Recombinant Expression and Molecular Elucidation of the Biochemical Functions of a Novel Linker Histone-like Protein from *Arabidopsis thaliana*

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DECLARATION

I, **Neo Mokgadi Mametja**, declare that the content of this dissertation submitted to the Department of Biological Sciences at North-West University, Mafikeng Campus, for the Doctor of Philosophy degree in Biology titled **Recombinant expression and molecular elucidation of the biochemical functions of a novel linker histone-like protein from *Arabidopsis thaliana;*** hereby submitted by me, is original except where cited and all references contained therein have been duly acknowledged. Neither the whole work, nor any part has been submitted for another degree at this or any other university or tertiary institution and/or examination body.

Student: Neo Mokgadi Mametja

Signature Date

Supervisor: Prof Oziniel Ruzvidzo

Signature Date

DEDICATION

I dedicate this work to God almighty my creator my everything. He has been the source of my strength throughout this program and only on His wings have I soared. To my family and my late father, mate it is done, you have a Dr in your household.

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DEFINITION 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).

Abiotic Stress: The negative impact of non-living factors on the living organisms in a specific environment or ecosystem.

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

***Arabidopsis thaliana*:** A small flowering plant that is widely used as a model organism in plant biology.

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

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

Chromatin: The material of which the chromosomes of organisms other than bacteria (i.e. eukaryotes) are composed, consisting of protein, RNA, and DNA.

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

Cloning: A process of producing similar copies of genetically identical materials and/or individuals.

Co-expression: The simultaneous expression of two or more genes.

Complementation: A genetic cross used to identify if two mutations are located within the same or different gene.

Cyclic Adenosine 3′,5′-monophosphate (cAMP): A second messenger cyclic nucleotide formed from adenosine triphosphate 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 evolves, functions, and exists independently of the rest of the protein chain.

Endocytosis: A process by which cells absorb molecules (such as proteins) by engulfing.

Endogenous: Originating from within an organism’s tissues or cells.

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

Expression Construct: A plasmid that carries and transmits specific genes for expression within target cells.

Expression Profile: A description of the (relative) expression levels of a gene across a set of experimental conditions.

Expressional Analysis: The determination of the pattern of genes expressed at the level of genetic transcription, under specific circumstances or in a specific cell.

Food Security: A measure of the availability of food and individuals' ability to access it.

Heterologous Expression: The expression of a gene or part of a gene in a host organism, which naturally does not have this gene or gene fragment.

Histones: Any of a group of five small basic proteins, occurring in the nucleus of eukaryotic cells, that organize DNA strands into nucleosomes by forming molecular complexes around which the DNA winds.

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

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

Linker Histones: A set of proteins binding to DNA that enters and exits the nucleosome and facilitate the shift in equilibrium of chromatin towards more condensed, higher order forms.

Linker Histone-like Protein: Chief components of the chromatin, acting as spools around which DNA winds playing a role in gene regulation.

MacConkey Lactose Agar: A selective and differential medium designed to isolate and differentiate enterics based on their ability to ferment lactose.

Microarray: A set of DNA/RNA sequences representing the entire set of genes of an organism, arranged in a grid pattern for use in genetic testing.

Motif: A sequence pattern of nucleotides in a DNA molecule or amino acids in a protein molecule.

Nucleosome: A structural unit of a eukaryotic chromosome, consisting of a length of DNA coiled around a core of histones.

Polymerase Chain Reaction (PCR): A technique of amplifying a single copy or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of that particular DNA sequence.

Primer: A strand of short nucleic acid sequences, generally about 10 base pairs that serves as a starting point for DNA/RNA synthesis.

Promoter: A region of DNA that initiates transcription of a particular gene.

Recombinant Protein Purification: A method used widely for purifying proteins of interest based on well-developed recombinant DNA and protein expression technologies.

Re-folding: A process that restores the biological activity of an unfolded or misfolded protein.

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.

