Spectrometric and *in-silico* assessment of the adenylyl cyclase activity of a recombinant clathrin assembly protein from *Arabidopsis thaliana*

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DECLARATION

I, Savannah Fazila Brandt, declare that the dissertation entitled "Spectrometric and *in-silico* assessment of the adenylyl cyclase activity of a recombinant clathrin assembly protein from *Arabidopsis thaliana*" and submitted to the Department of Botany at the North-West University, Mafikeng campus for the Master of Science in Biological Sciences (Botany) degree has never been submitted for any degree or examination at this university or any other university or institution elsewhere. This is my own work and all the sources used or quoted here have been properly indicated and sincerely acknowledged.

Student: Savannah Fazila Brandt

Signature: ..... **Date: .....**

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DEDICATION

This dissertation is gratefully dedicated to my family, close friends, the Plant Biotechnology Research Team, my supervisor Prof. O Ruzvidzo and my co-supervisors; Dr D Kawadza and Dr T Dikobe. Thank you for your support, knowledge, mentorship, and motivation, I honour and appreciate all the support throughout my studies.

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LIST OF TERMS

Abiotic stress: The negative impact of non-living factors on other living organisms in a specific environment.

Adenylate cyclase: An enzyme that synthesizes cyclic adenosine monophosphate (cAMP) from adenosine triphosphate (ATP).

Annotation: A determination of the locations of genes, their coding regions in a genome and the functions of such genes.

Bioinformatics: The collection, classification, storage, and analysis of biological information using computers.

Biotic stress: A stress aspect that occurs on plants as a result of damage done to them by other living organisms.

Clathrin assembly protein: A protein that stimulates the assembly of clathrin lattices onto cellular membranes.

Climate change: The long-term shift in weather patterns in a specific region or globally.

Electron cryotomography: An imaging technique used to produce high-resolution (~4 nm) three-dimensional views of samples, typically biological macromolecules and cells.

Cyclic adenosine 3′,5′-monophosphate (cAMP): A second messenger cyclic nucleotide molecule formed from adenosine triphosphate (ATP) by the action of the enzyme adenylate cyclase that participates in signal transduction.

Domain: A conserved part of a given protein sequence and structure that can evolve functions and exist independently of the rest of the protein chain.

Elucidate: An explanation that results from interpreting something.

Endocytosis: A process by which cells take up molecules (such as proteins) through invagination and engulfment.

Enzyme immunoassay: A test that combines antibody binding with enzymatic detection systems to quantify biological molecules of interest.

Epsin N-terminal homology (ENTH): A structural domain found in proteins that are involved in endocytosis and the cytoskeletal machinery.

Eukaryote: Any organism whose cells have a nucleus enclosed within membranes.

Genome: A full haploid set of chromosomes with all its genes; the total genetic constitution of a cell or organism.

Germination: A process by which an organism grows from a seed or similar structure.

Homeostasis: The tendency towards a relatively stable equilibrium between interdependent elements, especially as maintained by physiological processes.

***In-silico*:** A process performed on computer or via computer to read and process stored complex genetic sequences.

***In-vitro*:** Occurring or made to occur in a laboratory vessel or other controlled experimental environments rather than within a living organism or natural system.

***In-vivo*:** Occurring or made to occur within a living organism or natural setting.

Neurotransmission: The process by which signalling molecules called neurotransmitters are released by the axon terminal of a neuron (the presynaptic neuron), and bind to and react with the receptors on the dendrites of another neuron (the postsynaptic neuron)

Palindromic sequence: A nucleic acid sequence on double-stranded DNA or RNA wherein reading 5′ to 3′ forward on one strand matches the opposite sequence reading 5′ to 3′ on the complementary strand.

Prokaryote: A unicellular organism that lacks a membrane-bound nucleus or any other membrane-bound organelle.

Second messenger: A biological molecule capable of transmitting external cellular signals within the cell for the development of appropriate cellular responses through regulated gene expressional and metabolic events.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE): The separation of proteins according to their molecular weights, and based on their differential rates of migration through a sieving gel matrix under the influence of an applied electrical field.

Second messenger: Molecules that relay signals received at receptors on the cell surface to target molecules within the cytosol and/or nucleus.

Sequestered: A process of isolating, taking up or hiding away.

Signal transduction: The transmission of molecular signals from a cell's exterior to its interior or otherwise.

Spectrometry: An analytical technique that measures the mass-to-charge ratio of charged particles.

Transformation: The genetic alteration of a cell resulting from the direct uptake and incorporation of exogenous genetic material.

X-ray crystallography: A technique used for determining the atomic and molecular structure of a crystal, in which the crystalline atoms cause a beam of incident X-ray to diffract into many specific directions.

LIST OF ABBREVIATIONS

AC	**:**	Adenylate cyclase
ANOVA	**:**	Analysis of variance
ANTH	**:**	AP180 N-terminal homology
AtCAP	**:**	*Arabidopsis thaliana* clathrin assembly protein
AtCAP-AC	**:**	*A. thaliana* clathrin assembly protein-adenylate cyclase
ATP	**:**	Adenosine 5′-triphosphate
cAMP	**:**	Cyclic adenosine 3′,5′-monophosphate
CAP	**:**	Clathrin assembly protein
CCV	**:**	Clathrin-coated vesicle
CME	**:**	Clathrin mediated endocytosis
CRISPR	**:**	Clustered regularly interspaced short palindromic repeats
EDTA	**:**	Ethylene di-amine tetra-acetic acid
ENTH	**:**	Epsin N-terminal homology
IPTG	**:**	Isopropyl-β,D-thiogalactopyranoside
LB	**:**	Luria-Bertani
Ni-NTA	**:**	Nickel-nitrilotriacetic acid
OD	**:**	Optical density
Rpm	**:**	Revolutions per minute

SDS	**:**	Sodium dodecyl sulphate
TAIR	**:**	The Arabidopsis Information Resource
VHS	**:**	VPS-27, Hrs and STAM
YT	**:**	Yeast-tryptone

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ABSTRACT

Adenylate cyclases (ACs) are enzymes that catalyse formation of the second messenger molecule, 3′,5′-cyclic adenosine monophosphate (cAMP) from 5′-adenosine triphosphate (ATP). Although cAMP is recognised as an important role player in signalling pathways in higher plants, ACs are still yet to be fully consolidated as there are currently only eight experimentally confirmed such molecules in higher plants. In this study and in an effort to provide additional information on what is currently available about ACs in higher plants, we report the recombinant expression of an *Arabidopsis thaliana* clathrin assembly protein (AtClAP; At1g68110) and characterization of its function by means of mass spectrometry and *in silico* analysis. This AtClAP protein was initially annotated bionformatically as a putative AC candidate, followed by its recent practical confirmation as an AC, However, its AC function has not yet been fully elucidated. Our findings from this study have established the AtClAP protein as part of several cellular response systems responsible for growth, development and response to various environmental stress challenges in plants.

Our findings from this study have established the AtC1AP protein as part of several cellular response systems responsible for growth, development and response to various environmental stress challenges in plants. In our study we used the *E. cloni* BL21 (DE3) pLysS DUOs cells as the expression host strain of choice and as a result the desired and targeted recombinant AtClAP protein was successfully expressed at its expected molecular weight size of around 22.6 kDa. Consequently, our study succeeded to purify the expressed recombinant AtClAP protein using an established chromatographic approach. The successfully purified protein was assessed by mass spectrophotometry and this detected high cAMP activity and other molecular components. The detected molecular components are proved to be key elements of the cell signaling systems in most living organisms including plants- necessary for essential cellular processes such as growth, development and responses to various environmental stress factors.

After determining the enzymatic capacity of the recombinant AtC1AP protein we wanted to analyse its functional role in plants by means of an *in silico* analysis. The findings indicated AtClAP protein to be co-expressed with a wide array of other proteins of which the 25 top most proteins were specifically involved in peroxisomal transport, photoperiodic flowering, lipid modification, to mention a few. These are proven key cellular processes essential for plant growth, development and responses to various environmental stress factors.

Keywords: *Arabidopsis thaliana*, clathrin assembly protein, adenylate cyclase, cAMP, spectrometry, *in silico* analysis.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Plants are constantly faced with unfavourable or stressful conditions which affect their normal growth and development. These conditions include biotic stresses such as pathogen infections and herbivore attacks, and abiotic stresses such as droughts, high and low temperatures, nutrient deficiencies, and excess salts or toxic metals. Drought, salt and temperature stresses are believed to be the major environmental factors that affect the geographical distribution of plants in nature, limit plant productivity in agriculture, and threaten food security (Zhu, 2016). Apparently, agriculture remains the main thrust of many countries in Africa, as the principal source of food and livelihood, making it a critical component of programs that seek to reduce poverty and attain food security in the continent (Ogundari, 2014). However, in recent years, food security has become a serious concern in Africa, especially in sub-Saharan Africa (SSA) (Ogundari, 2014). Biotechnology plays an important role in solving problems associated with agricultural production as it has the potential to deal with specific problems such as increases in crop productivity, crop diversification, and enhancement of food nutritional values and reduction of environmental stress factors.

From all of the known species of flowering plants on earth, *Arabidopsis thaliana* stands out as one of the most thoroughly studied candidates for plant stress responses (Koornneef and Meinke, 2010). One of the main reasons why this plant is used as a model organism in Plant biology is because it possesses desirable characteristics such as a small genomic structure, extensive genetic and physical maps of all chromosomes, a rapid life cycle (about 6 weeks

from germination to mature plant), a prolific seed production system and easy cultivation in restricted spaces - thus making it very desirable for research studies, to fully understand plant genomes with a future anticipation of applying these knowledge to economical crops.

Recent studies suggest that genome editing using the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein9 nuclease (Cas9) system can be used to modify plant genomes. CRISPR/Cas9 system has been developed in model plants such as *Arabidopsis* and tobacco and is now utilized effectively in rice with high efficiency. It has also been utilized in other crop species, such as sorghum, wheat, maize, sweet orange, soybean and woody plants such as poplar. The results suggest further applications in molecular breeding to improve plant function using optimized plant CRISPR/Cas9 systems (Osakabe *et al*., 2016).

