Establishing Liposome Binding Studies for the GRP1 Protein Isolated from *Craterostigma plantagineum*

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Abstract

A wide array of environmental stresses can alter the physiological and biochemical responses in plants, yet dehydration is one of the most prevalent stresses that are contemporarily relevant to our changing dynamics in agricultural production due to global warming. Desiccation tolerance is exhibited by seeds and a small group in angiosperms termed resurrection plants. *Craterostigma piantagineum* is a representative of resurrection plants and used as a model plant to study desiccation properties. They are able to withstand repeated periods of desiccation and rehydration. During the dehydration process, the structural integrity of the plant is preserved via the induction of transcripts and proteins expresion responsible for modulating the shrinkage of leaves and their extensive folding. The process is facilitated by the presence of protective proteins, specific carbohydrates, restructuring of membrane lipids, and other regulatory mechanisms. Notwithstanding the evidence implicating plant Glycine-rich Proteins (GRPs) in responses to environmental stresses, the knowledge pertained to the underlying physiological mechanisms responsible for acclimation to dehydration stress remains largely unknown. The CpGRP1 gene was analysed and its role in binding to lipids was investigated. Three liposome binding assays were conducted on three protein fragments to evaluate the binding affinity of CpGRP1 to phosphatidic acid (PA), an important signaling molecule in plants.

Keywords: *Craterostigma plantagineum*, GRPS, GRP1, Liposome Binding Assay, Phosphatidic Acid

1 Introduction

In nature, not only biotic stresser are continuously consorting with plants, but also a plethora of abiotic stresses impact the upkeeping of plants' hemeostasis. Abiotic stresses represented by acute and severe environmental conditions immensly restrict the productivity of plants and availability of habitation. The latter threats constitutes a major constraint for agricultural production (Bartels and Salamini, 2001). Enviornmental stresses comprise a huge assortment of abiotic elements (Bohnert et al., 1995) including salinity stress (Ashraf and Foolad, 2013), cold stress associated with extreme latitudes (Thomashow, 1999), high temperature stress (Gillooly et al. 2001), nutrient stress (Lopez-Arredondo et al. 2013), and heavy metal stress (Khan et al. 2014, 2015). Nevertheless, among the different forms of stresses, the most pervasive form is drought stress which adversely impacts the growth and productivity of plants (Araus et al., 2002) in arid and semiarid areas (Ahmadizadeh et al., 2012). Drought stress constitutes a threat for sustainable crop production especially in a world that is experiencing abrupt temperature changes due to climate change (Tubiello et al., 2000). Drought manifested by low supply of water is either the outcome

of high temperatures (Tester and Bacic, 2005) or low precipitation (Ahmadizadeh et al., 2012). By the year 2025, the International Water Management Institute portends that one-third of the world's population will be living in regions that experience severe water scarcity (Bartels and Salamini, 2001).

The negative physiological and biochemical effects of drought stress encompass loss of water use efficacy, induction of heat stress, reduction stem extension (Kramer, 1974; Farooq et al., 2009), dwindling rates of photosynthesis (Kawamitsu et al., 2000), hindrance of stomatal conductance to conserve water yielding a substantial reduction in CO2 fixation and lessening assimilate production for growth and yield of plants (Boyer, 1970), induction of stomatal closure (Flexas et al., 2004), and the curtailment of leaf chlorophyll content (Ommen et al., 1999) due to the accumulation of active oxygen species which damage chloroplasts (Smirnoff, 1995). Subsequently, the aforementioned stresses collectively cause irreversible damage to the cellualr membranes and organelles followed by termination of growth and cessation of general metabolism (Hsiao, 1973).

However, since plants are sessile organisms and they are perpetually exposed to environmental stimuli (Al-Whaibi, 2011; Smékalová et al., 2014; Pan et al., 2016) they have evolved adaptations to subsist and survive challenging environmental stresses (Bartels and Salamini, 2001). Their responses and adaptations to dehydration stress is displayed by complex and multifaceted changes at the are evident at the biochemical, molecular, physiological, and morphological levels (Czolpinska and Rurek, 2018). The acclimation and deacclimation processes are meticulously mediated by regulating the expression of many genes (Gaff, 1971). Fluctuations in hemeostatic conditions favorable for plant growth bring about changes in the transcript levels of specific genes (Gaff, 1971), thereby altering gene expression and protein production. Outstanding progress has been made in the pursuit of identifying and characterizing drought stress responsive genes in plants (Joshi et al., 2016b). Several signaling pathways including those encoding ABA biosynthetic pathway (Sah et al., 2016) which mobilize a wide range of molecules (Al-Whaibi, 2011; Smékalová et al., 2014; Pan et al., 2016) and proteins that have either metabolic or regulatory roles are encoded by this group of salient genes (Joshi et al., 2016). These desiccation-induced genes encode for antioxidnt enzymes that are involved in detoxifying the cells from reactive oxygen species (ROS) (e.g., superoxide dismutase, catalase, ascorbate peroxidase, peroxidase, and glutathione reductase) (Foyer and Noctor, 2005), redox metabolites (e.g., ascorbic acid and glutathione) (Ashraf, 2009), osmolyte biosynthesis and accumulation (e.g., proline) (Sanchez et al., 1998; Alexieva et al., 2001), and transcritption factors regulating gene expression (e.g., AREB, AP2/ERF, NAC, bZIP, MYC, and MYB)

(Wani et al., 2013). Moreover, Röhrig et al. (2006) also showed the phosphorylation of dehydration-induced phosphoproteins CDeT11-24 and CDeT6-19, verifying that post-translational modifications play a vital role in desiccation-induced gene expression.

As previously stated, plant growth and development is frequently impaired by water deficit. Plants react to water scarcity by either reducing water loss or increasing water uptake (Bray, 1997). In lower plants, drought-tolerance mechanisms are commonly found. On the other hand, higher plants do not enjoy the same drought-resistance privilages of lower plants particularly when the relative water content of their cells is below 60% (Giarola et al., 2017), with two notable exceptions. First, seeds and pollen grains can withstand desiccation to an air-dried state with the former surviving for long periods of time in the desiccated state while the latter loosing its tolerance rather quickly (Walters et al., 2005). Second, resurrection plants, a minor group of vascular angiosperm plants, can lose most of their cellular water content (Gaff, 1971) and revive from an air-dried state within few hours of rewatering (Bartels, 2005).

It has been postulated that there are around 200 extant species of resurrection plants (Bartels and salamini, 2001). They can be found in regions which suffer from seasonal and sporadic rainfall and weather. They favor growing at low to moderate elevations on rocky outcrops in tropical and subtropical climates (Porembski and Barthlott, 2000). Nonetheless, the most painstakingly studied resurrection plant is the dicotyledonous South African *C. plantagineum* (Bartels et al., 1990). It is a member of the genus Linderniae, a tribus of the Scrophulariaceae which is a large heterogenous family of polyphyletic origin. It has been estimated that 170 Linderniae species have been discovered with most of them being endemic to Africa (Fischer, 2004).

C. plantagineum is a poikilohydrous plant meaning that its water content fluctuates as the relative humidity in the environment changes (Bartels and Salamini, 2001). It is capable of surviving substantial amount of water dficit in their roots and aerial tissues (Hartung et al., 1998). Even though *C. plantagineum* is well-suited for molecular analysis because gene expression of desiccation tolerant genes can be studied and compared in two genetically identical systems (undifferentiated callus cultures and differentiated plants) (Bartels, 1990), the physiological basis of desiccation tolerance in resurrection plants is governed by extremely intricate netwroks (Bartels and Salamini, 2001) and it was only until recently that the interconnectedness of these regulatory netwroks have been unraveled. Therefore**,** for the sake of developing plants that exhibit resistance to water-limited conditions, understanding the biochemical and

molecular responses to drought and dessication tolerance has become a task of utmost importance (Anjum et al., 2011).

Therefore, understanding the intricate mechanisms by which plant growth and development are affected can facilitate he discovery of economically and ecologically new solutions capable of mitigating losses in food production. Nonetheless, the integrity of cellular structures during severe water loss is a biological phenomenon which has not yet been completely understood (Röhrig et al., 2006). Drought deprives plants from water inducing desiccation tolerance mechanisms which then activates and mobilizes a large network of genes to help deal with mechanical stress imposed on cell walls. Wall collapse is detrimental to the survival of the most plant species (Walters et al., 2002). Thus, modulating the proper communication channels between cell walls and cytoplasm is vital for the perception of environmental changes (Giarola et al., 2015). Resurrection plants avoid collapse of cell wall and rupture of plasmalemma by actively engaging in reversible cell wall folding and replacement of water in the vacuoles with compatible solutes (Farrant, 2007). Among the studies which have supported a role for GRP proteins in cell wall interactions in resurrection plants are two studies done by Neale et al. (2000) and Wang et al. (2008) who recorded the upregulation of GRP proteins upon desiccation in Sporobolus stapfianus and Boea hygrometrica respectively. There are 5 major classes of GRP proteins. Class I GRPs are chiefly comprises high-glycine-content region with (GGX)n repeats whereas Class V GRPS are characerized by the presence of mixed patterns of glycine repeats. Class II GRPs contain a characteristic C-terminal cysteine-rich region. Class III GRPs retains Low glycine content and the possible presence of an oleosin domain. Class IV GRPs also termed RNA-binding GRPs have either a cold-shock domain with some members possesing ancilliary zinc-finger motifs or an RNA-recognition motif (Mangeon et al., 2010).

