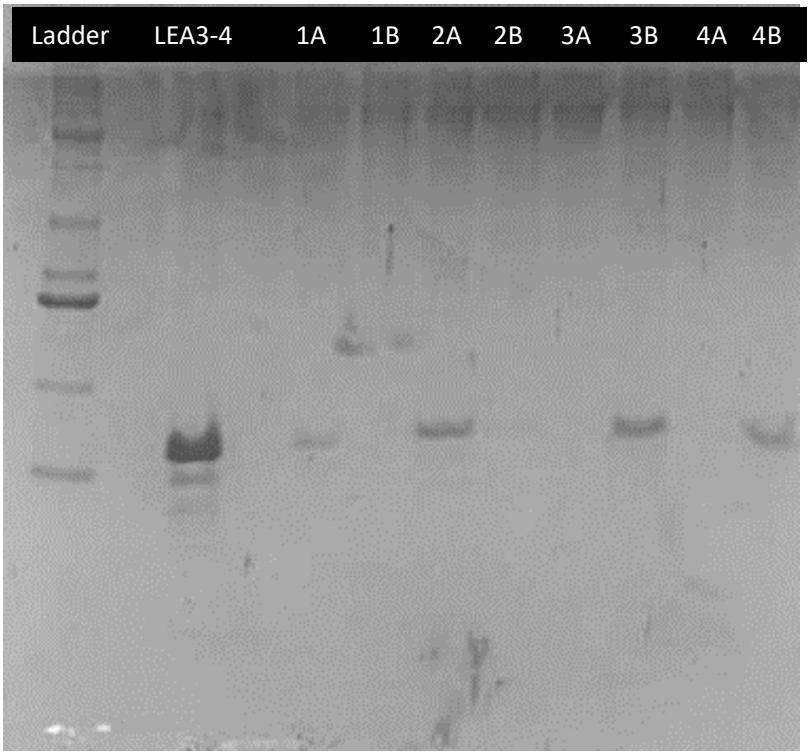

Figure 3. SDS-PAGE gel showing Immobilized metal ion affinity chromatography (IMAC) of LEA3-4. Ca^{2+} with EQ Buffer (1A), Ca^{2+} with EDTA Buffer (1B), Co^{2+} with EQ Buffer (2A) Co^{2+} with EDTA Buffer (2B), Cu^{2+} with EQ Buffer (3A), Cu^{2+} with EDTA Buffer (3B), Fe^{2+} with EQ Buffer (4A), Fe^{2+} with EDTA Buffer (4B).