This study addressed plant enzymatic molecules known as adenylate cyclases (ACs), in an effort to elucidate how plants respond to stressful conditions. To date, there are only nine experimentally tested and functionally confirmed AC's in plants; eight in higher plants and one in lower plants. Those in higher plants are the *Zea mays* pollen protein (ZmPSiP; AJ307886) responsible for the polarized pollen tube growth (Moutinho *et al*., 2001), the *A. thaliana* pentatricopeptide repeat protein (AtPPR-AC; At1g62590) responsible for cellular responses during pathogen attack and gene expressions (Ruzvidzo *et al*., 2013), the *Nicotiana benthamiana* adenylyl cyclase protein (NbAC; ACR77530) responsible for the tabtoxinine-β-lactam-induced cell deaths during wildfire diseases (Ito *et al*., 2014), the *Hippeastrum hybridum* adenylyl cyclase protein (HpAC1; ADM83595) involved in stress signalling (Świeżawska *et al*., 2014), the *A. thaliana* K^+-uptake permease 7 (AtKUP7; At5g09400) responsible for facilitating transport of potassium ions across cells (Al-Younis *et al*., 2015), the *A. thaliana* clathrin assembly protein (AtC1AP; At1g68110) involved in plant defense (Chatukuta *et al*., 2018), the *A. thaliana* K^+-uptake permease 5 (AtKUP5; At4g33530) involved in potassium ion transport (Al-Younis *et al*., 2018) and the recently identified *A. thaliana*

leucine rich repeat protein (AtLRRAC1; At14460) involved in responses to pathogens (Bianchet *et al*., 2018). The one in lower plants is the class III AC (MpCAPE; Mp0068s0004) in the liverwort *Marchantia polymorpha*, whose AC activity is combined with the phosphodiesterase activity (PDE) and has an essential function in the male reproductive process (Kasahara *et al*., 2016). Among all these confirmed ACs, the AtC1AP is part of the ENTH/ANTH/VHS domain, located in the Golgi apparatus, mitochondrion, clathrin-coated pit, and clathrin-coated vesicle and is involved in clathrin coat assembly; endocytosis (TAIR:http://www.arabidopsis.org).

Naturally, cells are activated by molecular signals at the cell surface to trigger a flow of biochemical reactions that lead to the appropriate responses. These responses will allow the cells to grow and develop and adapt to stress associated factors such as salinity and drought. Some of these molecular signal molecules include cyclic monophosphates such as 3′, 5′- cyclic guanosine monophosphate (cGMP) and 3′, 5′- cyclic adenosine monophosphate (cAMP), which play an important role in cellular signal responses (Xu *et al*., 2018).

Cytosolic homeostasis is crucial to optimal cell metabolism and the activity of various cytosolic enzymes in many cellular processes such as photosynthesis and protein synthesis to mention but a few (Al-Younis *et al*., 2018). Endocytosis is a fundamental process for engulfing external materials as well as regulating the abundance and distribution of plasma membrane proteins and lipids. Plant genomes have many genes that are similar to endocytosis-related genes in animals and yeasts but, very few have been observed and thus often poorly understood (Fujimoto *et al*., 2010). Clathrin-mediated endocytosis is the uptake of material into the cell using clathrin-coated vesicles. This process is fundamental to the essential eukaryotic cell processes such as neurotransmission, signal transduction and the regulation of many plasma membrane activities (McMahon and Boucrot, 2011). This process is used by all known eukaryotic cells, although other clathrin-independent entry portals exist, but their molecular

details and cargo specificity being not well defined as is the clathrin-mediated endocytosis (McMahon and Boucrot, 2011).

The clathrin assembly protein is involved in many cellular processes, endocytosis being the main one and annotated as a functional AC (Chatukuta *et al*., 2018). However, despite the progress made in recent years regarding the structure and function of the ANTH/ENTH/VHS family, further elucidation of this protein as an AC is still essential and necessary of which this study was specifically focusing on. On the other hand, we are still far from fully understanding the mechanisms involved and hence, the undertaken spectrometric and *in-silico* analyses were poised to help broaden our understanding on how plant ACs function and the role they play in cell communication and signalling.

1.2 LITERATURE REVIEW

1.2.1 Cyclic Adenosine Monophosphate (cAMP) and its Role in Cellular Function

The second messenger, cAMP is possibly one of the most widely studied, and a great amount of research suggests its pivotal role in cell metabolism, cell growth and differentiation of both prokaryotic and eukaryotic organisms. It is believed that cellular responses and signals are mediated by cAMP, thus acting as an intracellular second messenger. The molecule cAMP is produced from ATP and the enzyme that catalyzes this process is known as an AC. In order to recognize changes in the environment and to respond to them appropriately, all organisms rely on signal transduction pathways. These pathways include signal recognition, transfer of the signal and the response. Second messengers are therefore very essential and key components of many signal transduction pathways (Commichau *et al*., 2015).

1.2.2 The Clathrin Assembly Proteins

According to Chen *et al*. (2011), a clathrin consists of heavy and light chains, which function as a coat protein during vesicle generation (Chen *et al.*, 2011). Clathrin, a self-assembling protein, is recruited to membranes from the cytoplasm of eukaryotic cells to form a protein coat (Brodsky, 2012). The purpose of this protein coat is to sort proteins in the membrane and it contributes to membrane deformation, thus resulting in transport of sequestered cargo in a clathrin-coated vesicle or segregation of proteins in a clathrin-coated membrane patch. Clathrin sorts proteins at the plasma membrane, at endosomal membranes, and in the trans-Golgi network (TGN) for organelle biogenesis as well as in numerous specialized pathways with physiological relevance (Brodsky, 2012). According to a study by Daboussi *et al.* (2012), the progression of adaptor-specific clathrin coat formation reveals a previously unrecognized process of TGN maturation (Daboussi *et al*., 2012).

A process that occurs in all eukaryotic cells called endocytosis, is a cellular process by which external molecules or plasma membrane-localized proteins are internalized to early endosomes (Nguyen *et al*., 2018). Endocytosis and subsequent trafficking pathways are complex processes involving many proteins such as coat proteins, adapter proteins, and accessory proteins. These processes include various steps such as the collection of cargoes, assembly of vesicles, only to mention a few (Nguyen *et al*., 2018).

The exchange of material between cellular compartments is mostly conducted by coated transport vesicles, and the formation of transport vesicles is mediated by cytosolic coat proteins (Faini *et al*., 2013). According to Faini *et al.* (2013), recent advances using structural biology approaches including X-ray crystallography, cryoelectron tomography (cryo-ET) and cryoelectron microscopy (cryo-EM), have given new structural insights into protein complexes

implicated, thus presenting that clathrin-coated vesicle (CCV) act in the latter secretory pathway and in the endocytic pathway (Faini *et al*., 2013).

1.2.3 The Function of the Domains of the Clathrin Assembly Proteins

The clathrin protein falls under the class of ANTH/ENTH/VHS domain-containing proteins and it is believed that more than 30 *A. thaliana* proteins contain this domain (Zouhar and Sauer, 2014). Presumably, the ANTH/ENTH/VHS domain-containing proteins are important factors in CCV generation. The ENTH domain contains 8 helices comprising roughly 130 to 150 amino acids and the ANTH domain contains 9 to 10 helices and is considerably longer, at 250 to 300 amino acids. In addition, the VHS domain is very similar to the ENTH domain, the VHS domain contains 8 helices and is 150 amino acids in length (Zouhar and Sauer, 2014). Studies suggest that the ANTH/ENTH/VHS domain proteins are involved in various instances of clathrin-related endomembrane trafficking in plants (Zouhar and Sauer, 2014). Furthermore , Zouhar and Sauer (2014), proposed, based on recent studies through phylogenetic analysis and through findings from the literature a functional classification in which ENTH-containing proteins such as EPSINs, play roles in post-Golgi (presumably vacuolar) transport, while ANTH-containing proteins are involved in endocytosis, and TOLs are required for the selection of PM-derived cargo for vacuolar degradation (Zouhar and Sauer, 2014).

Based on the *in-silico* analyses of the ANTH/ENTH/VHS superfamily by De Craene (2012), results suggest that metabolism, cytokinesis and intracellular trafficking pathways co-evolved with membrane trafficking at the centre of this co-evolutionary process. Furthermore, the study suggests that membrane trafficking and compartmentalization were not only key features for the emergence of eukaryotic cells but also drove the separation of eukaryotes in the different taxa (De Craene *et al*., 2012).

1.2.4 Clathrin-dependant Endocytosis

Clathrin-mediated endocytosis (CME) is a developmental endocytic pathway that plays a key role in the regulation of protein abundance at various sites during signalling events and re-targeting or degradation of membrane proteins (Wang *et al*., 2013). Furthermore, Dannhauser and Ungewickell (2012) suggest that during the process of clathrin-mediated endocytosis, a detailed area of the membrane undergoes a gross deformation to form a spherical bud. In addition, literature also suggests that there are three ways by which the membrane can be induced to form this bud; one of which is the formation of a protein scaffold over the surface and such a scaffold being spontaneously generated by clathrin (Dannhauser and Ungewickell, 2012). According to McMahon and Boucrot (2011), clathrin-mediated endocytosis controls receptor signalling, responses to channel activation and transporter activity, and this is all due to its ability to influence the surface protein composition of cells (McMahon and Boucrot, 2011).

1.2.5 Clathrin-independent Endocytosis

As previously mentioned, endocytosis is used by cells to internalise different molecules. Not all of these molecules go through the same pathways, as their molecular compositions differ. Before any endocytic pathway is initiated, mechanisms for specific cargo selection must first be established at the cell surface (Mayor and Pagano, 2007). Clathrin-mediated endocytosis has provided an important insight for the analysis of membrane trafficking in cells, however, several endocytic pathways that do not use clathrin have also been discovered (Figure 1.1) (Mayor and Pagano, 2007).

A research study by Boucrot *et al.* (2015) shows that the BAR (Bin/amphiphysin/Rvs)-domain-containing protein endophilin A, an endocytic protein that recruits dynamin and evidently shows an involvement in CME is also involved in clathrin-independent (CI) endocytic pathways. Boucrot *et al*. (2015) also discovered a new route called the fast endophilin-mediated endocytosis (FEME). In order to construct an endocytic vesicle, cargo recruitment adaptors, membrane scission machinery and others are needed, but endophilin possesses all these characteristics in one protein, thus reporting a fast CI endocytosis (Boucrot *et al*., 2015).