Polysaccharides (e.g., cellulose, hemicelluloses, and pectins) are not the only components of cell walls but also structural proteins (Ryser et al., 2003) add another layer of complexity to cell wall-protein interactions. The third major class of structural wall proteins is represented by Glycine-rich proteins (GRPs) (Keller, 1993). GRPs enhances the structural flexibility of cell walls and their synthesis is induced under stress conditions (Mousavi and Hotta, 2005). They are proteins whose final Gly composition ranges between 50 and 70% of the total amino acidin the protein sequence (Keller, 1993). The majority of GRPs are characterized by exhibiting a pairwise arrangement of the amino acids that can be written as (Gly-X)n where X is frequently Gly (Condit and Meagher, 1986). Previous research have shown that two GRP

proteins are part of cell walls including grp-1 from petunia (Condit et al., 1990) and GRP 1.8 from *Phaseolus vulgaris* (Keller et al., 1988; Ryser and Keller, 1992).

Pectin is a primary component of cell walls in dicot land plants. It encompasses a wide range of galacturonic-acid rich polysaccharides. It was shown that cell wall-associated protein kinases (WAKs), through their galacturonan-binding domain (GUB), bind to pectins (Decreux and Messiaen, 2005; Decreux et al., 2006). This binding triggers downstream signalling pathways (Kohorn et al., 2009; Brutus et al., 2010). Giarola et al. (2015) suggested a sensory and down-stream transduction role for WAKs to sense cell wall status based on their ability of WAKs to bind pectins. Cell wall-associated protein kinases 1 (CpWAK1) is bound to cell wall pectins and regulates cell expansion. Giarola et al. (2015) also found that water deprivation brings about an increase in the expression of CpGRP1 and modifications of pectin, which ultimately begets the formation of the dehydration-specific signalling complex. CpGRP1 and CpWAKs are selectively expressed in leaves and they exert their function in this tissue. Eventually, CpGRP1, which is secreted in the cell wall, binds to CpWAK1 and partake an essential role in CpWAK1-mediated growth regulation (Giarola et al., 2015).

In this report, we suggest a role for GRP1 in binding to membrane lipids by using the liposome binding assay. My results verify the reults of a previous experiment done on GRP1 using the lipid binding assay.

2 Materials and Methods

2.1 Proteins of Interest

GRP1 full protein, long fragment (all regions excised except N-terminal region), the arginine-mutated N-terminal fragment (two arginines were mutated), and short fragment (all regions excised except C-terminal region). A formerly over-expressed sample for Cp11-24 was provided by Niklas Jung was used as a positive control.

2.2 Bacterial Culture and Over-expression (Studier et al., 1990)

Recombinant *Escherichia coli* BL23 (D3) strain transformed with genes containing our ptoteins of interest (refer to section) were provided by Niklas Jung to conduct the experiments. Recombinant *E. coli* was stroken on petri-dishes and was left to grow overnight in an incubator at 37ºC. For pre-culture, a tip which was used to inoculate a portion of a grown colony was dispensed in 20 mL LB media infused with 20 µL Kanamycin (1:1000 dilution) in a 200 mL flask. The pre-culture was left to grow in a shaking incubator

overnight (15 to 18 hours of incubation period) at 37ºC. The main culture was prepared by mixing 200 mL LB media, 200 µL Kanamycin (1:1000 dilution), and 3 mL of pre-culture in a 2 L flask to maintain the optimum growth condition for bacteria. The Recombinant *E. coli* was left to grow in a shaking incubator. The OD (Optical Density) of the main culture was intermittently measured at wavelength 600 nm until the OD value reached 0.5. Then, the main culture was induced with IPTG. IPTG was freshly prepared by adding 0.238 g IPTG to 10 mL dH_2O. A 1 mL sample (**t_0**) was taken before adding the IPTG. Afterwards, 2 mL of the prepared IPTG was added to 200 mL of main culture (ratio: 0.5 mL IPTG must be added to 50 mL of main culture). The main cuulture was left to grow in a shaking incubator at 37ºC and samples (1 mL) were taken at regular nitervals (**t_1**= after 1 hour, **t_3**=after 3 hours, and **t_5**=after 5 hours). The obtained samples, after their withdrawal, were subsequently centrifuged (10 min; 4ºC; 10,000 × g). The supernatant was discarded and the pellet was stored at -20ºC. Similarly, the remaining bacterial suspension was used for large scale purification of the desired protein by using Ni-NTA column. The suspension was centrifuged (10 min; 4ºC; 10,000 × g), the supernatant was discarded, and the pellet was stored at -20ºC.

2.3 Protein Purification Using Ni-NTA Column (Porath et al., 1975)

Columns that are based on the principle of chelate affinity chromatography (Porath et al., 1975) was used to purify proteins obtained from bacterial pellets via the attachment of the his-tag connected to the proteins to the Ni-NTA column. The required buffers were prepaed prior to starting the experiment. To concoct 200 mL of Buffer A, 50 mM HEPEs/NaOH (pH 7.4), 300 mM NaCl, 5 mM imidazole, 10% (v/v) glycerol, and 0.1% (V/v) Triton X-100 were mixed and kept at room temperature (RT). Before using Buffer A, 1.04 µL of 1.5 mM ß-Mercaptoethanol (ß-ME) was freshly added to 10 mL of the already prepared Buffer A. To make 200 mL of Buffer B, 50 mM HEPEs/NaOH (pH 7.4), 300 mM NaCl, 20 mM imidazole, 10% (v/v) glycerol, and 0.1% (V/v) Triton X-100 were mixed and kept at room temperature (RT). Likewise, before using Buffer B, 1.258 µL of 1.5 mM ß-ME was freshly added to 12 mL of the already prepared Buffer B. Finally, to prepare 50 mL of Buffer B, 50 mM HEPEs/NaOH (pH 7.4), 300 mM NaCl, 250 mM imidazole, 10% (v/v) glycerol, and 0.1% (V/v) Triton X-100 were mixed and kept at room temperature (RT). Before using Buffer C, 0.419 µL of 1.5 mM ß-ME was freshly added to 4 mL of the already prepared Buffer C. All ß-ME-infused buffers were kept on ice. The frozen bacterial pellets were resuspended in 5 mL of Buffer A, 5 mg of Lyzozyme was added, and suspensions were incubated on ice for 30 minutes. Next, the suspension was transferred to 15 mL falcon tubes, sonicated 4 times (amplitde 35) for 30 seconds

using an Ultrasonic Processor, centrifuged (30 min; 4ºC; 14,00 0 rpm). The supernatant was used for further steps while the pellet was discarded. To commence the purification steps, the 30% ethanol used for storing the Ni-NTA column was drained. After that, the Ni-NTA column was washed with 3 mL of dH_2O, 5 mL of 6M Guanidine-HCL (pH 7.5), and 3 mL of dH_2O consecutively. Then, to recharge the Ni-NTA column, 4 mL of 0.1 M EDTA (pH 8.0), 3 mL of dH_2O, 4 mL of 1% (w/v) $NiSO_4$, and 2 mL of dH_2O were added sequentially. The Ni-NTA column was equilibrated with 3 mL of Buffer A (without ß-ME). Afterwards, a 15 mL falcon was placed below the colmn and the supernatant was filtrated into the funnel of the column using a syringe attached to a filter (0.22 µm). The funnel was allowed to completely drain. The flow-through was loaded into the column for a second round of purification to obtain higher yields of the protein. The flow-through was discarded. The column was washed two times with 5 mL of Buffer A and two times with 6 mL of Buffer B. Proteins were eluted with 4 mL of Buffer C and collected in 6 fractions, each containing approximately 600-700 µL of eluted proteins. The fractions were stored at -20ºC overnight and were used on the following day to determine protein concentrations using the Bradford Assay (Bradford, 1976). The column was washed with 2 mL of dH_2O, 5 mL of 6M Guanidine-HCL (pH 7.5), 3 mL of dH_2O), 3 mL of 30% ethanol successively. Finally, 1 mL of 30% ethanol was added to the bottom-capped column which was eventually stored at 4ºC for later use.

2.4 Desalting and Buffer Exchange (GE Healthcare, 2019)

For downstream experimental steps, it is important to replace the buffer containing the his-tag proteins with a non-interferring buffer which won't affect the running of his-tag purified proteins on SDS-PAGE gels. Salts, free labels, and other impurities are efficiently removed by using a buffer exchange column. The gravity protocol was used to exchange the buffer ebcause its recovery (in the range 70% to 95%) and desalting capacities (98% of salts removed) are outstanding when compared to other buffer exchange protocols. The Sephadex G-25 In PD-10 Desalting Columns were used which work by gel filtration (Porath and Foldin, 1959). The principle of the protocol is simple as it is divided into four segments. First, the top cap was removed and the sealed end at the bottom was cut. Second, column was equilibrated by washing the column five times with 5 mL 100 mM Na_2CO_3. 100 mM Na_2CO_3 (pH 9.6) was used because it has volatile ingredients which will vanish during the freeze-drying process in vaccum. Third, 2.5 mL of samples with highest protein concentrations were collected in a 15 mL falcon tube and then applied to the desalting column. The column was left to drain and the flow-through was discarded. Molecules that are smaller than the pores in the sephadix matrix are going to pass through the pores while molecules larger

than pore sizes are going to penetrate the column to varying extents due to their different moelcualr weights. Fourth, a 15 mL falcon tube was placed beneath the tip of the column and 3.5 mL of equilibration buffer was added. The eluted proteins were collected for freeze-drying.