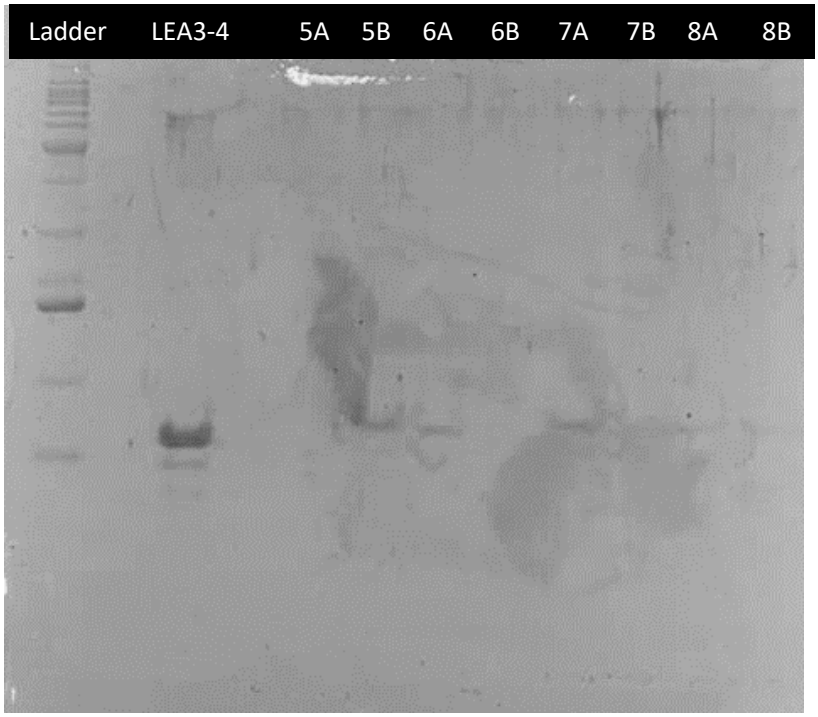

Figure 4. SDS-PAGE gel showing Immobilized metal ion affinity chromatography (IMAC) of LEA3-4. Fe^{3+} with EQ Buffer (5A), Fe^{3+} with EDTA Buffer (5B), Mg^{2+} with EQ Buffer (6A), Mg^{2+} with EDTA Buffer (6B), Ni^{2+} with EQ Buffer (7A), Ni^{2+} with EDTA Buffer (7B), Zn^{2+} with EQ Buffer (8A), Zn^{2+} with EDTA Buffer (8B).

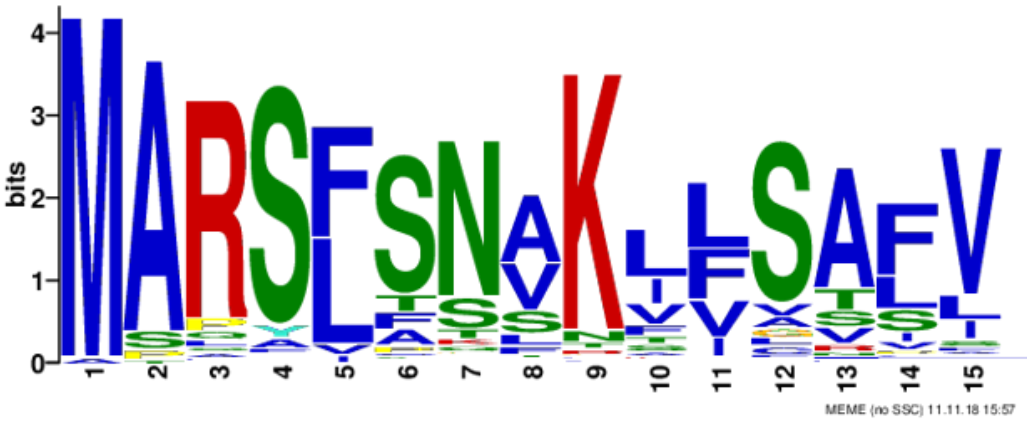

Figure 5. LOGO representation of the MARS-motif created by MEME program. Amino acids are coloured based on their group type. Blue – hydrophobic (A, V, L, I, F, M,W), Green – polar (S, T, N, Q), Purple – negative (D, E), Red – positive (K, R, H), Cyan – Y, Orange – G, Yellow - P

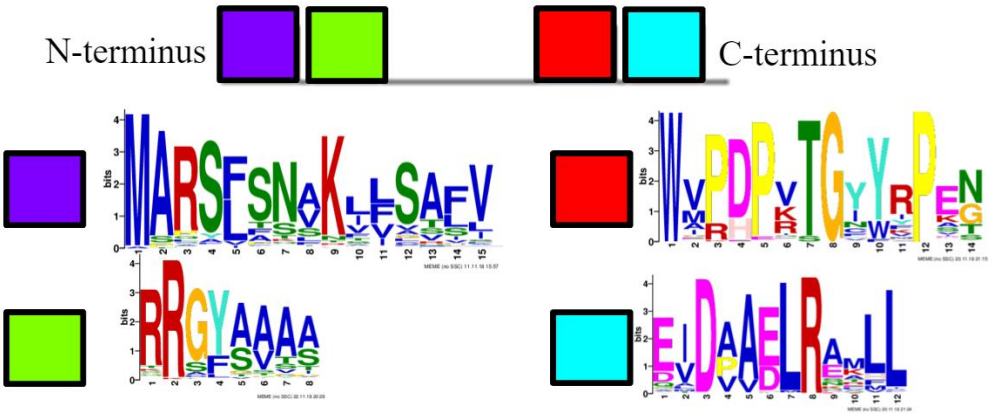

Figure 6. Position of the motifs generated by MEME program. Each motif is color coded. Red – W motif, Blue – LL motif , Green – RA motif and Purple – MARS motif.