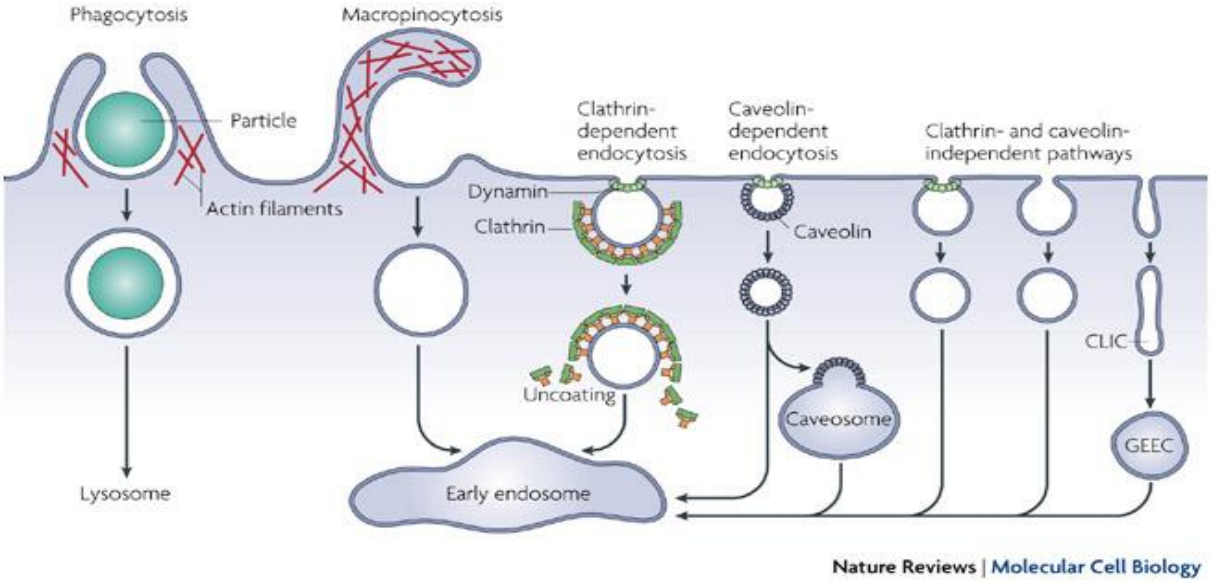

Figure 1.1: Different pathways of entry into a cell. Large particles can be taken up by the cell through phagocytosis, whereas fluids can be taken up by a process called macropinocytosis. In both cases, the plasma membrane remodelling is actin-mediated. Several cargoes can be taken up by the cell through mechanisms that are independent of clathrin coat proteins. Clathrin-independent pathways show independence of dynamin. This review shows that cargoes can also be delivered to early endosomes via tubular intermediates (known as clathrin- and dynamin-independent carriers (CLICs)) that are derived from the plasma membrane (taken from Mayor and Pagano, 2007).

1.2.6 Osmotic Stress in Plants

Osmotic variations in the soil can hinder plant growth and development. Several agricultural losses are caused by limiting factors for crop growth and development such as drought stress. In recent years, plant producers have found means to reduce chemical fertilizers by new ways such as increasing crop inoculation with plant growth promoting rhizobacteria (PGPR) (García *et al*., 2017). The use of these innovative breakthrough technologies can help plants growing under these extreme situations face at least, some types of stress factors such as drought by increasing root length, which allows for better access to water (García *et al*., 2017).

Osmotic stress can affect different plant species differently. Experiments with *Triticum aestivum* L. by Ahanger and Agarwal (2017), whereby plant species when grown under restricted and normal irrigation conditions with potassium applied in varying doses, results suggested that supplementing these plants with potassium increased plant growth and activity of antioxidant enzymes in both experimental and control plant cell lines. The outcome showed that this occurred as a result of the potassium increasing the total phenols and tannins, thus strengthening components of the enzymatic and non-enzymatic antioxidant system (Ahanger and Agarwal, 2017).

1.2.7 Clathrin-mediated Endocytosis and Osmotic Stress

During the process of endocytosis, a mechanism called scaffolding is used to create membrane curvature by shaping the membrane to form the spherical shape that is the clathrin cage. A study by Saleem *et al.* (2015) show that membranous factors such as membrane tension, affects clathrin polymerization. By altering the osmotic conditions, Saleem and colleagues found that

the clathrin coats cause extensive budding, polymerization into shallow pits, and complete inhibition of polymerization under low membrane tension, moderate tension and high tension respectively (Saleem *et al*., 2015).

Plant cells need to maintain a perfect balance between the extracellular and intracellular surroundings, but it is known that it is not always possible as plants are faced with different environmental conditions. During osmotic stress, the plasma membrane integrity is gravely affected and this causes a huge problem as the plasma membrane serves as the main means by which the cell communicates with its surroundings. Osmotic stress can be categorized into two conditions: hypo-osmotic conditions, which increase cell volume and hyper-osmotic conditions, which decrease cell volume (Zwiewka *et al*., 2015).

A study by Zwiewka *et al.* (2015) show that when cells are treated with hyper-osmotic stress factors, the situation promotes the intake of plasma membrane proteins by clathrin-mediated endocytosis. The cells of other species such as animals have been shown to immediately adapt to increase in cell volume by using other mechanisms but only for a short while until the cell requires the addition of membrane materials to the plasma membrane, thus stressing the importance of membrane trafficking mechanisms (Zwiewka *et al*., 2015).

Even though the AC activity of the AtC1AP protein has been partially characterised, its comprehensive characterization is still necessary. Therefore, the specific focus of this study was to create a better understanding of this protein in plants with respect to molecular trafficking and responses to the various environmental stress factors.

1.3 PROBLEM STATEMENT

The clathrin assembly protein (C1AP) from *A. thaliana* (AtClAP) is an important biomolecule encoded by the gene At1g68110 from the ENTH/ANTH/VHS superfamily. The protein has previously been associated with stress response (De Vos and Jander, 2009, McLoughlin *et al*., 2013) and recently, confirmed as a functional AC with a role in endocytosis (Chatukuta *et al*., 2018). However, more information regarding its role as an AC still needs to be explored and carefully unpacked to enhance our insight of how plants respond to various biotic and abiotic stress factors . More so, this would also create a good platform for acquiring knowledge to be applied into our own agricultural crops for enhanced crop production and sustainable food security.

1.4 RESEARCH AIM

The aim of this research study was to assess the known AC activity of a recombinant clathrin assembly protein (AtClAP) from *A. thaliana* by means of spectrometric and *in-silico* analyses.

1.5 OBJECTIVES

The following objectives were set to address the aim of the study:

1. To recombinantly express the heterologous AtClAP protein from *A. thaliana*.
2. To purify the recombinant AtClAP protein under native non-denaturing conditions through affinity purification.

3. To characterize the AC activity of the recombinant AtClAP protein through spectrometry.

4. To further characterize the AC activity of the recombinant AtClAP protein using *in silico* analysis.

5. To infer the probable mechanism by which AtClAP participates in stress response and adaptation mechanisms in Arabidopsis and other closely related plants.

1.6 SIGNIFICANCE OF STUDY

The research study was intended to provide additional insights into the AC activity of a recombinant clathrin assembly protein (AtClAP), encoded by the annotated At1g68110 gene. The study is significant as it would contribute towards a better understanding of the general mechanisms through which plants respond and adapt to adverse environmental conditions. The knowledge obtained from this study of such genes responsible for stress response and adaptation mechanisms in plants would subsequently contribute towards an improved management and sustainability of most agronomically important crops in the Southern African region and particularly South Africa as a developing country.

CHAPTER TWO

MATERIALS AND METHODS

2.1 PARTIAL EXPRESSION AND AFFINITY PURIFICATION OF AtClAP

2.1.1 Recombinant Expression of the AtClAP

A single colony of the *E. cloni* BL21 (DE3) pLysS DUOs cells harbouring the pTrcHis2-TOPO:AtC1AP fusion construct was collected from the Plant Biotechnology Research Laboratory – North-West University (where it was previously designed).

The fusion construct was used to inoculate 10 ml of the selective LB broth (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1.0% (w/v) sodium chloride, 0.5% (w/v) glucose, 100 µg/ ml ampicillin, 34 µg/ml chloramphenicol, pH: 7.0) followed by incubation of the culture overnight at 37° C in an orbital shaker shaking at 200 rpm. The next day, 1 ml of the overnight culture was used to inoculate 20 ml of the selective LB broth. The cells were incubated at 37° C with low to moderate shaking at 200 rpm until an OD_{600} of 0.6 was reached, and was measured by a Helios Spectrophotometer (Merck, Gauteng, RSA). The culture was immediately split into two equal portions so that one portion could be induced to express the intended or desired AtClAP recombinant protein through the addition of 1 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma-Aldrich Corp., Missouri, USA) while the other was left un-induced (control). Both cultures were allowed to grow for a further 3 hours at 37° C with low to moderate shaking at 200 rpm. After incubation, 500 µl of each of the cell cultures was separately collected for analysis by sodium dodecyl sulphate-polyacrylamide gel

electrophoresis (SDS-PADE), while the rest of the cells were harvested by centrifugation at 16300 x *g* for 5 minutes and the pellet stored at -20°C for downstream use.

2.1.2 Affinity Purification of the Recombinant AtClAP

The harvested induced bacterial cell culture, containing the expressed recombinant AtC1AP was re-suspended in 1 ml phosphate saline (PBS) buffer (140 mM NaCl, 3 mM KCl, 4 mM $Na_2HPO_4.2H_2O$, 1.5 mM KH_2PO_4) supplemented with 10 mM imidazole and 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and sonicated for 6 minutes (10 seconds pulsing and 10 seconds chilling cycles) to solubilise the cell contents. The solubilised cell contents were centrifuged at 9 200 x *g* for 10 minutes and the supernatant kept on ice as cleared lysate. Concurrently, about 50 µl of the nickel-nitrilotriacetic acid (Ni-NTA) slurry matrix/beads (Thermo Scientific, Rockford, USA) were washed twice with 1 ml sterile distilled water on a rotary mixer for 5 minutes. The beads were then equilibrated with 1 ml of PBS supplemented with 10 mM imidazole. The equilibrated beads were then sedimented down through a low-speed centrifugation for 15 seconds and the supernatant was discarded. The cleared lysate were then mixed with the Ni-NTA beads (binding) on a rotary mixer for 1 hour at 4°C. After an hour, the beads were then washed three times with 1 ml of PBS supplemented with 10 mM imidazole and 0.5 mM PMSF, and after each wash, a small portion (20 µl) of the wash buffer was collected and kept aside for analysis by SDS PAGE. After the three washes, the bound beads were kept at 4° C for further use while a small portion (20 µl) was analysed together with the small portions of the different wash buffers using SDS PAGE.

2.1.3 Chemical Elution of the Recombinant AtClAP

The bound and fully purified AtC1AP recombinant protein was eluted off the Ni-NTA beads by adding 5 ml of the elution buffer (200 mM NaCl, 50 mM Tris-Cl (pH 8.0), 250 mM imidazole, 0.5 mM PMSF, and 20% (v/v) glycerol) into the Ni-NTA beads-protein matrix and allowed to settle for 10 minutes. The resultant supernatant containing the eluted AtC1AP was

then collected and stored at 4° C for further use while a small portion (20 µl) of it was kept aside for analysis by SDS PAGE.