2.5 Freeze-drying (Chang and Patro, 2004)

The buffer-exchanged proteins were subjected to freeze drying also called protein lyophilisation, a term that is attributed to the work of Rey LR in 1976. The process of lyophilisation generates a dry porous structure whose high porosity and exposed surface swiftly resorb the solvent (water, vapour or moisture) in the surrounding environment. Hence, the freeze-dried materials exhibit a lyophil characteristics towards the solvent (Varshney and Singh, 2015). The first attempt to freeze dry a specimen was accomplished by Altmann in 1890 in order to make histological sections. Altman prior to drying pieces of frozen tissue, he placed them in a vacuum desiccator at -20 °C (Altmann, 1890).The rationale of lyophilisation using volatile compounds (e.g., Na_2CO_3 and NH_4HCO_3) or non-aqueous solvents (e.g., ammonia, carbon dioxide, carbon tetrachloride, and benzene) is simple. Drying materials can be easily sublimed from the solid state at low temperature under vaccum in the same manner that ice is sublimed. This technique is principally used for the preparation of easily soluble powders of materials not soluble in water (Rey, 1964). In the same sense, the proteins suspended in Na_2CO_3 equilibration buffer were lyophilised in a freeze dryer. The freeze dryer was pre-cooled to -50ºC. Prior to placing the suspended proteins inside the freeze dryer, the samples were immersed in liquid nitrogen and placed inside slightly tilted on the inner rim of iron plate. Generation of vaccum was initiated and the sample was left to freeze dry for 3 to 4 days till all the ice crystals has disappeared. Finally, the highly concentrated protein powder was dissolved in 100 µL of dH_2O, OD was measured at wavelength 595 nm using Bradford assay, and protein concentration was determined. Subsequently, the freeze-dried proteins were run on SDS gels.

2.5 Liposome-binding Assay (Zhang et al., 2004)

The protocol for liposome-binding Assay was adapted from Zhang et al. (2004) and modified based on the concentration of our freeze-dried proteins samples. One of two buffers are needed to conduct the experiment. 20 mL of Liposome buffer A was prepared and it is composed of 20 mM MES, 50 mM Tris-HCL (pH 7), and 100 mM NaCl. The optimum spectrum of pH for using this buffer ranges between 5 and 9. Before immediate usage, 15 mg of 1 mM DTT was freshly added to 10 mL of lipososme buffer A. To prepare 20 mL of Liposome-binding buffer B, 20 mM MES, 30 mM Tris-HCL (pH 7), 0.5 mM NaCl, 2 M Urea, and 0.5% CHAPS were mixed. Similarly, before immediate usage, 15 mg of 1 mM DTT was

freshly added to 10 mL of lipososmebinding buffer B. The two tested lipids, Phosphatidic acid (PA) and phosphatidylcholine (PC) were dissolved in chloroform/methanol (2:1) solution with final concetration of 4 µg/µL. The prepared lipids were stored separately at -20ºC. For each assay, 150 µg PC (37.5 µL) and 100 µg of the tested lipid PA (25 µL) transferred to 1.5 mL eppendorf tubes under the laminar flow hood. Solvents were allowed to evaporate under the laminar flow hood, and were resuspeneded in 125 µL of DTT-infused liposome buffer B (0.5 µg/µL). This mixture was incubated at on a shaker set to 250 rpm at 37ºC for 1 hour. The resulting liposomes were vortexed for 5 minutes and centrifuged (10 min; 4ºC; 20,000 × g). the supernatant was discarded. The obtained liposomes can either be used directly or stored at -20ºC for no longer than 2 days. The obtained liposomes were resuspended in µL of lipososme-binding buffer B containing 0.1 µg/ µL of the protein of interest. The total volume of the resulting sample must be 125 µL. Therefore, for GRP1 (having a concetration of 0.270 µg/µL) 4.62 µL of the protein was mixed with 120.4 µL of liposome-binding buffer. For N-terminal fragment (having a concetration of 0.149 µg/µL) 8.3 µL of the protein was mixed with 116.7 µL of liposome-binding buffer; and for N-terminal fragment with two mutated Arginines (having a concetration of 0.101 µg/µL) 12.4 µL of the protein was mixed with 112.6 µL of liposome-binding buffer. The resulting mixtues were incubated at 30ºC for 30 minutes and centrifuged (10 min; 4ºC; 10,000 × g). The supernatants were transferred to new 1.5 L eppendorf tubes. The obtained pellets and supernatants can be directly used for analysis on SDS-PAGE gels or stored at -20ºC for no longer than 2 days. For performing the SDS-PAGE analysis, 125 µL of 2X Laemmli loading buffer was added to the supernantants whereas 50 µL of 1X Laemmli loading buffer was added to the pellets. 15 mg of DTT was added to 1 mL of the loading buffer before it was used. The samples were subsequently incubated at 95ºC for 10 minutes, centrifuged (10 min; 4ºC; maximum speed), and stored at -20ºC. Once the samples are taken from -20ºC, they were incubated 70ºC for 5 minutes and spun down (1 min; 4ºC; maximum speed).

2.6 Determination of Protein Concentration (Bradford, 1976)

The determination of protein concentration was conducted for samples obained from his-tag purification and freeze-drying based on the Bio-Rad Protein Assay (Bradford, 1976) and before adding the Laemmli sample buffer to them. BSA-stock solution (1 mg/ml) was prepared and the BSA standard curve was evaluated based on the values supplied in the table below. To prepare the bradford solution, a dilution of 1:5 of bradford stock was made. Finally, the OD was measured at wavelength 595 nm. For his-tag purified

and freeze-dried samples, the criteria mentioned in the figure below was followed to evaluate protein concentration.

BSA Standard Cruve		
BSA (µg)	Bradford (µL)	dH2O (µL)
0	200	800
1	200	799
2	200	798
4	200	796
8	200	792
12	200	788
16	200	784
20	200	780
For his-tag purified samples		
Sample (µL)	Bradford (µL)	dH2O (µL)
10	200	790
For freeze-dried samples		
Sample (µL)	Bradford (µL)	dH2O (µL)
2	200	798

Figure 1. The volumes of protein samples that were used for performing the bradford assay.

2.7 SDS-PAGE (Laemmli, 1970)

The previously obtained samples were kept on ice to prevent damaging the proteins. To preapre 10 mL of 1X Laemmli sample buffer (Laemmli, 1970), 15 mg of DTT was freshly added to 10 mL of 1X SDS. Before running the samples on SDS-PAGE, 200 µL of 1X Laemmli sample buffer was added to the IPTG-induced samples where as for freeze-dried protein only 18 µL of 1X Laemmli sample buffer was mixed with 2 µL of protein. When the final samples were preapred, they were not kept without direct heating. Since endogenous proteases are very active in SDS sample buffer, they can acutely degrade the proteins. Therefore, the samples were heated at 95ºC for 10 min on a heating block. If the samples were previously frozen at -20ºC, they were heated at 70ºC for 5 min before use. Nevertheless, once the protein infused laemmli buffer is heated, the samples could sit at RT for a short period of time until loading. For analysis of the proteins 4% stacking gel was used, however, one attempt to use 10% resolving gel for SDS-PAGE did not yield good resolution of the desired bands, thus, 15% resolving gel was utilized to optimize band

resolution. The gel cassettes was assembled on a clean bench. To prepare 15 mL of the resolving gel (a sufficient volume for two resolving gels): 3.4 mL dH_2O, 7.5 mL of 30%acrylamide mix, 3.8 mL of 1.5 mM Tris (pH 8.8), 150 µL of 10% SDS, 150 µL of 10% ammonium persulfate (APS), and 6 µL of TEMED were mixed in the inscribed order because APS and TEMED intiate the polymerization of the gel. The mixture was mixed thoroughly in a 50 mL falcon tube. Then, the solution was pipetted into the gel cassettes, 1.5 cm below the top border of glass plate, and 1 mL of dH_2O was pipetted ontop of the resolving gel to ensure the smooth polymerization of the two-gel interface. The gel was left to polymerize. Later, the stacking gel was made. To prepare 4 mL of the stacking gel (a sufficient volume for two resolving gels): 2.89 mL dH_2O, 530 µL of 30 %acrylamide mix, 500 µL of 1.5 mM Tris (pH 6.8), 40 µL of 10% SDS, 40 µL of 10% ammonium persulfate (APS), and 4 µL of TEMED were mixed in the inscribed order and mixed thoroughly in a 50 mL falcon tube. Then, the solution was pipetted into the gel cassettes and a comb was inserted cranial to the resolving gel between the two glass plates. The stacking gel was left to polymerize. The gels can be stored at 4ºC for 3 to 4 days until further use is desired. When the gels polymerized, the protein-containing laemmli buffer was loaded on the gel, and run at an intial current of 15 mA. Tris/Glycin running buffer with the following composition (25 mM Tris; 192 mM Glycin; and 0.1% SDS) was used to run the SDS-PAGE gels. When the proteins has migrated through the stacking gel, the current was increased to 30 mA.

2.8 Western Blotting (Towbin et al., 1979)

Western blotting was used to transfer the proteins from the SDS-PAGE gels to nitrocellulose membranes. The blotting sandwich was assembled. Before assembling each piece of the sandwich, the pieces were soaked with towbin buffer with the fllowing composition (25 mM Tris; 192 mM Glycin; and 20% (v/v) methanol). The assembly followed this order: support grid (Black), whatman paper, gel, nitrocellulose membrane, whatman paper, support grid (Red). The sandwich was placed in the blotting chamber along with a magnet and an ice bag. The blotting chamber was placed on top of a magnetic stirror and run setting the amperes to maximum for 50 minutes. The optimum voltage for running the blotting process ranges between 70 and 50 V. Therefore, towbin buffer can be optimally used for 3 times only.