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

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

LIST OF ABREVIATION

AC : Adenylate cyclase

AMP : Adenosine monophosphate

ANOVA : Analysis of variance

AtCAP : *A. thaliana* clathrin assembly protein

ATP : Adenosine 5′-triphosphate

AtKUP5 : *A. thaliana* K$^+$-uptake permease 5 protein

AtKUP7 : *A. thaliana* K$^+$-uptake permease 7 protein

AtLHL : *A. thaliana* linker histone like protein

AtPPR : *A. thaliana* pentatricopeptide protein

BLAST : Basic Local Alignment Search Tool

Bp : Base pairs

cAMP : 3’,5’-Cyclic adenosine monophosphate

cDNA : Copy deoxyribonucleic acid

cGMP : 3′,5′-Cyclic guanosine monophosphate

cNGC : Cyclic nucleotide-gated channels

cNMP : Cyclic nucleotide monophosphate

C-terminal : Carboxyl terminal

Cv : Column volume

***cya*A**	**:**	Adenylate cyclase gene
DNA	**:**	Deoxyribonucleic acid
EDTA	**:**	Ethylene di-amine tetra-acetic acid
EST	**:**	Expresses sequence tag
EWE	**:**	Extreme weather events
FAO	**:**	Food and agriculture organization
GC	**:**	Guanylate cyclase
GTP	**:**	Guanosine triphosphate
HpAC1	**:**	*Hippeastrum hybridum* adenylate cyclase 1
IBMX	**:**	3-Isobutyl-1-methylxanthine
IPTG	**:**	Isopropyl β-D-1-thiogalactopyranoside
kDa	**:**	KiloDalton
KEGG	**:**	Kyoto encyclopedia of genes and genomes
LB	**:**	Luria-Bertani
LHL	**:**	Linker histone like
MALDI-TOF	**:**	Matrix laser desorption ionization time-of-light
mRNA	**:**	Messenger ribonucleic acid
MS	**:**	Murashige and Skoog
NbAC	**:**	*Nicotiana benthamiana* adenylate cyclase

NCBI	:	National Centre for Biotechnology Information
Ni-NTA	:	Nickel-nitrilotriacetic acid
NRL	:	Nucleosome repeat length
N-terminus	:	Amino-terminus
PAGE	:	Pulse-field gel electrophoresis
PAL	:	Phenylalanine ammonia lysate
PCR	:	Polymerase chain reaction
PSiP	:	Pollen signaling protein
RefSeq	:	NCBI reference sequence database
RNA	:	Ribonucleic acid
RT-PCR	:	Reverse transcriptase-polymerase chain reaction
sAC	:	Soluble adenylate cyclase
SAR	:	Systemic acquired resistance
SDS	:	Sodium dodecyl sulphate
SDS-PAGE	:	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SOC	:	Super optimal broth
SREC	:	School research and ethics committee
TAIR	:	The Arabidopsis Information Resource
TBE	:	Tris/borate/EDTA

TBS	**:**	Tris-buffered saline
tmAC	**:**	Trans-membrane adenylate cyclase
tRNA	**:**	Transfer ribonucleic acid
UN	**:**	United Nations
UniProt	**:**	Universal protein resource
UV	**:**	Ultraviolet
VIC	**:**	Voltage independent channels

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RESEARCH SUMMARY

Plants are sessile organisms and have evolved to live in environments exposed to various stress factors that may occur singly and/or in combination and as such, plants have to respond and adapt accordingly for survival. The threat of global climatic change has caused serious concerns among scientists as crop growth could be severely affected by changes in key climatic variables. In this regard therefore, food security thus has to heavily depend on the development of crop plants with increased resistance to environmental stress. As a result of this reason, plant biotechnologists have strategically began focusing on those plant molecules involved in maintaining and sustaining functional homeostasis. Cyclic 3′,5′-adenosine monophosphate (cAMP) and its generating enzyme, adenylate cyclase (AC), are one group of such plant molecules that plant biotechnologists are currently focusing on. ACs have previously been experimentally proven to be centrally involved in a number of stress response systems in prokaryotes, higher eukaryotes and animals, while their existence and/or functional properties in higher plants have until recently, been a very serious matter of debate and elusiveness. To date, only eight *A. thaliana* protein ACs have since been confirmed in higher plants while only one has been confirmed in lower plants. Those in higher plants are the *Zea mays* pollen signaling protein, the *Arabidopsis* pentatricopeptide repeat-containing protein, the *Nicotiana benthamiana* adenylyl cyclase protein, a *Hippeastrum hybridum* adenylyl cyclase protein, two *A. thaliana* K^+-uptake permeases, an *Arabidopsis* clathrin assembly protein *and* an *A. thaliana* leucine-rich repeat protein. Apparently, with the knowledge that a single AC per plant cannot account for all the cAMP-dependent processes in plants, this study was thereof set out to enzymatically and functionally characterize a novel probable AC candidate from *A. thaliana* in the form of a putative LHL

protein (AtLHL: At3g18035), with a view of elucidating the elusiveness of its exact biological, functional and physiological roles in higher plants.