2.1.4 Concentration and Desalting of the Recombinant AtC1AP

The eluted AtC1AP was freed from the buffering salts and excess water by transferring 5 ml of the eluent into the upper chamber of a Spin-X UF de-salting and concentrating device (Corning, Rockford, USA). The device was centrifuged at 2 540 x *g* at 4° C for 4 hours or until the final volume was down to 100 µl. The concentrated and desalted protein fraction was then removed from the device and transferred into a new Eppendorf tube. Protein concentration was then subsequently determined using a 2000 Nanodrop spectrophotometer (Thermo Scientific, California, USA) and the final eluent stored at -20° C. A small portion (20 µl) of the protein was also analyzed by SDS-PAGE to determine the level of purity.

2.2 ACTIVITY ASSAYING

2.2.1 Characterization of the AC Activity of AtC1AP by Mass Spectrometry

The *in vitro* enzymatic ability of the purified AtC1AP recombinant protein to convert ATP into cAMP in a Tris-HCl-buffered system was characterized through assessment by mass spectrometry. Mass spectrometry is a method equally and analytically sensitive to the commonly used enzyme immunoassay.

2.2.1.1 Generation and preparation of the cAMP sample

In this regard, 10 µg of the recombinant AtC1AP was incubated with 1 mM ATP, 2 mM IBMX, and 5 mM Mn^{2+} in a final volume of 200 µl of 50 mM Tris- HCl (pH 8.0) buffer. Any residual cAMP levels resulting from the non-AC activity on the substrate were also measured in tubes that contained the incubation medium with no protein added. All reaction incubations were performed for 20 minutes at 25° C and terminated by the addition of 10 mM of ethylene di-amine tetra-acetic acid (EDTA) and boiled for 3 minutes. The reaction mixture were cooled on ice for 2 minutes followed by centrifugation at 9 200 x *g* for 3 minutes and supernatant was collected.

2.2.1.2 Analysis of the generated cAMP sample by mass spectrometry

The collected supernatant in 2.2.1.1 was introduced into a Waters API Q-TOF Ultima mass spectrometer (Waters Microsep, Johannesburg, RSA) with a Waters Acquity UPLC at a flow rate of 180 ml/min. Separation was achieved in a PhenomenexSynergi (Torrance, CA) 4 µm Fusion-RP (250 × 2.0 mm) column when a gradient of solvent ‘‘A’’ (0.1% (v/v) formic acid) and solvent ‘‘B’’ (100% (v/v) acetonitrile) was applied over an 18 minute duration. During the first 7 minutes, the solvent composition was kept at 100% (v/v) ‘‘A’’ followed by a linear gradient of up to 80% (v/v) ‘‘B’’ for 3 minutes, and then a re-equilibration to the initial conditions. An electrospray ionization in the negative (W) mode was used at a cone voltage of 35 V, to detect molecules and generate chromatograms. All results and the generated data were then subjected to the statistical analysis of variance (ANOVA) in triplet forms.

2.2.2 Further Characterization of the AC Activity of AtC1AP by *in silico* Analysis

In order to augment the spectrophotometric assaying findings and elucidate the probable functional roles of the AtClAP in *A. thaliana* and other related higher plants, web-based software packages and computer-based programs were used to analyze the expressional profiles of the At1g68110 gene in *A. thaliana*.

2.2.2.1 Co-expressional analysis of the AtClAP

In order to determine the co-expressional profile of the At1g68110 gene with the other related genes in *Arabidopsis*, the *Arabidopsis* co-expression tool (ACT) (http://www.genevestigator.com) was used. This analysis was carried out across all microarray experiments using At1g68110 as the search gene, and leaving the gene list limit blank to obtain a full correlational list. The tool makes use of signal intensities from microarray experiments to obtain the Pearson correlation co-efficient (r-value), which indicates the linear associations of various expressions between a reference gene (At1g68110) and all other Arabidopsis genes represented on the selection chip. The tool then calculates both the positive and negative correlations (ranges from -1 to +1), which are measures of statistical significance, expressed as probability (P) and expectation (E) values.

2.2.2.2 Stimuli-specific analysis of the AtClAP

Following the co-expressional analysis of the AtClAP protein in Arabidopsis, an *in-silico* global expression analysis of the At1g68110 gene with its 25 top most co-expressed genes in the Arabidopsis plant (*AtClAP-ECGG-25*) was performed to identify specific experimental conditions (both biotic and abiotic) that induced differential expression of these genes. The

expression profiles of this *AtCAP-ECGG-25* gene set was initially screened against the available ATH1:22K array Affymetrix public microarray data in the Genevestigator V3 (https://www.genevestigator.com) using the stimulus and mutation tools (Zimmermann *et al*., 2004). Additionally, and in order to obtain a greater resolution of the gene expression profiles of this set, the normalized microarray data was subsequently downloaded and analyzed for experiments that are found to induce differential expression of these genes. The data were then downloaded from the following repository sites; the GEO (NCBI) (http://www.ncbi.nlm.nih.gov/geo/) (Slotta *et al*., 2009), the NASC Arrays (http://affymetrix.arabidopsis.info/narrays/experimentbrowse.pl) (Craigon *et al*., 2004), and the TAIR-ATGenExpress (http://www.ebi.ac.uk/microarray-as/ae/). The downloaded array data was then analysed and the fold change (log2) values for each experiment calculated. Expression heat maps were then generated using the Multiple Array Viewer program from the Multi-Experiment Viewer (MeV) software package (version 4.2.01) created by The Institute for Genomic Research (TIGR) (Saeed *et al*., 2003), for both presentation and subsequent interpretation.

CHAPTER THREE

RESULTS

3.1 RECOMBINANT EXPRESSION OF THE AtClAP

In order to facilitate expression of the desired recombinant AtClAP , the obtained *E. cloni* BL21 (DE3) pLysS DUOs cells transformed with the pTrcHis2-TOPO:AtClAP fusion construct were induced with 1 mM IPTG. As is shown in Figure 3.1 , the desired recombinant AtClAP with a molecular size of 22.6 kDa was successfully expressed as a His-tagged (C-terminus) fusion product.

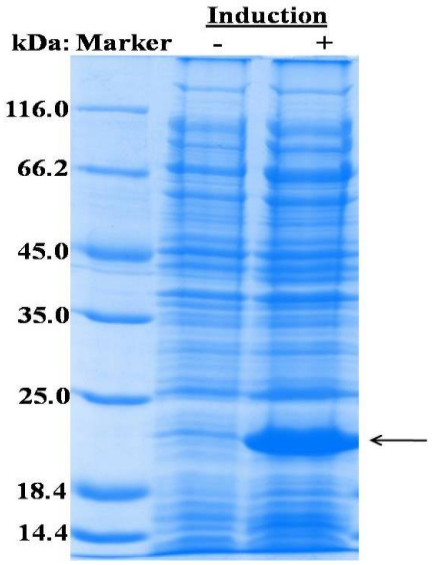

Figure 3.1: Recombinant expression of the AtClAP. The recombinant AtClAP fragment was expressed in chemically competent *E. cloni* EXPRESS BL21 (DE3) pLysS DUOs cells through their induction with 1 mM IPTG. The expressed protein was then resolved by SDS-PAGE on a 12% poly-acrylamide gel, where Marker is the unstained protein molecular weight marker (ThermoFisher Scientific Inc., New York, USA), while **(-)** and **(+)** represent the un-induced and induced cell cultures respectively. The arrow is marking the expressed and desired recombinant AtClAP with a size of approximately 22.6 kDa.

3.2 PURIFICATION OF THE RECOMBINANT AtClAP

Expression of recombinant proteins in various prokaryotic systems such as *E. coli* usually results in aggregation of the recombinant protein into inclusion bodies (Rudolph and Lilie, 1996). In this study and after determining that the recombinant AtClAP was wholly expressed in form of inclusion bodies, this poly-histidine C-tagged fusion product was then purified under native non-denaturing conditions using a Ni-NTA affinity system (Hochuli *et al*., 1987) and following the manufacturer's protocol (Catalogue # P6611; Sigma-Aldrich Inc., Missouri, USA). As is shown in Figure 3.2 , the protein was properly purified as a result of the use of a Ni-NTA affinity system, that contains a correspondingly 6xNi positively charged platform complementary to the negatively charged 6xHis-tag on the recombinant AtC1AP.

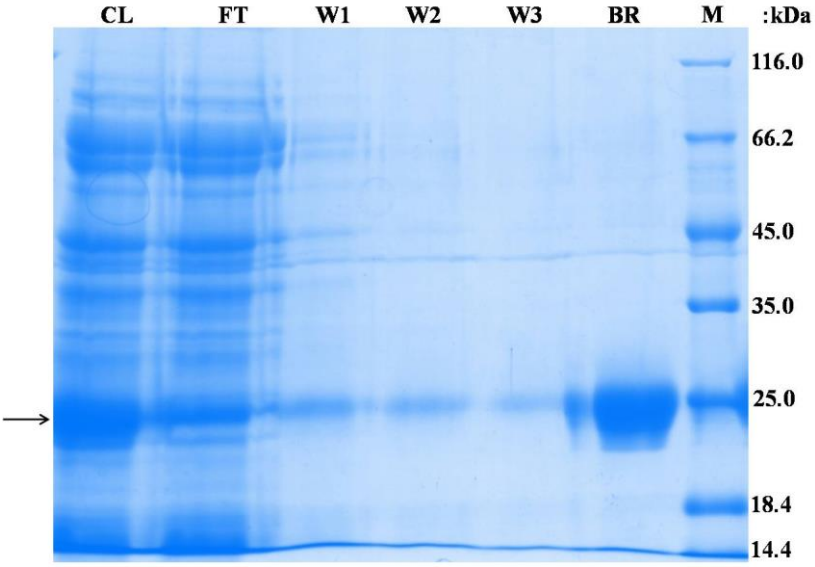

Figure 3.2: Purification of the recombinant AtClAP under native non-denaturing conditions. An SDS-PAGE of various fractions of the recombinant AtClAP that were collected at the different stages of its purification process. **CL** represents the cleared lysate generated through clarification of the induced cell cultures after rapturing by sonication; **FT** represents the flow-through of the cleared lysate after it had been passed through the equilibrated Ni-NTA matrix; **W1**, **W2** and **W3** represent the three successive washes of the bound AtClAP protein on the matrix; and **BR** represents the purified and bound recombinant AtClAP protein. The arrow is marking the recombinant AtClAP protein while **M** is

representing the unstained low molecular weight marker (ThermoFisher Scientific Inc., New York, USA).