2.9 Coomassie Staining (Zehr et al., 1989)

The separated proteins on SDS-PAGE gels were detected using Colloidal Coomassie R-250 protein dye which is a sensitive detection method capable of detecting protein concentrations as low as 10 ng. The SDS-PAGE gels were transferred to plastic petri-dishes and incubated with fixation solution (10% acetic

acid; and 40% methanol) and placed on an orbital shaker for at least 1 hour. The fixation solution was removed and the gels were washed 3× times (10 minutes each) with dH_2O. Next, the gel was incubated in Coomassie stain (0.25g Coomassie Brilliant Blue R250 dye; 125 mL methanol; 25 mL galcial acetic acid; and 100 mL dH_2O) overnight. Afterwards, the gels were washed with dH_2O. the gels were destained with destaining solution (100 mL methanol; 100 mL glacial acetic acid; and 800 mL dH_2O) till the bands became visible. The gels were washed with dH_2O.

2.10 Ponceau-S Staining (Stochaj et al., 2006)

Ensuing the western blotting, ponceau-S staining was performed to check if th transfer was conducted properly. Even though the method is inensitive because it only detects the most abundant proteins, the method is favorable not only because it is used to easily visualize the tranfer of samples (at least the protein marker) onto the membranes but also due to the reversible nature of the stain which permits the stain to be completely removed with dH_2O before moving further with processing the blot (Stochaj et al., 2006). The membanes were stained with cooled ponceau-S staining (0.2% Ponceau-S in 3% (w/v) TCA) for 10 minutes, the bands of the protein markers were labelled with a lead pencil, then the membranes were destained with dH_2O.

2.11 Immunodetection

To immunodetect the proteins two solutions are needed: 10X TBS and 1X TBST. To prepare 10X TBSL: 200 mM Tris-HCL was added to 1.5 M NaCl and the pH was adjusted to 7.5 using 37% HCL buffer. To prepare 1X TBST: 1X TBS was added to 0.1% (V/v) Tween-20and the pH was adjusted to 7.5 using 37% HCL buffer. After washing off the ponceau-S stain, the membranes were incubated for at least 30 minutes in 50 mL of Blocking Solution (1X TBS; 0.1% (v/v) Tween-20 (TBST); 4% (w/v) skimmed milk powder). Next, for the detection of the positive control (Cp11-24) the blocknig solution was removed and the membranes were incubated in 50 mL blocking solution mixed with 10 µL of anti-serum of the investigated protein (1:5000 dilution) overnight. However, for the detection of his-tagged proteins, the blocknig solution was removed and the membranes were incubated in 15 mL blocking solution mixed with 1.5 µL of anti-serum of the investigated protein (1:5000 dilution) overnight. The membranes were washed with TBST following this washing order: 1× rapid rinse, 1× wash for 15 minutes, and 3× wash for 5 minutes each. The same secondary antibody was used for the detection of positive control and his-tagged proteins. After washing the membranes with TBST, the membranes were incubated in 50 mL blocking solution

mixed with 10 µL of peroxidase-linked anti-rabbit antibody (1:5000 dilution) for 45 minutes. The membranes were washed as before.

2.12 ECL Detection (Mruk and Cheng, 2011)

After the final wash, two pieces of whatman paper and plastic lab-grade Saran wrap were cut according to the size of the membrane. The membranes were dried one at a time using two whatman papers. The following solutions were mixed (1:1 ratio) on the Saran wrap in the following order: 500 µL ECL solution A (luminol) and 500 µL ECL solution B (H_2O_2). Then the top side of the dried membrane (which contians the proteins) was placed onto the combined ECL solutions and incubated for 60 seconds. All air bubbles were removed to ensure proper distribution of ECL solution to all parts of the membrane. Then, the membranes were dried again. Eventually, the protein marked on the membranes were marked with the Lumi-pen and subsequently the chemiluminescence was detected in a Lumi-Imager (Azure c300, Azure biosystems).

3. Results

3.1. Protein overexpression

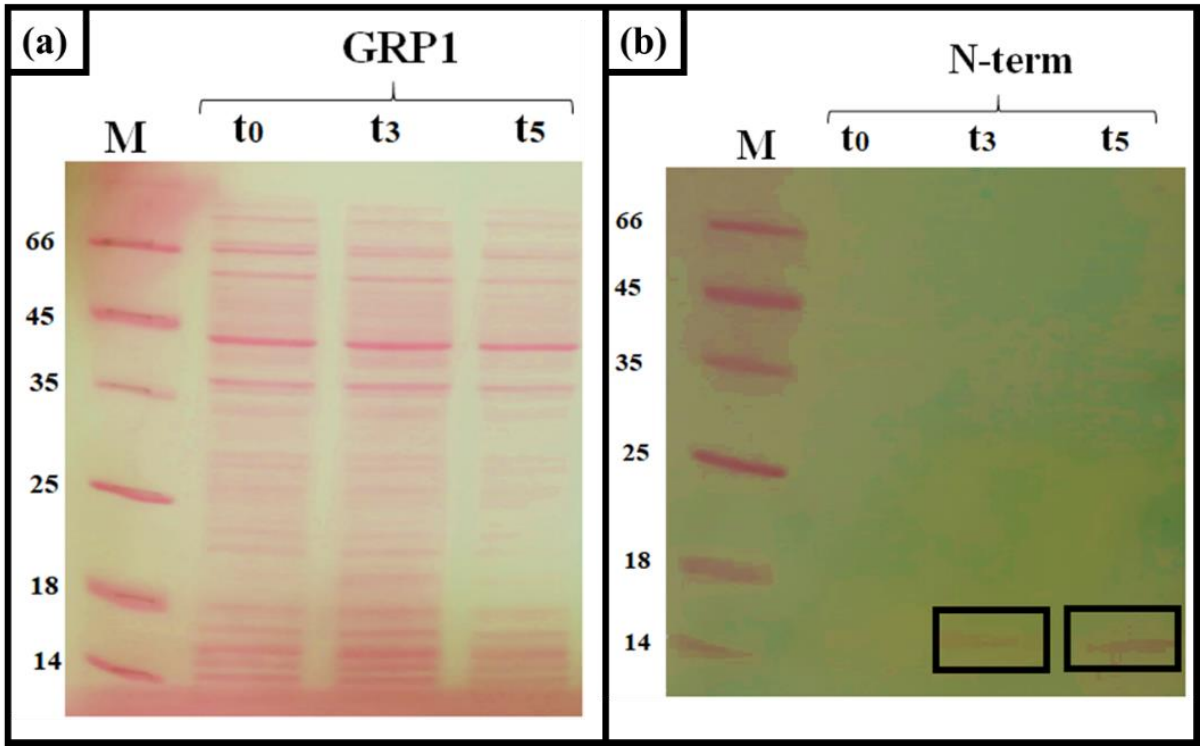

Figure 2. Ponceau S staining of the overexpressed proteins (GRP1 protein and N-terminal fragment) at three time intervals (t_0= 0 hour before IPTG induction, t_3= 3 hours after IPTG induction, and t_5= hours after IPTG induction). Black boxes indicate the desired bands.

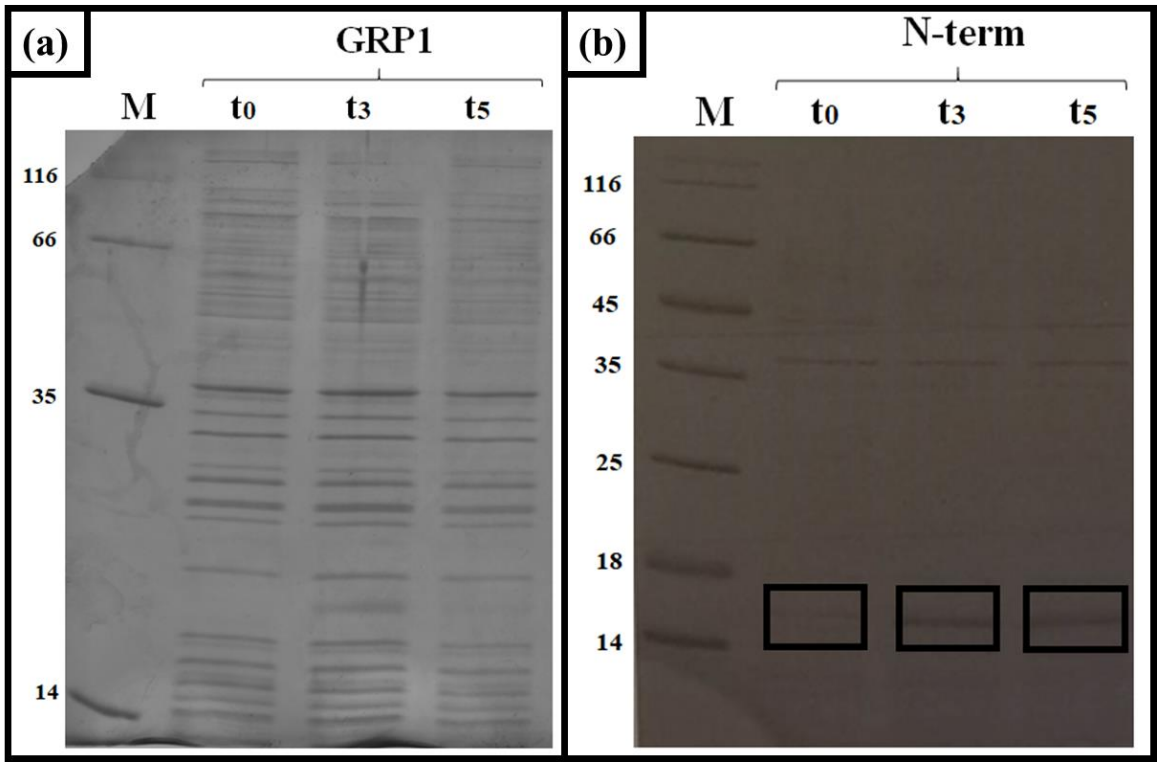

Figure 3. Coomassie staining of the overexpressed proteins (GRP1 protein and N-terminal fragment) at three time intervals (t_0= 0 hour before IPTG induction, t_3= 3 hours after IPTG induction, and t_5= hours after IPTG induction). Black boxes indicate the desired bands.

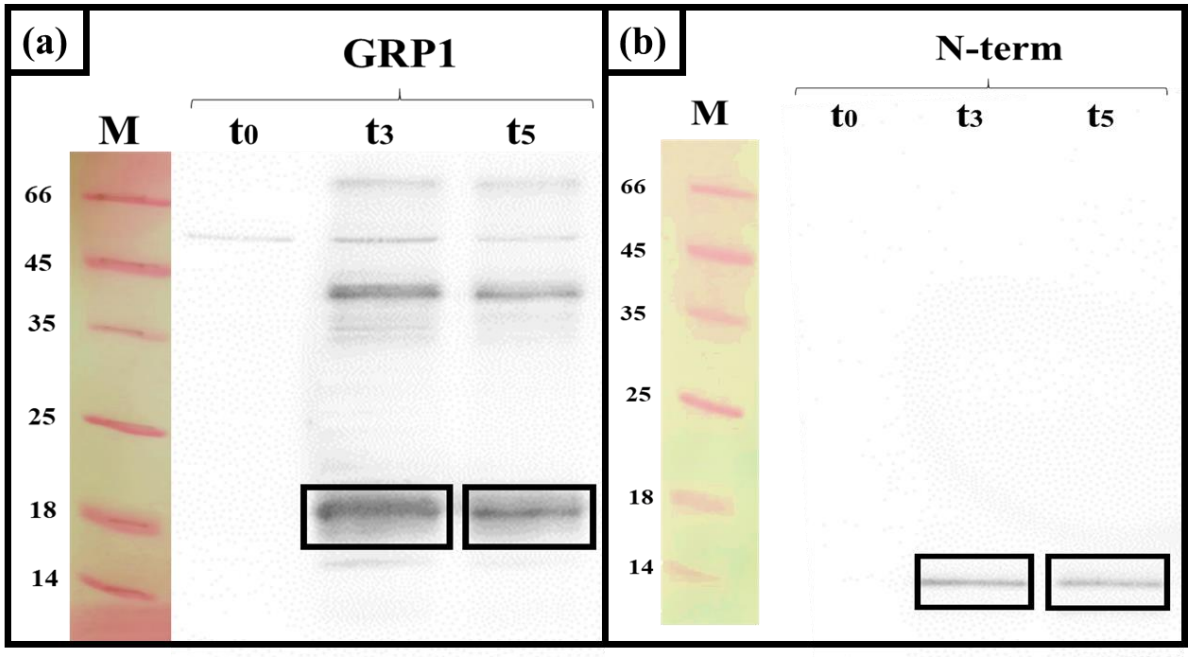

Figure 4. Immunoblotting of the overexpressed proteins (GRP1 protein and N-terminal fragment) at three time intervals (t_0= 0 hour before IPTG induction, t_3= 3 hours after IPTG induction, and t_5= hours after IPTG induction) using two antibodies (his-tag antibody as the primary antibody; and a secondary antibody). Black boxes indicate the desired bands.

After performing the pre-cultre and main culture, overexpression was conducted. When the O.D. of the main culture reached 0.5, a sample was taken at time t=0. IPTG induction followed. Three samples were taken at t=1, 3, and 5 hours. Both in Coomassie (figure 3) and Ponceau S (figure 2) stainings yielded similar results. For GRP1 protein a large number of proteins were detected whereas for the N-terminal only the desired bands were observed to run at 15 kDa at t_3 and t_5, which is 5 kDa more than the actual value (10.2 kDa). Immunoblotting (figure 4) revealed better results for the GRP1 protein clearly showing two bands at t_3 and t_5 running at 19 kDa, which is 4 kDa more than the expected value (14.4 kDa). No bands were detected in t_0 for both proteins.

3.2. Determination of Protein Concentration

Bradford assay was made to determine the concentration of extracted protein fractions and freeze-dried proteins. Figure (5a) shows the OD of BSA standard while figure (5b) shows the values of OD as a function of BSA (µg). However, for calculating the concentrations of the proteins, the Bradford assay provided by Niklas was used (Figure 6).

(a)

BSA (µg)	OD
0	0
1	0.043
2	0.122
4	0.163
8	0.263
12	0.335
16	0.402
20	0.452

(b)

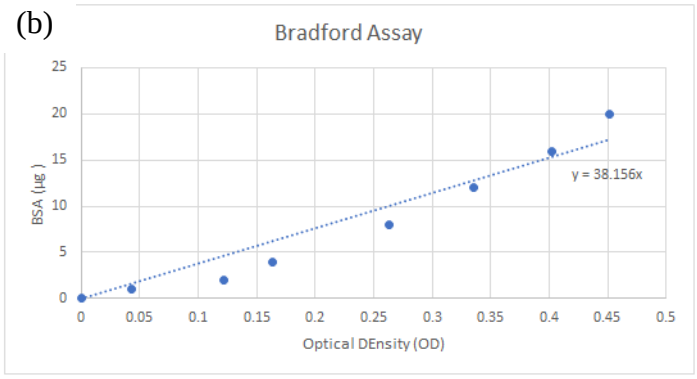

Figure 5. Bradford assay showing OD (595 nm) as a function of BSA (µg).

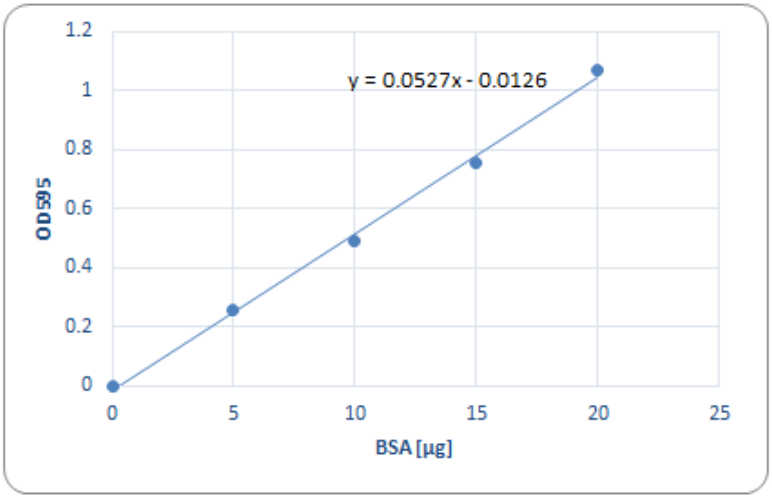

Figure 6. Bradford assay showing OD (595 nm) as a function of BSA (µg) was provided by Niklas.

		Proteins			
		GRP1		N-terminal	
		OD	Concentration (µg/µL)	OD	Concentration (µg/µL)
Fractions	Fraction 1	0.059	0.880455408	0.027	0.273244782
	Fraction 2	0.092	1.506641366	0.125	2.132827324
	Fraction 3	0.093	1.525616698	0.351	6.421252372
	Fraction 4	0.095	1.563567362	0.285	5.168880455
	Fraction 5	0.104	1.734345351	0.193	3.423149905
	Fraction 6	0.062	0.937381404	0.138	2.379506641
		Arg Mutation		C-Terminal	
		OD	Concentration (µg/µL)	OD	Concentration (µg/µL)
Fractions	Fraction 1	0.052	0.747628083	0.06	0.89943074
	Fraction 2	0.206	3.669829222	0.068	1.051233397
	Fraction 3	0.282	5.111954459	0.104	1.734345351
	Fraction 4	0.187	3.309297913	0.25	4.504743833
	Fraction 5	0.146	2.531309298	0.373	6.838709677
	Fraction 6	0.091	1.487666034	0.501	9.267552182

Figure 7. The values of OD and concentrations (µg/µL) for the his-tag purified GRP1 protein, N-terminal fragment, Arg Mutated N-terminal fragment, and C-terminal fragment.

Proteins

GRP1	
OD	Concentration (µg/µL)
0.281	0.27

N-terminal	
OD	Concentration (µg/µL)
0.155	0.149

Arg Mutation	
OD	Concentration (µg/µL)
0.105	0.101

Figure 8. The values of OD and concentrations (µg/µL) for the freeze-dried GRP1 protein, N-terminal fragment, Arg Mutated N-terminal fragment, and C-terminal fragment.

3.3. Dry-freezing

Subsequent to the purification of the proteins, the buffer was exchanged and the samples were lypholized in a freeze-dryer. This was followed by running the freeze-dried fractions on SDS-PAGE gels. Equal fractions were loaded on the gels. Figure 9 shows Ponceau S staining of GRP1 protein and N-terminal fragment. No band was detected using this technique for GRP1 whereas a band was detected at 15 kDa for N-terminal fragment. Notwithstanding, using Coomassie staining (figure 10) a band was successfully identified for GRP1 at 19 kDa. Similarly, Coomassie staining showed a band for N-terminal running at 15 kDa. Moreover, the desired bands were identified using Immunoblotting (figure 11). Several additional bands were identified on the Coomassie stained gels, we don't know what these bands represent, but they might be multimers. More experiments need to be conducted to identify the nature of these bands.