3.3 ELUTION, DESALTING AND CONCENTRATION OF THE AtClAP

After purification of the recombinant AtClAP on the Ni-NTA affinity matrix under native non-denaturing conditions, the affinity purified recombinant AtClAP was the eluted off the Ni-NTA affinity matrix with an imidazole supplemented buffer. The eluted protein was then removed of all saltsand protein concentrated was increased as shown in Figure 3.3 , in preparation for the subsequent functional characterization steps. The elution and concentration step is important as they purify and concentrate the recombinant protein and that is indicated by a sharp, clear sigle band as shown in Figure 3.3.

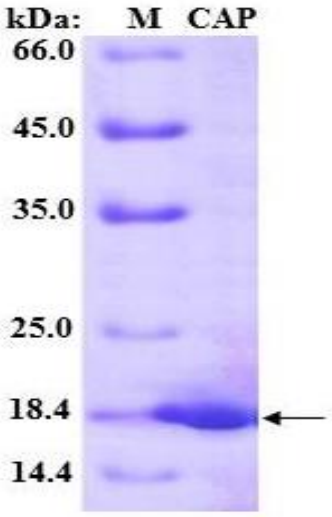

Figure 3.3: Elution, desalting and concentration of the purified recombinant AtClAP. An SDS-PAGE of the eluted, desalted and concentrated purified recombinant AtClAP . **M** represents the low molecular weight marker (ThermoFisher Scientific Inc., Missouri, USA), **CAP** represents the eluted, desalted and concentrated purified recombinant AtClAP while the arrow is marking the final and resultant protein product.

3.4 SPECTROMETRIC ANALYSIS OF THE RECOMBINANT AtClAP ACTIVITY

After elution, desalting and concentration, the AtClAP's ability to generate cAMP and other related molecular species from ATP was then assessed by mass spectrometry.. Mass spectrometry is a method of choice to enzyme immunoassay, capable of specifically and sensitively detecting cAMP and other related molecules at femtomolar levels as seen in Figure 3.4. The mass chromatogram showsvarious molecules such as cAMP, AMP, ADP and adenine generated by AtC1AP in the reaction mixture containing Tris-HCl; pH 8.0, IBMX, Mn^{2+} and ATP.

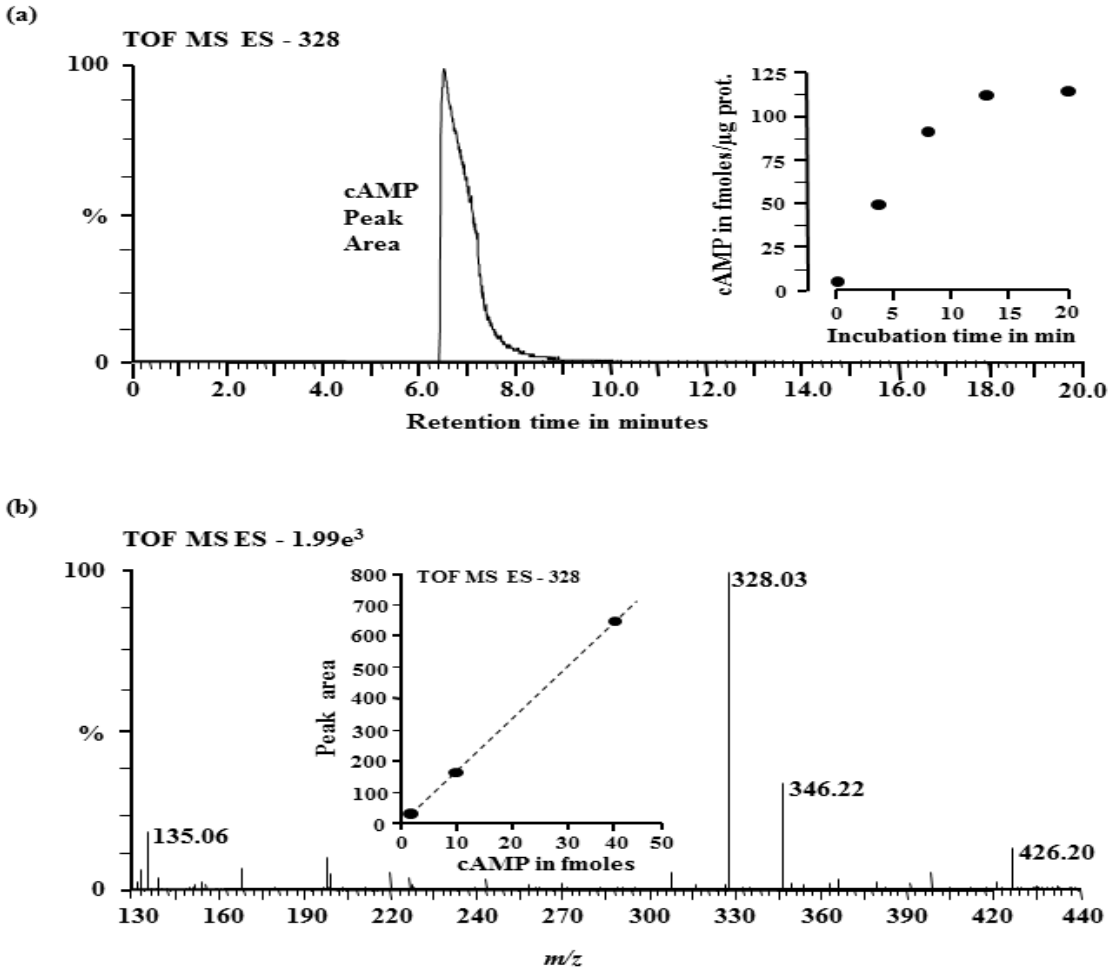

Figure 3.4: Spectrometric analysis of the AC activity of the recombinant AtClAP protein. (**a**) An extracted mass chromatogram of the *m/z* 328 $[M-1]^{-1}$ ion of cAMP generated by 10 µg of the AtC1AP

in a reaction system containing 50 mM Tris-HCl; pH 8.0, 2 mM IBMX, 5 mM Mn^{2+} and 1 mM ATP. Inset: The incubation time course. (**b**) Masses of peaks of the various molecules generated by AtClAP in the reaction mixture and detected by the system. The detected molecules are, from left to right: adenine, cAMP, AMP and ADP. Inset: The detection calibration curve. Samples were analyzed by ANOVA as means of three independent and representative assays ($n = 3$; $p < 0.05$).

3.5 CO-EXPRESSIONAL ANALYSIS OF THE AtClAP

After realizing the potential of AtClAP to generate various signalling molecules, including cAMP, its probable function in plants was then assessed by analyzing its co-expressional status in connection with other related proteins of known function in Arabidopsis and/or other closely related plants. As is shown in Table 3.1, a list of the 25 top most co-expressed proteins ($-0.71 \leq r \geq 0.81$) were retrieved and used to infer function for the AtClAP. The AtC1AP was found to be co-expressed with a wide array of other proteins of which most were specifically involved in cellular processes essential for plant growth such as peroxisomal transport, intracellular protein transmembrane import, peroxisome organization, photoperiodic flowering to mention a few.

Table 3.1: List of the top 25 co-expressed proteins with AtClAP (At1g68110).

Num	Locus and Gene Ontology terms[a]	r-value	Annotation and Functional Description
1	AT1G05840[PT, IPTI, PO, PF, EPLP, PLP, LM, FABO, PTP, MACP, IPTT, LO, PI, FAO, FACP, I-GV-MT, LT, VMT, LL, PTT, CLCP, PIPM, LIT, PP, VRTM]	0.81	Involved in proteolysis
2	AT1G58030[PT, IPTI, PO, PF, EPLP, PLP, LM, FABO, PTP, MACP, IPTT, LO, PI, FAO, FACP, CLCP, PIPM, PP, VRTM]	0.73	Catalase-2, Cationic amino acid transporter 2, vacuolar
3	AT2G28910[PT, IPTI, PF, EPLP, PLP, PTP, IPTT, PI, I-GV-MT,LT, VMT, LL, PTT, PIPM, LIT, PP, VRTM]	0.73	CAX-interacting protein 4
4	AT3G07560[PT, IPTI, PO, EPLP, PLP, LM, FABO, PTP, MACP, IPTT, LO, PI, FAO, FACP, I-GV-MT, LT, VMT, LL, PTT, CLCP. PIPM, LIT	0.72	Peroxisomal membrane protein 13
5	*AT1G26670[PT, IPTI, EPLP, PLP, PLV, EPLV, PTP, PTV, IPTT, PI, I-GV-MT, LT, VMT, SB, SRA, LL, PTT, PIPM, LIT	0.81	Vesicle transport V-SNARE family protein
6	*AT1G15880[PT, I-GVMT, PLP, EPLP, PTP, PO, IPTI, IPTT, PI, LT, VMT, SB, SRA, LL, PTT, PIPM, LIT	0.80	Golgi snare 11
7	AT5G66160[PT, IPTI, PO, EPLP, PLP, PTP, IPTT, PI, I-GV-MT, LT, VMT, LL, PTT, PIPM, LIT	0.73	Receptor homology region, transmembrane domain protein 1
8	AT1G64230[PT, IPTI, PO, EPLP, PLP, LM, FABO, PTP, MACP, IPTT, LO, PI, FAO, FACP, VMT, PTT, CLCP, PIPM	0.74	Ubiquitin-conjugating enzyme E2 28
9	AT1G05790[PT, IPTI, PO, EPLP, PLP, LM, FABO, PTP, MACP, IPTT, LO, PI, FAO, FACP, ,PTT, CLCP, PIPM	0.71	Lipase Class 3 family protein
10	AT3G19860[PT, IPTI, EPLP, PLP, LM, FABO, PTP, MACP, IPTT, LO, PI, FAO, FACP, PTT, CLCP, PIPM	0.77	Transcription factor bHLH121
11	AT1G16240[PLV, EPLV, PTV, I-GV-MT, VMT, SB, SRA	0.79	Syntaxin of plants 51
12	AT2G45980[PLV, EPLV, PTV, VMT	0.80	ATG8-interacting protein 1
13	AT5G06140[PLV, EPLV, PTV, VMT	0.78	Sorting nexin 1
14	*AT4G22750[PLV, EPLV, PTV, VMT	0.77	Probable protein S-acyltransferase 13
15	AT5G66030[I-GV-MT, VMT	0.74	Protein GRIP
16	*AT2G36900[VMT, SB, SRA	0.81	Membrin 11
17	*AT4G32150[VMT, SB, SRA	0.80	Vesicle-associated membrane protein 711
18	*AT4G17730[VMT, SB, SRA	0.78	Syntaxin of plants 23
19	AT2G28370[VMT	0.81	CASP-like protein 5A2
20	AT1G13450[VMT	0.78	Trihelix trancription factor GT-1
21	AT1G49240[VMT	0.77	Actin-8
22	AT4G24520[VMT	0.75	NADPH-cytochrome P450 reductase 1
23	AT1G53400[VMT	0.75	Uncharacterized protein
24	AT3G24315[VMT	0.72	AtSec20 family protein
25	AT5G39590[VMT	0.71	TLD-domain containing nucleolar protein

PT: Peroxisomal transport ($P = 3.05e^{-08}$), IPTI: Intracellular protein transmembrane import ($P = 2.93e^{-07}$), PO: Peroxisome organization ($P = 3.05e^{-08}$), PF: Photoperiodic flowering ($P = 1.85e^{-15}$), EPLP: Establishment of protein localization to peroxisome ($P = 3.05e^{-08}$), PLP: Protein localization to peroxisome ($P = 3.05e^{-08}$), LM: Lipid modification ($P = 3.22e^{-02}$), FABO: Fatty acid beta-oxidation ($P = 3.79e^{-03}$), PTP: Protein targeting to peroxisome ($P = 3.05e^{-08}$), MACP: Monocarboxylic acid catabolic process ($P = 1.88e^{-02}$), IPTT: Intracellular protein transmembrane transport ($P = 3.82e^{-07}$), LO: Lipid oxidation ($P = 4.68e^{-03}$), PI: Protein import ($P = 2.76e^{-04}$), FAO: Fatty acid oxidation ($P = 4.00e^{-03}$), FACP: Fatty acid catabolic process ($P = 1.55e^{-02}$), I-GV-MT: Intra-Golgi vesicle-mediated transport ($P = 2.52e^{-07}$), LT: Lipid transport ($P = 1.02e^{-03}$), and VMT: Vesicle mediated transport ($P = 8.4^{-03}$).

3.6 STIMULUS-SPECIFIC ANALYSIS OF THE AtClAP

When the bioinformatic analysis was extended further to identify conditions that induce expression of AtClAP and its correlated proteins, it was noted that this whole set of proteins is strongly and transiently induced by biotrophic pathogens such as *Pseudomonas syringae* and the associated pathogen effector molecules such as syringolin A and flagellin 22 as seen in Figure 3.5.

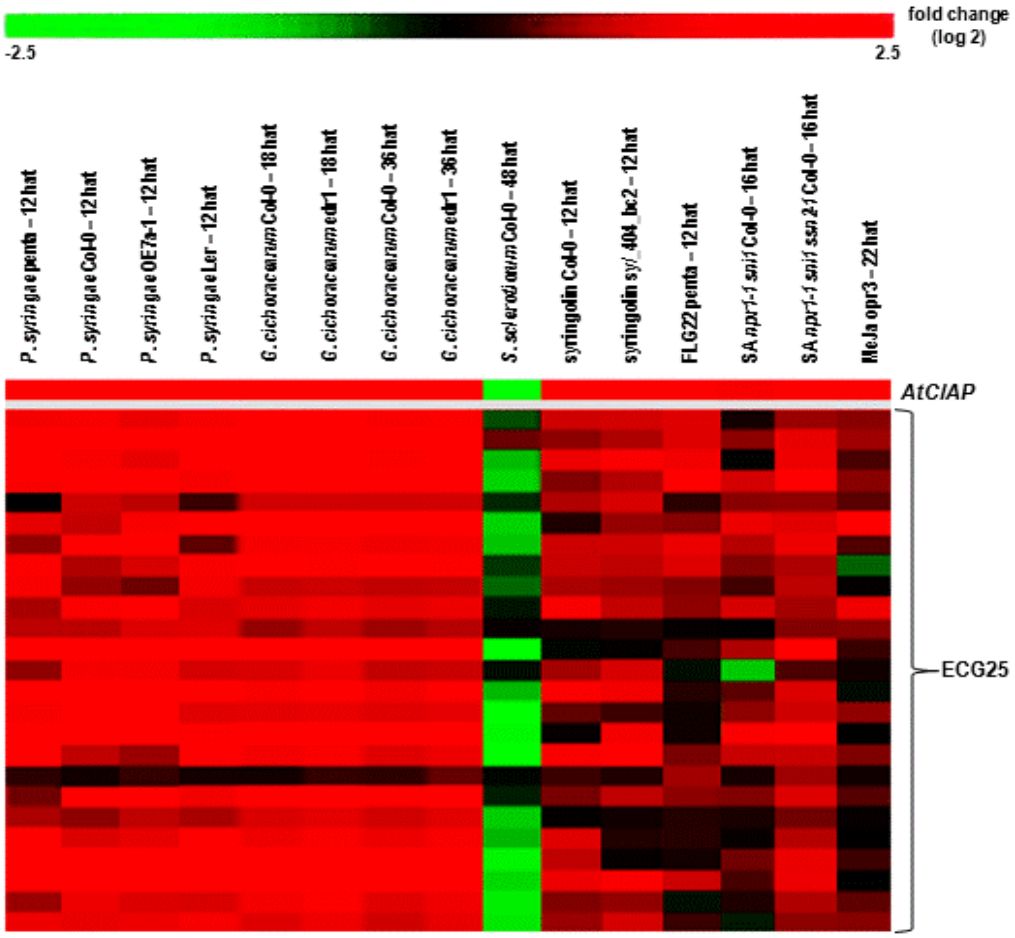

Figure 3.5: Stimulus-specific analysis of the AtClAP protein. The heatmap is a microaray analysis of the AtClAP constructed to illustrate the fold change (log2) in expression of AtClAP and 25 of its top most co-expressed proteins in response to selected arrays of experiments. The experiments presented include; *P. syringae* (12 h after treatment (hat), GSE17464 and E-MEXP-1094), *G. cichoracearum* (18 and 36 hat, GSE26679), *S. sclerotiorum* (48 hat, E-MEXP-3122), syringolin (12 hat, AT-00258/FGCZ and E-MEXP-739), flg22 (12 hat, GSE17464), salicylic acid *npr1-1 sni1* double and *npr1-1 sni1 ssn2-1* triple mutants (16 hat, GSE23617) and methyl jasmonate (22 hat, GSE17464).

CHAPTER FOUR

DISCUSSION, CONCLUSION AND RECOMMENDATION

4.1 DISCUSSION

The study reported herein dealt with the heterologous expression of a recombinant AtC1AP-AC protein, which was previously isolated from *Arabidopsis thaliana* plant and expressed in the Plant Biotechnology Research Lab at the North-West University (Republic of South Africa). In modern labs, plant genes that encode specific proteins can be expressed in bacterial, fungal or animal cellular systems, although isolation of genomic DNA for protein expression may result in production of non-functional protein product due to the presence of introns. Such a challenging situation, however, can be easily avoided or circumvented by expressing those desired plant genes in fungal and/or animal cells which, as a result of their eukaryotic nature, are able to process the required post-translational modifications that are required for the functionality of the target plant proteins (Clark and Pazdernik, 2011). On the other hand, an easier approach would be to clone and express plant copy DNA (cDNA) in bacterial or prokaryotic systems so that the problem of introns is thus resolved.

The most preferred prokaryotic host for heterologous protein expressions is *Escherichia coli* that is commonly used for the high-yield expression of most recombinant proteins, usually as a result of the high promotor specificities and transcriptional activities of its accompanying bacteriophage T7 RNA polymerase (Robichon *et al*., 2011). In this regard, in our study, we therefore, used the *E. cloni* BL21 (DE3) pLysS DUOs cells as the expression host strain of choice, as it possess the same DE3 chromosomal genotype as in the *E. coli* BL21 strain. As a

result of this approach, the desired and targeted recombinant AtClAP was successfully expressed as a 6xHis-tagged fusion product at its expected molecular weight size of around 22.6 kDa (Figure 3.1).

Normally, the affinity purification of most His-tagged recombinant proteins such as the AtC1AP expressed in *E. coli* systems is necessary to ensure that all other contaminating and/or toxic impurities are purged away (Franken *et al*., 2000). In this study, the affinity purification of the recombinant AtC1AP-AC was practically enhanced as a result of the use of an Ni-NTA affinity system, that contains a correspondingly 6xNi positively charged platform complementary to the negatively charged 6xHis-tag on the recombinant AtC1AP protein. Accordingly, our study succeeded to purify the expressed recombinant AtClAP protein using this established chromatographic approach Figure 3.2. The purification process is typically quick and simple as it facilitates a tailored association between the protein fused tag and its corresponding affinity matrix, thus allowing for unwanted protein contaminants to be easily washed off, even under strict denaturing conditions required sometimes to solubilize inclusion bodies (Crowe *et al*., 1994). In order to carry out the intended subsequent *in vitro* functional characterization of the recombinant AtC1AP, the purified protein had to be eluted off the Ni-NTA affinity matrix asFigure 3.3.

When the AC activity of the purified AtClAP was assessed by mass spectrometry, tospecifically and sensitively detect cAMP and other related molecules at femtomolar levels, several molecular components were detected (Figure 3.4). The parent molecule detected was a species component of the relative molecular weight size of 328.03, representing cyclic adenosine monophosphate or cAMP ($C_{10}H_{12}N_5O_6P$), followed by a species component of the relative molecular weight size of 135.06, represented adenine or A ($C_5H_5N_5$), followed by a species component of the relative molecular weight size of 346.22, representing adenosine monophosphate or AMP ($C_{10}H_{14}N_5O_7P$) and then a species component of the relative

molecular weight size of 426.20, representing adenosine di-phosphate or ADP ($C_{10}H_{15}N_5P_2$) (Figure 3.4). One way or the other, all these molecular components are key elements of the cell signaling systems in most living organisms including plants necessary for essential cellular process such as growth, development and responses to various environmental stress factors (Xu *et al*., 2018). Therefore, by considering this capacity of the AtC1AP protein to generate all these critical signalling elements, it strongly indicated to us how essential this protein is in the overall life cycles of plants and their biochemical and physiological processes.

After ascertaining the enzymatic capacity of the recombinant AtClAP protein to generate several essential signalling elements in plants, we then sought to use an *in silico* analysis to assess and possibly determine its possible functional roles in plants. Using this approach, the AtClAP was found to be co-expressed with a wide array of other proteins of which the 25 top most proteins were specifically involved in peroxisomal transport, intracellular protein transmembrane import, peroxisome organization, photoperiodic flowering, establishment of protein localization to peroxisome, protein localization to peroxisomes, lipid modification, fatty acid beta-oxidation, protein targeting to peroxisomes, monocarboxylic acid catabolic process, intracellular protein transmembrane transport, lipid oxidation, protein import, fatty acid oxidation, fatty acid catabolic process, intra-Golgi vesicle-mediated transport, lipid transport, and vesicle mediated transport (Table 3.1).