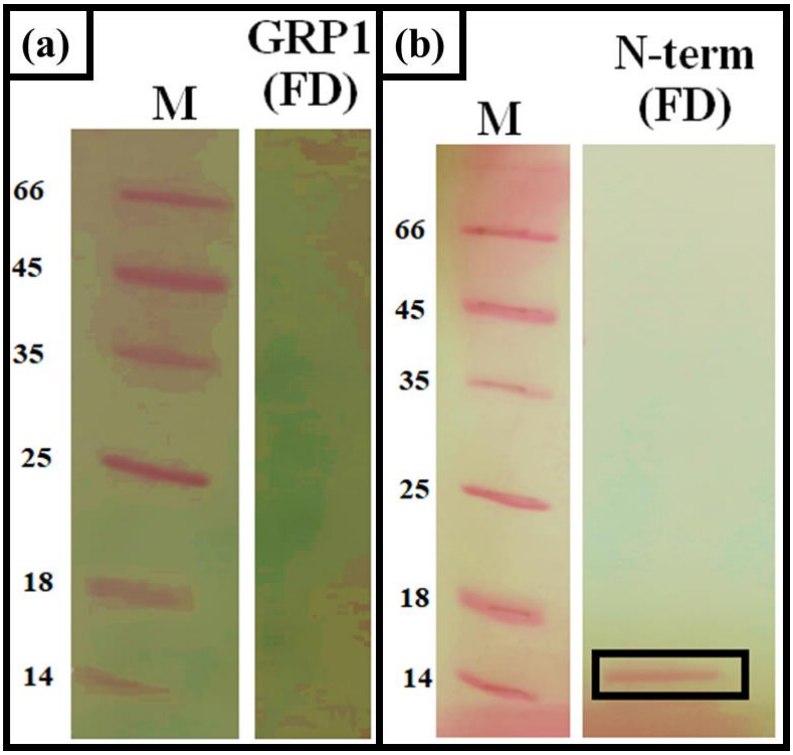

Figure 9. Ponceau S staining of the freeze-dried GRP1 protein (a) and N-terminal fragment (b). Black boxes indicate the desired bands. FD=Freeze-Dried.

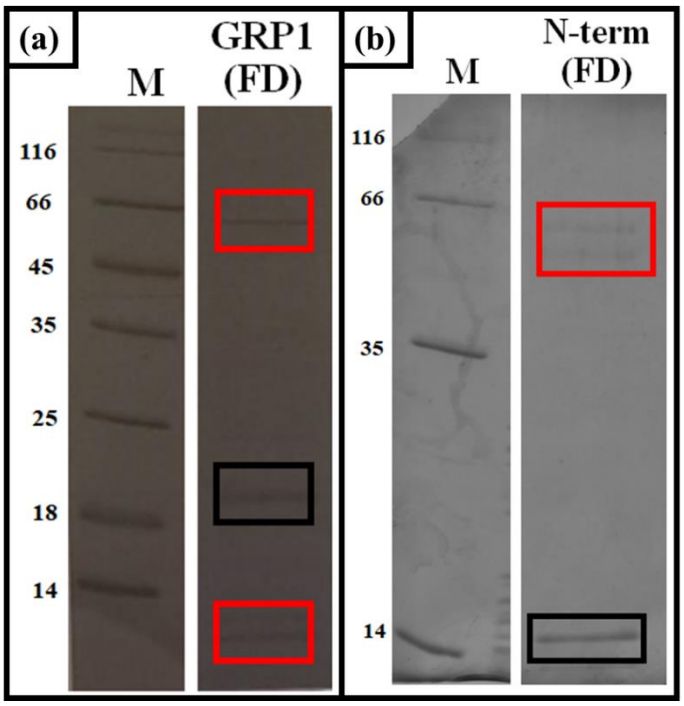

Figure 10. Coomassie staining of the freeze-dried GRP1 protein (a) and N-terminal fragment (b). Black boxes indicate the desired bands. Red boxes indicate additional bands whose nature is still unknown. FD=Freeze-Dried.

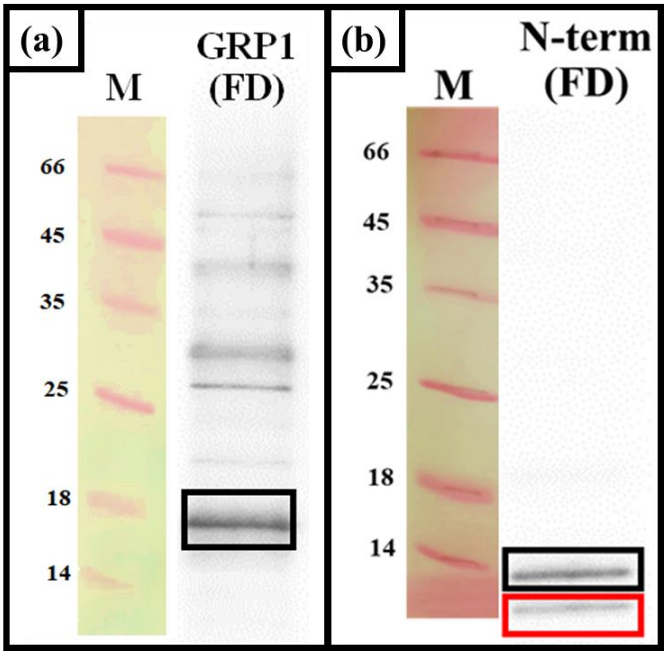

Figure 11. Immunoblotting of the freeze-dried GRP1 protein (a) and N-terminal fragment (b) using the his-tag antibody (as the primary antibody) and a secondary antibody. Black boxes indicate the desired bands. Red boxes indicate additional bands whose nature is still unknown. FD=Freeze-Dried.

3.4. Liposome Binding Assay

The freeze-dried protein fractions were subjected to binding liposomes in order to identify whether they bind lipids in vitro.The bound protein fractions were visualized using Ponceau S staining (figures12a and 13), Coomassie staining (figures12b and 14), and Western Blot (figures). Equal fractions of proteins were loaded on the gels to examine protein-lipid binding affinity and specificity. Regarding the Ponceau S- and Coomassie-stained membranes and gels, no bands were detected.

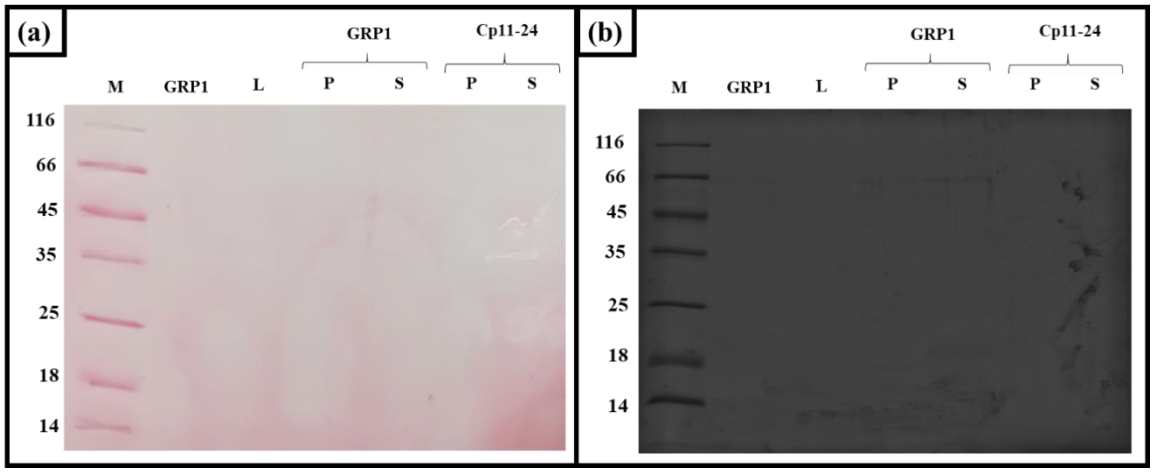

Figure 12. (a) Ponceau S staining of the freeze-dried GRP1 protein followed by liposomes (L) as negative control, Pellet (P) and Supernatant (S) obained centrifuging the GRP1 liposome bound sample, Pellet (P) and Supernatant (S) obained centrifuging the Cp11-24 liposome bound sample. Cp11-24 was used as positive control. (b) Coomassie sataining of SDS-PAGE gel with samples arranged acoording to the same previous order.

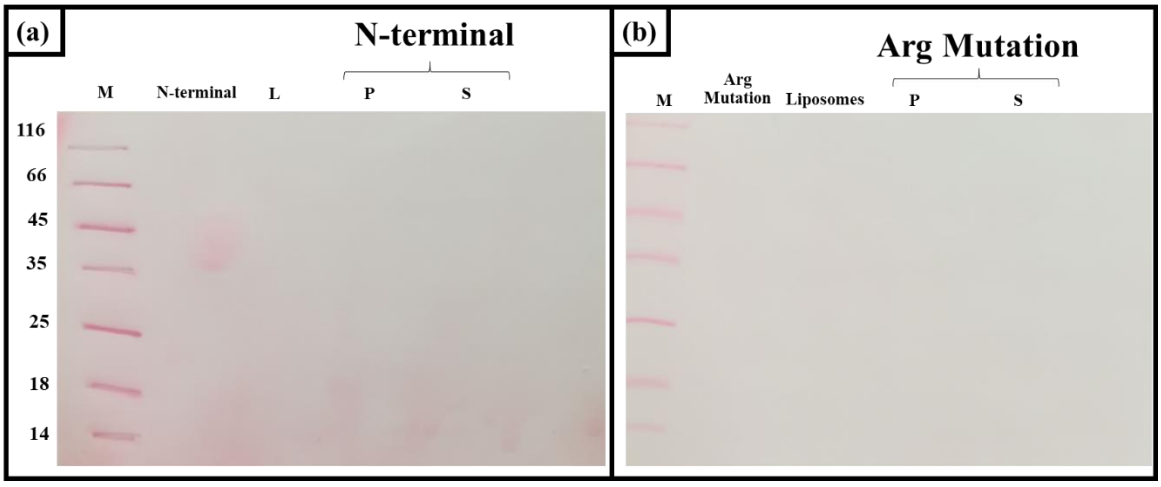

Figure 13. (a) Ponceau S staining of the freeze-dried N-terminal fragment followed by liposomes (L) as negative control, Pellet (P) and Supernatant (S) obained centrifuging the N-terminal liposome bound sample. (b) Ponceau S staining of the freeze-dried Arginine-mutated N-terminal followed by liposomes (L) as negative control, Pellet (P) and Supernatant (S) obained centrifuging the dried Arginine-mutated liposome bound sample.

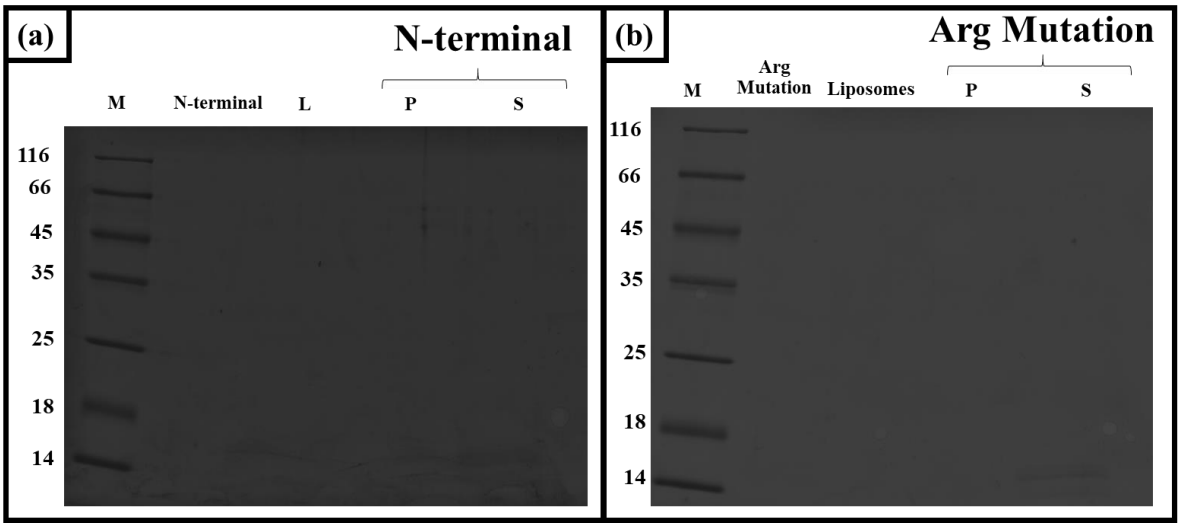

Figure 14. (a) Coomassie staining of the freeze-dried N-terminal fragment followed by liposomes (L) as negative control, Pellet (P) and Supernatant (S) obained centrifuging the N-terminal liposome bound sample. (b) Ponceau S staining of the freeze-dried Arginine-mutated N-terminal followed by liposomes (L) as negative control, Pellet (P) and Supernatant (S) obained centrifuging the dried Arginine-mutated liposome bound sample.

Nevertheless, liposome binding assay (figure 15a) shows the detected bands after applying the his-tag primary antibody. No band was detected in the pellet of Cp11-24. Therefore, after washing the previous membrane, the primary antibody of Cp11-24 was applied to it. A band can be clearly observed in the membrane's pellet slot (figure 15b). Additionally, no bands were in the Ponceau S stained membranes and Coomassie stained gels of N-terminal (figure 13a and 14a) and Arginine mutation (figure 13b and 14 b). The absence of detection in Ponceau S and Coomassie stainings might be atributed to the loading of samples with low concentrations, which can't be detected by either staining methods.

The strong intensity of the GRP1 protein band in the pellet (figure 15a) reflects the large size of the protein fragment. When the aforementioned band is compared with that of N-terminal (figure 16a), the intensity of the latter is fainter, reflecting the smaller size of the N-terminal fragment. C-terminal binding to membranes is expected to subside (results not provided because work was primarily done on the N-terminal fragment). This also shows that the N-terminal is the primary component which binds to membrane lipids. Remarkably, the binding of the Arginine mutated N-terminal fragment, despite having the same szie as the non-mutated N-terminal fragment, to lipids acutely abated (figure 16b). This notes that the two Arginines of the N-terminal play a central role in binding to membrane lipids.

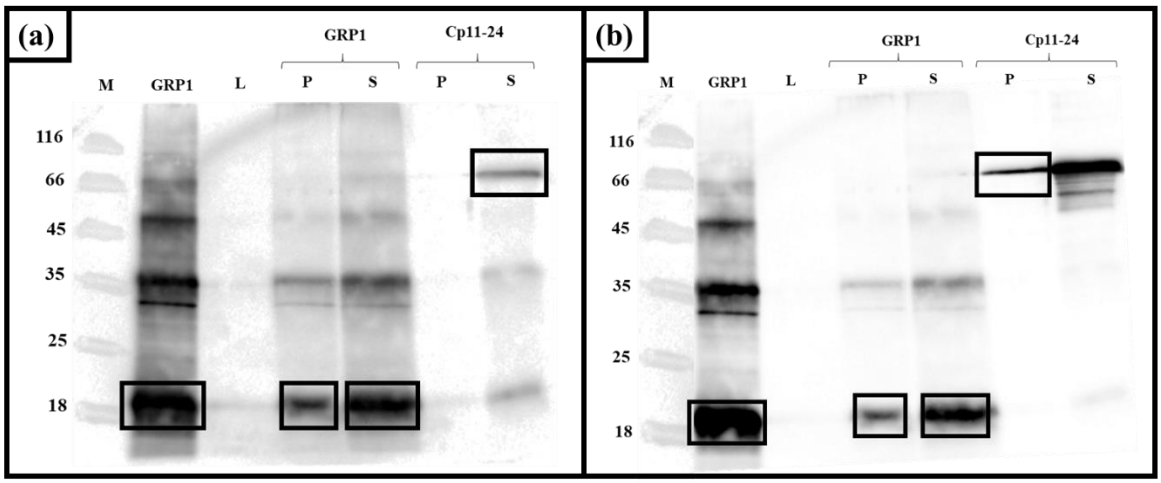

Figure 15. (a) Immunodetection of the freeze-dried GRP1 protein followed by liposomes (L) as negative control, Pellet (P) and Supernatant (S) obained centrifuging the GRP1 liposome bound sample, Pellet (P) and Supernatant (S) obained centrifuging the Cp11-24 liposome bound sample. Cp11-24 was used as positive control. Immunodetection was commenced using the his-tag antibody (as the primary antibody) and a secondary antibody. (b) Immunodetection conducted on the same membrane in (a), however, immunodetection was proceeded using the Cp11-24 antibody and a secondary antibody. Black boxes indicate the desired bands.

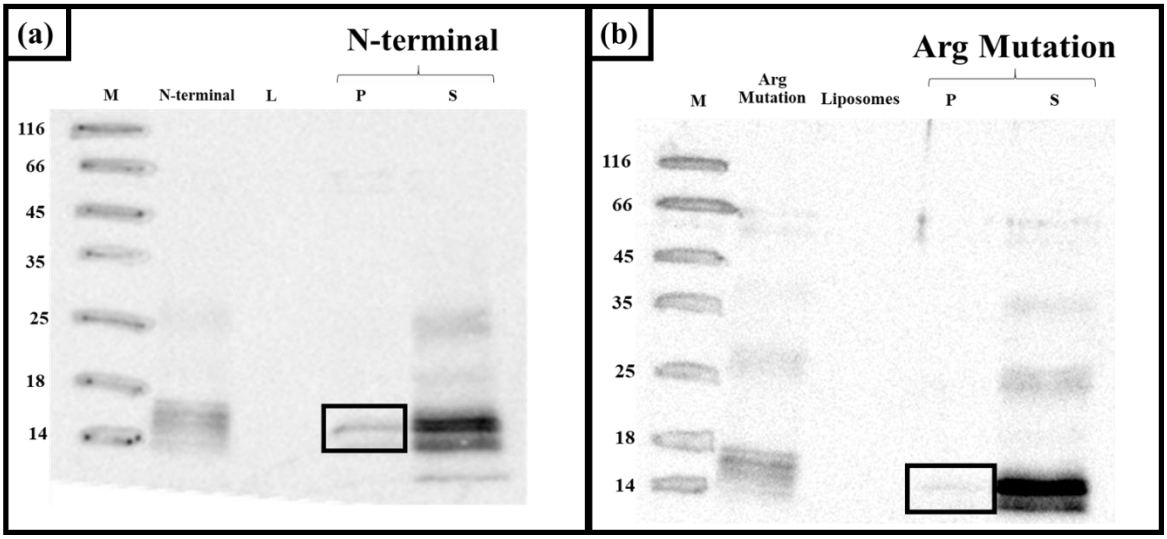

Figure 16. (a) Immunodetection of the freeze-dried N-terminal fragment followed by liposomes (L) as negative control, Pellet (P) and Supernatant (S) obained centrifuging the N-terminal liposome bound sample. (b) Immunodetection conducted on the Arginine-mutated N-terminal fragment. Immunodetection was commenced for the two membranes using the his-tag antibody (as the primary antibody) and a secondary antibody.