Incidentally, all the above listed processes are key cellular processes essential for plant growth, development and responses to various environmental stress factors (Wang *et al*., 2008), thus highlighting the significance of the AtClAP in the overall life cycles of plants and their biochemical and physiological processes. This is also entirely consistent with the annotation of the AtClAP protein as an ENTH (Epsin NH_2 terminal homology)/ANTH/VHS superfamily protein with a role in clathrin assembly and endocytosis (Wang *et al*., 2008). It is also worth to note that the AtClAP is transiently expressed in germinating pollens during tube growth in

Arabidopsis and clathrin-dependent endocytosis is a key to pollen tip function (Boavida *et al.*, 2011). Clathrin-dependent endocytosis in turn is regulated by phosphoinositides (Zhao *et al*., 2010) and yet phospholipids have been shown to affect cellular cAMP levels e.g. in ciliary transport (Chávez *et al*., 2015).

After ascertaining the key functional roles of the AtClAP , the protein was further analyzed by stimulus-specific analysis, whereby the expression profile of the AtClAP and its 25 top most co-expressed proteins were assessed and determine against a variety of environmental purtabations and stimuli. The approach showed that the expression of the AtClAP and its 25 top most co-expressed proteins (Table 3.1) were relatively induced by the biotrophic pathogens (Grant and Lamb, 2006) e.g. *Pseudomonas*. *syringae* and elicitors (syringolin A and flagellin 22) (Figure 3.5). Syringolin and flagellin are pathogen effector molecules produced by biotrophic bacteria such as *Pseudomonas* and they trigger the innate immune responses in plants (Hayashi *et al*., 2001, Schellenberg *et al*., 2010). The AtClAP and its 25 top most co-expressed proteins (Table 3.1) were also found to be up-regulated in the *npr1-1 sni1* double and *npr1-1 sni1 ssn2-1* triple mutants (Figure 3.5). Firstly, *npr1-1* is a mutant with defects in the NPR1 gene (Cao *et al*., 1997) that controls the onset of systemic acquired resistance (SAR) that is dependent on salicylic acid signaling (SA) (Yang *et al*., 1997) Secondly, in *npr1-1*, pathogenesis-related (PR) genes are suppressed and susceptibility to infections increased (Cao *et al*., 1997). Thirdly, both *sni1* and *ssn2-1* are regulators of the SAR that suppress effects of the *npr1-1* by triggering expression of PR genes and conferring resistance to pathogens. However, while *sni1* is SA- and NPR1-dependent, *ssn2-1* is SA- and NPR1-independent (Shah *et al*., 2001, Li *et al*., 1999). Lastly, in both the *npr1-1 sni1* double and *npr1-1 sni1 ssn2-1* triple mutants, *sni1* is SA-inducible and therefore, a strong induction of the AtClAP protein and its co-expressed proteins (Table 3.1) in these two mutants, together with their differential

expression in response to biotrophs and elicitors (Figure 3.5), would imply an SA-dependent role of the AtClAP protein in pathogen defense responses.

While plant infections by biotrophs trigger SA-dependent responses, infections by necrotrophs e.g. *Botrytis cinerea*, are dependent on jasmonic acid (JA) or its methyl ester, methyl jasmonate (MeJa) (Grant and Lamb, 2006), whereby expression of; for example, the defensin (PDF1) or proteinase inhibitors I and II (PI I and PI II) genes is involved (Manners *et al*., 1998). The JA precursor, 12-oxo-phytodienoic acid (OPDA), has also been implicated in plant defense systems, particularly mechanical wounding and looper infestation (Chehab *et al*., 2011). A plant mutant deficient in JA production but capable of accumulating OPDA is termed *opda reductase3* or simply *opr3*. The *opr3* contains a 17-kb T-DNA insertion in its second intron reported to block the JA biosynthesis pathway downstream of OPDA . Surprisingly, upon infection by *B. cinerea*, *opr3* is capable of accumulating substantial levels of JA potentially through a successful removal of the T-DNA harbouring intron and consequently, producing full-length OPR3 transcripts (Chehab *et al*., 2011). In this *opr3* mutant, the AtClAP protein and its co-expressed protein (Table 3.1) are strongly induced (Figure 3.5), thereby implying a JA-dependent role of the AtClAP in pathogen defense responses.

Incidentally, elevation of concentrations of cAMP through the administration of elicitors has been demonstrated in French bean (*Phaseolus vulgaris*), carrot (*Daucus carota*) (Kurosaki *et al*., 1993), alfalfa (*Medicago sativa*) exposed to *Verticillium alboatrum* glycoprotein (Cooke *et al*., 1994), Mexican cypress (*Cupressus lusitanica*) cell culture treated with yeast oligosaccharides (Zhao *et al*., 2004) and *A. thaliana* treated with *Verticillium dahliae* toxins, resulting in improved disease resistance of the plants (Jiang *et al*., 2005).

4.2 CONCLUSION

Based on the findings of this study, it is conceivable to conclude that the expression of cAMP-generating signalling molecules such as the AtClAP is part of the cellular response system to growth, development and stress challenges in plants.

4.3 RECOMMENDATIONS

Even though this study has explicitly established the role of the AtClAP as a key molecule in essential cAMP-mediated signalling cellular processes such as growth, development and response to both biotic and abiotic stress factors, it is however, still necessary to further characterize this protein molecule, mostly *in vivo* and/or *in planta* through purtabations and/or mutational studies so as to further expand our current knowledge on its functional roles in plants.

More so, since the AtClAP has been previously established as a functional AC together with several other plant proteins (Moutinho *et al*., 2001, Ruzvidzo *et al*., 2013, Ito *et al*., 2014, Świeżawska *et al*., 2014, Al-Younis *et al*., 2015, Chatukuta *et al*., 2018, Al-Younis *et al*., 2018, Bianchet *et al*., 2018), it is therefore, imperative that those other proteins are also equally functionally characterized as was the AtClAP protein in this study so that the gained knowledge may be effectively applied into our own agronomically important practises for the improvement of crop yields and thus food security against the currently prevailing climate change.

REFERENCES

Ahanger, M. A. & Agarwal, R. 2017. Potassium up-regulates antioxidant metabolism and alleviates growth inhibition under water and osmotic stress in wheat (*Triticum aestivum* L). *Protoplasma,* 254, 1471-1486.

Al-Younis, I., Wong, A. & Gehring, C. 2015. The *Arabidopsis thaliana* K^+-uptake permease 7 (AtKUP7) contains a functional cytosolic adenylate cyclase catalytic centre. *FEBS Letters,* 589, 3848-3852.

Al-Younis, I., Wong, A., Lemtiri-Chlieh, F., Schmöckel, S., Tester, M., Gehring, C. & Donaldson, L. 2018. The *Arabidopsis thaliana* K^+-uptake permease 5 (AtKUP5) contains a functional cytosolic adenylate cyclase essential for K^+ transport. *Frontiers in Plant Science,* 9.

Bianchet, C., Wong, A., Quaglia, M., Alqurashi, M., Gehring, C., Ntoukakis, V. & Pasqualini, S. 2018. An *Arabidopsis thaliana* leucine-rich repeat protein harbors an adenylyl cyclase catalytic center and affects responses to pathogens. *Journal of Plant Physiology*, 232, 12-22.

Boavida, L., Borges, F., Becker, J. & Feijó, J. 2011. Whole genome analysis of gene expression reveals coordinated activation of signaling and metabolic pathways during pollen-pistil interactions in Arabidopsis. *Plant Physiology,* 155, 2066-2080.

Boucrot, E., Ferreira, A. P., Almeida-Souza, L., Debard, S., Vallis, Y., Howard, G., Bertot, L., Sauvonnet, N. & Mcmahon, H. T. 2015. Endophilin marks and controls a clathrin-independent endocytic pathway. *Nature,* 517, 460.

Brodsky, F. M. 2012. Diversity of clathrin function: new tricks for an old protein. *Annual Review of Cell and Developmental Biology,* 28.

Cao, H., Glazebrook, J., Clarke, J. D., Volko, S. & Dong, X. 1997. The Arabidopsis NPR1 gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. *Cell,* 88, 57-63.

Chatukuta, P., Dikobe, T. B., Kawadza, D. T., Sehlabane, K. S., Takundwa, M. M., Wong, A., Gehring, C. & Ruzvidzo, O. 2018. An Arabidopsis clathrin assembly protein with a predicted role in plant defense can function as an adenylate cyclase. *Biomolecules,* 8, 15.

Chávez, M., Ena, S., Van Sande, J., De Kerchove D'Exaerde, A., Schurmans, S. & Schiffmann, S. N. 2015. Modulation of ciliary phosphoinositide content regulates trafficking and sonic hedgehog signaling output. *Developmental Cell,* 34, 338-350.

Chehab, E. W., Kim, S., Savchenko, T., Kliebenstein, D., Dehesh, K. & Braam, J. 2011. Intronic T-DNA insertion renders Arabidopsis opr3 a conditional jasmonic acid-producing mutant. *Plant Physiology,* 156, 770-778.

Chen, X., Irani, N. G. & Friml, J. 2011. Clathrin-mediated endocytosis: the gateway into plant cells. *Current Opinion in Plant Biology,* 14, 674-682.

Clark, D. P. & Pazdernik, N. J. 2011. *Biotechnology: Academic Cell Update Edition,* Academic Press.

Commichau, F. M., Dickmanns, A., Gundlach, J., Ficner, R. & Stülke, J. 2015. A jack of all trades: the multiple roles of the unique essential second messenger cyclic di-AMP. *Molecular Microbiology,* 97, 189-204.

Cooke, C. J., Smith, C. J., Walton, T. J. & Newton, R. P. 1994. Evidence that cyclic AMP is involved in the hypersensitive response of *Medicago sativa* to a fungal elicitor. *Phytochemistry,* 35, 889-895.

Craigon, D. J., James, N., Okyere, J., Higgins, J., Jotham, J. & May, S. 2004. NASCArrays: a repository for microarray data generated by NASC's transcriptomics service. *Nucleic Acids Research,* 32, D575-D577.

Crowe, J., Dobeli, H., Gentz, R., Hochuli, E., Stiiber, D. & Henco, K. 1994. 6xHis-Ni-NTA chromatography as a superior technique in recombinant protein expression/purification. *Protocols for Gene Analysis.* Springer, 31:371-387

Daboussi, L., Costaguta, G. & Payne, G. S. 2012. Phosphoinositide-mediated clathrin adaptor progression at the trans-Golgi network. *Nature Cell Biology,* 14, 239.

Dannhauser, P. N. & Ungewickell, E. J. 2012. Reconstitution of clathrin-coated bud and vesicle formation with minimal components. *Nature Cell Biology,* 14, 634.