4 Discussion

HRGPs (hydroxy-Pro-rich glycoproteins), PRPs (Pro-rich proteins), and GRPs represent the three major classes of structural cell wall proteins (Keller, 1993; Showalter, 1993; Cassab, 1998). GRP genes exhibit tisue-specific and developmentally regulated expression patterns. GRPs have been discovered in all species of higher plants (Ryser and Keller, 1992; Ryser et al., 1997) and more than 150 distincet genes enccoding members of the GRP superfamily were discovered in a large number of seed plant genomes including sugarcane (*Saccharum officinarum*), eucalyptus (*Eucalyptus* sp.), rice (*O. sativa*), and *Arabidopsis* (Mangeon et al., 2009).

The presence of additional motifs, as well as the nature of the glycine repeats, groups them in different classes. The diversity in structure as well as in expression pattern, modulation and sub cellular localization have always indicated that these proteins, although classified as members of the same superfamily, would perform different functions in planta. (Mangeon et al., 2010). In general, the expression of these genes is influenced and moderated by a plethora of abiotic and biotic factors (Mehrel et al., 1990). The strucurally different families of plant GRPs display a wide array of functions in planta. But it was only recently, two decades later after the discovery of GRPs in plants, that our understanding of their functional characterization and their involvement in diverse biological and biochemical processes has been unraveled (Mangeon et al., 2010). Ever since, muliple studies and reviews have exploed tissue expression pattern shed the light on the various aspects of subcellular localizations modulation of gene expression (Ringli et al., 2001; Mehrel et al., 1990), and functional characterization of plant GRPs (Mangeon et al., 2010). HRGPs, PRPs, and GRPs have been shown to have mechano-chemical properties influencing the extracellular matrix (Keller, 1993; Showalter, 1993; Cassab, 1998). Moreover, it has been found that GRPs display cell-type specific expression, showing that GRPs are involved in conferring specific properties to some cell walls only instead of being part of all cell walls. In the species studied by Ringli et al. (2001), GRPs were expressed only in a small number of cells, principally expressed in cells of the xylem tissue.

It has been demonstrated that GRP1 from petunia (Condit et al., 1990) and GRP 1.8 from bean (*Phaseolus vulgaris*) (Keller et al., 1988; Ryser and Keller, 1992) are parts of the cell wall and they are wound induced (Condit and Meagher, 1986). GRP 1.8 prevents water loss by forming a hydrophobic protein layer which hampers the diffusion of water across cell walls of the elongating protoxylem vessels (Ringli et al., 2001). Mangeon et al. (2009) showed that *Arabidopsis thaliana* AtGRP5 is a vacuolar GRP which mediates

organ growth by promoting cell elongation processes. Yang et al. (2014) demonstrated that transgenic rice plants expressing AtGRP2 or AtGRP7 under drought stress conditions presented high recovery rates and grain yields as compared to wild-type plants, suggesting that GRPs can be exploited to improve the yield potential of crops under stress conditions. Previous studies conducted on maize (Gomez et al., 1988) and rice (Mundy and Chua, 1988) have shown that production of GRPs is up-regulated during dehydration. The aforementioned GRPs didn't contain a signal peptide, thereby eliminating the possibility of localization to cell walls. Nonetheless, the impacts of water stress on mechanical properties of cell walls are well documented (Sakurai et al., 1987; Sakurai and Kuraishi, 1988). Harrak et al., (1999) was the first to associate PTGRP (proline-, threonine-, and glycine-rich protein), a drought-regulated protein isolated from the wild tomato species (*Lycopersicon chilense*), with cell wall localization. Furthermore, osmotically stressed wheat coleoptiles expressed a substantial reduction in cell wall stiffening (Wakabayashi et al., 1997) implying that different GRP genes may have opposing roles (Mangeon et al., 2009).

It has been coroborrated that dehydration did not only induce the up-regulation of GRPs in non-resurrection plants but also in resurrection plants (de Oliveira et al., 1990). Elongation and shrinkage of cell walls are an integral part of acclimation to drought stress in desiccation plants. Concomittent with this observation, the *C. plantagineum* CpGRP1 which is a transcriptionally reglated GRP has been shown to accumulate in the apoplast of desiccated leaves. CpGRP1 works in synchrony with the CpWAK1 kinase to induce revesible morphological changes in the cell wall during dehydration (Giarola et al., 2017). GRP may have the ability to specifically interact with a WAK kinase in desiccation tolerant plant *Sporobolus stapfianus* (Blomstedt et al., 2010). Likewise, cell wall profiling by Fourier transform infrared spectroscopy revealed that the expression of the dehydration-induced BhGRP1 gene in the desiccation tolerant *Boea hygrometrica* was increased upon desiccation and plateaued after re-watering. BhGRP1 is involved in cell wall maintenance and mediates the repair processes during dehydration and rehydration (Wang et al., 2009; Wu et al., 2009).

Upon dehydration, the structure of the plasma membranes are significantly altered, affecting intracellular metabolism as well as lipid-protein and protein-protein associations (Navari-Izzo and Rascio, 1999; Leprince et al., 2000; Navari-Izzo et al., 2000; Kerkeb et al., 2001) which is essential for preserving the integrity of plasma membranes (Hernandez and Cooke, 1997). Studies conducted on resurrection plants Boea hygroscopica and Sporobolus stapanus have showed that after sever dehydration, the lipid content

drastically diminishes to low levels (Navari-Izzo et al., 1995; Quartacci et al., 1997). The reduction is attributed to the decrease in the total membrane area of the cells (Hernandez and Cooke, 1997). Therefore, resurrection plants evolved several measures which would allow them to maintain the integrity of plasma membranes after dehydration and effortlessly recover after rehydration (Quartacci et al., 1997; Navari-Izzo et al., 2000). Strategies to counteract the dehydration-induced mechanical stress effects to prevent the rupture of the plasma membrane include the accumulation of non-polar lipids (Navari-Izzoet al., 1990; Quartacciet al., 1997), decrease in the degree of fatty acid desaturation (Monteiro de Paula et al., 1990; Daklma et al., 1995), and storage of of proteins, lipids, and carbohydrates within vacuoles which would ultimately replace lost water (Vicre´ et al., 2004). Moreover, *Myrothamnus flabellifolius* employs another method to minimize mechanical damage to cell walls by promoting cell wall folding (Moore et al., 2006). High levels of free sterols were discovered in the plasma membranes of dehydrated Ramonda serbica leaves (Quartacci1 et al., 2002). Sterol enrichment can be interpreted as an adaptation mechanism to alter membrane fluidity and permeability to water, two responses which are commonly associated with sterol-induced membrane rigidifcation (Yoshida and Uemura, 1990; Quartacci et al., 2001).

Members of the phospholipase D (PLD) family are characterized by their involvement in salt and dehydration mediated responses. PLDs catalyzes the hydrolysis of structural phospholipids to produce phosphatidic acid (PA) which acts downstream as a second messenger in signal transduction pathways (Munnik, 2001) eventually recruiting protein targets and thereby moderating their activities (Hou et al., 2016). PLDα1 and its derived PA have been shown to not only decrease transpirational water loss but also promote ABA-mediated stomatal closure (Sang et al., 2001; Zhang et al., 2004).

Several GRPs have been shown to have a huge role in responding to enviornmental stresses. For instance, the expression GRP7 is mediated by environmental stresses (Carpenter et al., 1994; Kwak et al., 2005). Cao et al.(2006) demonstarted that GRP7 of *A. thaliana* plays an essential role in the regulation of abscisic acid (ABA) and stress responses. Due to the differentil expression of the family of GRP proteins the up-regulation of GRP7 expression was detected under freeze-stress while its down-regulation was promoted by high salinity or dehydration stresses (Kwak et al., 2005). This implies that under salt or dehydration stressconditions, GRP7 elicits a negative effect on seed germination and seedling growth (Kim et al., 2008). Mayfield et al. (2001) has identified an oleopollenin termed GRP17 in the pollen of *A. thaliana*. Illustrating how members of GRP family are important in acclimating seeds and grown plants to dehydration and drought stress.

In this study the binding affinity of GRP1 protein along with its truncated and mutated Long fragments to PA was examined. In silico screenings have lead to the identification of CpGRP1 as a putative novel gene (Giarola et al., 2015b). Furthermore, the analysis conducted on the domain organization of GRP1 protein discerned CpGRP1 as a member of the class II secreted glycine-rich proteins (Giarola et al., 2016). Lipid binding assays conducted in the Lab have shown that GRP1 binds PA (figure). The truncated long fragement (N-terminal) has exhibited stronger binding to PA as compared to the truncated short fragment (C-terminal) conferring on the long fragment a more important role in binding to membrane phospholipids. Different amino acid mutations were examined to identify the ones mediating PA-binding. Proteins with mutated cysteines displayed a small decline in binding to PA whereas mutating the arginines immennsly curtailed the binding to PA (figure). To validate the results of lipid binding assay, I conducted liposome binding assay on the GRP1 protein, truncated long fragement (N-terminal), and the arginine-mutated N-terminal fragment. My results showed that binding affinity to PA weakened in the arginine-mutated N-terminal fragment as compared to truncated long fragement. The decrease in binding affinity in the arginine mutated N-terminal fragment associates the role of modulating lipid-binding to the two arginines.

Conclusion

Mechanisms governing stress perception the downstream pathways regulating gene expression are universal in the plant kingdom. Bray (1993) indicated that the expression of genes subsequent to drought-stress exposure highly resemble gene expression operating during seed desiccation. Thus, the study of resurrection plant adaptations to water deficit has become an important domain of science aiding scientists in developing new plants that can withstand severe water loss. Here we propose that the characterization of the structure and function of novel GRPs such as CpGRP1 can provide insightful data pertinent to enhancing stress tolerance mechanisms, thereby facilitating the production of new genotypes with enhanced stress tolerance or rapid growth and development under sub-optimal environmental conditions (Miao et al., 2015; Pandey et al., 2015; Wang et al., 2016; Rihan et al., 2017).

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