De Craene, J.-O., Ripp, R., Lecompte, O., Thompson, J. D., Poch, O. & Friant, S. 2012. Evolutionary analysis of the ENTH/ANTH/VHS protein superfamily reveals a coevolution between membrane trafficking and metabolism. *BMC Genomics,* 13, 297.

De Vos, M. & Jander, G. 2009. *Myzus persicae* (green peach aphid) salivary components induce defence responses in *Arabidopsis thaliana*. *Plant, Cell & Environment,* 32, 1548-1560.

Faini, M., Beck, R., Wieland, F. T. & Briggs, J. A. 2013. Vesicle coats: structure, function, and general principles of assembly. *Trends in Cell Biology,* 23, 279-288.

Franken, K.L., Hiemstra, H.S., van Meijgaarden, K.E., Subronto, Y., Den Hartigh, J., Ottenhoff, T.H. and Drijfhout, J.W., 2000. Purification of his-tagged proteins by immobilized chelate affinity chromatography: the benefits from the use of organic solvent. *Protein expression and purification*, *18*(1), 95-99.

Fujimoto, M., Arimura, S. I., Ueda, T., Takanashi, H., Hayashi, Y., Nakano, A. & Tsutsumi, N. 2010. Arabidopsis dynamin-related proteins DRP2B and DRP1A participate together in clathrin-coated vesicle formation during endocytosis. *Proceedings of the National Academy of Sciences*, 107, 6094-6099.

García, J. E., Maroniche, G., Creus, C., Suárez-Rodríguez, R., Ramirez-Trujillo, J. A. & Groppa, M. D. 2017. *In vitro* PGPR properties and osmotic tolerance of different *Azospirillum* native strains and their effects on growth of maize under drought stress. *Microbiological Research*, 202, 21-29.

Grant, M. & Lamb, C. 2006. Systemic immunity. *Current Opinion in Plant Biology*, 9, 414-420.

Hayashi, F., Smith, K. D., Ozinsky, A., Hawn, T. R., Eugene, C. Y., Goodlett, D. R., Eng, J. K., Akira, S., Underhill, D. M. & Aderem, A. 2001. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature*, 410, 1099.

Hochuli, E., Döbeli, H. & Schacher, A. 1987. New metal chelate adsorbent selective for proteins and peptides containing neighbouring histidine residues. *Journal of Chromatography A*, 411, 177-184.

Ito, M., Takahashi, H., Sawasaki, T., Ohnishi, K., Hikichi, Y. & Kiba, A. 2014. Novel type of adenylyl cyclase participates in tabtoxinine-β-lactam-induced cell death and occurrence of wildfire disease in *Nicotiana benthamiana*. *Plant Signaling & Behavior*, 9, e27420.

Jiang, J., Fan, L. W. & Wu, W. H. 2005. Evidences for involvement of endogenous cAMP in Arabidopsis defense responses to *Verticillium* toxins. *Cell Research*, 15, 585.

Kasahara, M., Suetsugu, N., Urano, Y., Yamamoto, C., Ohmori, M., Takada, Y., Okuda, S., Nishiyama, T., Sakayama, H. & Kohchi, T. 2016. An adenylyl cyclase with a

phosphodiesterase domain in basal plants with a motile sperm system. *Scientific Reports,* 6, 39232.

Koornneef, M. & Meinke, D. 2010. The development of Arabidopsis as a model plant. *The Plant Journal,* 61, 909-921.

Kurosaki, F., Kaburaki, H. & Nishi, A. 1993. Synthesis and degradation of cyclic AMP in cultured carrot cells treated with forskolin. *Archives of Biochemistry and Biophysics,* 303, 177-179.

Li, X., Zhang, Y., Clarke, J. D., Li, Y. & Dong, X. 1999. Identification and cloning of a negative regulator of systemic acquired resistance, SNI1, through a screen for suppressors of npr1-1. *Cell,* 98, 329-339.

Manners, J. M., Penninckx, I. A., Vermaere, K., Kazan, K., Brown, R. L., Morgan, A., Maclean, D. J., Curtis, M. D., Cammue, B. P. & Broekaert, W. F. 1998. The promoter of the plant defensin gene PDF1. 2 from Arabidopsis is systemically activated by fungal pathogens and responds to methyl jasmonate but not to salicylic acid. *Plant Molecular Biology,* 38, 1071-1080.

Mayor, S. & Pagano, R. E. 2007. Pathways of clathrin-independent endocytosis. *Nature reviews Molecular Cell Biology,* 8, 603.

Mcloughlin, F., Arisz, S. A., Dekker, H. L., Kramer, G., De Koster, C. G., Haring, M. A., Munnik, T. & Testerink, C. 2013. Identification of novel candidate phosphatidic acid-binding proteins involved in the salt-stress response of *Arabidopsis thaliana* roots. *Biochemical Journal,* 450, 573-581.

Mcmahon, H. T. & Boucrot, E. 2011. Molecular mechanism and physiological functions of clathrin-mediated endocytosis. *Nature reviews Molecular Cell Biology,* 12, 517.

Moutinho, A., Hussey, P. J., Trewavas, A. J. & Malho, R. 2001. cAMP acts as a second messenger in pollen tube growth and reorientation. *Proceedings of the National Academy of Sciences,* 98, 10481-10486.

Nguyen, H. H., Lee, M. H., Song, K., Ahn, G., Lee, J. & Hwang, I. 2018. The A/ENTH Domain-Containing Protein AtECA4 Is an adaptor protein involved in cargo recycling from the trans-golgi network/early endosome to the plasma membrane. *Molecular Plant,* 11(4), 568-583.

Ogundari, K. 2014. The paradigm of agricultural efficiency and its implication on food security in Africa: what does meta-analysis reveal? *World Development,* 64, 690-702.

Osakabe, Y., Watanabe, T., Sugano, S. S., Ueta, R., Ishihara, R., Shinozaki, K. & Osakabe, K. 2016. Optimization of CRISPR/Cas9 genome editing to modify abiotic stress responses in plants. *Scientific Reports,* 6, 26685.

Robichon, C., Luo, J., Causey, T. B., Benner, J. S. & Samuelson, J. C. 2011. Engineering BL21 (DE3) derivative strains to minimize *Escherichia coli* protein contamination after IMAC purification. *Applied and Environmental Microbiology,* AEM. 00119-11.

Rudolph, R. & Lilie, H. 1996. In vitro folding of inclusion body proteins. *The FASEB Journal,* 10, 49-56.

Ruzvidzo, O., Dikobe, B. T., Kawadza, D. T., Mabadahanye, G. H., Chatukuta, P. & Kwezi, L. 2013. Recombinant expression and functional testing of candidate adenylate cyclase domains. *Cyclic Nucleotide Signaling in Plants.* Springer, 13-25.

Saeed, A., Sharov, V., White, J., Li, J., Liang, W., Bhagabati, N., Braisted, J., Klapa, M., Currier, T. & Thiagarajan, M. 2003. TM4: a free, open-source system for microarray data management and analysis. *Biotechniques,* 34, 374.

Saleem, M., Morlot, S., Hohendahl, A., Manzi, J., Lenz, M. & Roux, A. 2015. A balance between membrane elasticity and polymerization energy sets the shape of spherical clathrin coats. *Nature Communications,* 6, 6249.

Schellenberg, B., Ramel, C. & Dudler, R. 2010. *Pseudomonas syringae* virulence factor syringolin A counteracts stomatal immunity by proteasome inhibition. *Molecular Plant-microbe Interactions,* 23, 1287-1293.

Shah, J., Kachroo, P., Nandi, A. & Klessig, D. F. 2001. A recessive mutation in the Arabidopsis SSI2 gene confers SA-and NPR1-independent expression of PR genes and resistance against bacterial and oomycete pathogens. *The Plant Journal,* 25, 563-574.

Slotta, D. J., Barrett, T. & Edgar, R. 2009. NCBI Peptidome: a new public repository for mass spectrometry peptide identifications. *Nature Biotechnology,* 27, 600.

Świeżawska, B., Jaworski, K., Pawełek, A., Grzegorzewska, W., Szewczuk, P. & Szmidt-Jaworska, A. 2014. Molecular cloning and characterization of a novel adenylyl cyclase gene, HpAC1, involved in stress signaling in *Hippeastrum hybridum*. *Plant Physiology and Biochemistry,* 80, 41-52.

Wang, C., Yan, X., Chen, Q., Jiang, N., Fu, W., Ma, B., Liu, J., Li, C., Bednarek, S. Y. & Pan, J. 2013. Clathrin light chains regulate clathrin-mediated trafficking, auxin signaling, and development in Arabidopsis. *The Plant Cell,* 25, 499-516.

Wang, H., Yang, P., Liu, K., Guo, F., Zhang, Y., Zhang, G. & Jiang, C. 2008. SARS coronavirus entry into host cells through a novel clathrin-and caveolae-independent endocytic pathway. *Cell Research,* 18, 290.

Xu, N., Zhang, C., Lim, L. L. & Wong, A. 2018. Bioinformatic analysis of nucleotide cyclase functional centers and development of ACPred webserver.

Yang, Y., Shah, J. & Klessig, D. F. 1997. Signal perception and transduction in plant defense responses. *Genes & Development*, 11, 1621-1639.

Zhao, J., Zheng, S. H., Fujita, K. & Sakai, K. 2004. Jasmonate and ethylene signalling and their interaction are integral parts of the elicitor signalling pathway leading to β-thujaplicin biosynthesis in *Cupressus lusitanica* cell cultures. *Journal of Experimental Botany*, 55, 1003-1012.

Zhao, Y., Yan, A., Feijó, J. A., Furutani, M., Takenawa, T., Hwang, I., Fu, Y. & Yang, Z. 2010. Phosphoinositides regulate clathrin-dependent endocytosis at the tip of pollen tubes in Arabidopsis and tobacco. *Plant Cell*, 22(12), 4031-4044.

Zhu, J.-K. 2016. Abiotic stress signaling and responses in plants. *Cell*, 167, 313-324.

Zimmermann, P., Hirsch-Hoffmann, M., Hennig, L. & Gruissem, W. 2004. GENEVESTIGATOR. Arabidopsis microarray database and analysis toolbox. *Plant Physiology*, 136, 2621-2632.

Zouhar, J. & Sauer, M. 2014. Helping hands for budding prospects: ENTH/ANTH/VHS accessory proteins in endocytosis, vacuolar transport, and secretion. *The Plant Cell*, 26, 4232-4244.

Zwiewka, M., Nodzyński, T., Robert, S., Vanneste, S. & Friml, J. 2015. Osmotic stress modulates the balance between exocytosis and clathrin-mediated endocytosis in *Arabidopsis thaliana*. *Molecular Plant*, 8, 1175-1187.