Findings from our preliminary analysis of this protein using various bioinformatic programs and tools showed that this putative candidate is actually a binding signalling candidate with a possible role in cAMP-dependent adaptation and stress response mechanisms. More so, when this putative protein was cloned and recombinantly expressed in some chemically competent *E. cloni* BL21 (DE3) pLysS DUOs cells, the protein demonstrated its ability to generate endogenous cAMP in this prokaryotic expression system thereby raising the cAMP levels to over 2 folds. Again, the recombinant protein when expressed in the SP850 *Escherichia coli* mutant, it demonstrated its catalytic ability to generate cAMP from ATP whereby it functionally rescued this AC-deficient *E. coli* mutant from a non-lactose fermenter into a lactose fermenter – data that unequivocally provided evidence that the protein is indeed a functional AC. Additionally and when purified, the recombinant demonstrated to generate significant cAMP *in vitro* AC activity that was Mn^{2+}-, Ca^{2+} and CO_3^{2-}-dependent, reminiscent of all known soluble ACs (sACs). In summary, we therefore, concluded that the LHL protein is an essential plant protein candidate that may play an essential role in the possible management of plant stress-related factors for the enhancement of cultivar status and ultimately, crop productivity.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

The Food and Agriculture Organization (FAO) defines food security as occurring when "all people, at all times, have physical, social, and economic access to sufficient, safe and nutritious food, which meets their dietary needs and food preferences for an active and healthy life". Food security therefore, is upheld by four pillars including availability, access, utilization, and stability (Acharya *et al.*, 2007; Ai *et al.*, 2008; FAO, 2017). It is expected that climate change, including extreme weather events (EWEs), will impact the stability of food security within all four pillars across the world (Brown *et al.*, 2015; Niles *et al.*, 2017). If food availability, access, utilization, and stability in the regional food systems are disrupted by the EWE, then the ability to have safe and nutritious food becomes challenging, and the rising food prices may cause community members to be more vulnerable to issues such as individual and community health issues (Dodge, 2013). Food security at all times depends heavily on the development of crop plants with increased resistance to both biotic and abiotic environmental stress factors (Atkinson & Urwin, 2012). One of the societal, economic, and scientific challenges of the modern world is the constantly growing human population. Global population has been estimated to exceed 9 billion people by 2050, and unfortunately, food production levels of current state are not sufficient to meet the demand of such a large population. The ability to achieve global food security by 2050 will be challenging unless otherwise vital actions are considered and employed to prevent the devastating ripple effects of food shortage. The agriculture sector will be affected by changes in climate and soil deterioration that will further challenge the productivity of the world's agricultural resources (Fedoroff *et al.*, 2010; Tyczewska *et al.*, 2018).

Climate change has increased the risks and uncertainties associated with agriculture, particularly in developing countries. Climate change is among the principal limiting factors causing a decline in agricultural productivity. The threat of global climate change has caused concern among scientists as crop growth could be severely affected by changes in key climatic variables (i.e. rainfall and temperature) thus compromising agricultural production and food security both locally and globally (Altieri & Nicholls, 2017; Imbach *et al.*, 2017). One key of the impact would be the projected increase of extreme weather events, defined as a weather occurrence "rare or rarer than the 10th or 90th percentile", and "typically include floods and droughts" throughout the 21st century (Herring *et al.*, 2015; Pachauri *et al.*, 2014).

Plants play a very central and vital role in the ecosystem of the earth because they produce food that is later on made available to consumers such as humans and animals, and for that one reason, farming of crop plants for both food and feeding purposes make agriculture an important system to the sustainable existence of both humankind and the animals that solely depend on it. However, the productivity of crop plants is somewhat becoming increasingly threatened, and in some cases, rather diminished by the various stress factors induced by climate change. Climate change is projected to affect virtually every region in the world though with varying impacts and uncertainties (NASA, 2017; Pachauri *et al.*, 2014). Most importantly, climate change impacts are predicted even if the most plausible and effective climate change mitigation strategies have been deployed to date. Climate change is predicted to influence food security globally across the food system among many other potential impacts, with impacts ranging from reduced yields in agricultural production to storage and disruptions in food transportation (Barnosky *et al.*, 2012; Brown *et al.*, 2015; Rockström *et al.*, 2009).

Nakashima and team (2014) have described plants as sessile organisms that have evolved to live in environments where they are often exposed to different stress factors that affect them in combination; and as such, need to respond and adapt to such severe environmental conditions for survival. Plants have unique cellular systems involved in environmental responses for acquiring stress resistance (Nakashima *et al.*, 2014). Unlike animals, plants do not have a central nervous system, thus by connecting roots and shoots, and the vasculature is thought to be important for integrating environmental information from underground to aerial parts of the plant. More so, certain mobile molecules function through the plant vasculature for the integration of environmental responses (Takahashi & Shinozaki, 2019).

The word stress is well known to most biologists, as it has proven to be a very elusive concept, and is currently being used in the scientific literature in many different ways and contexts. With respect to plants, stress is defined as any change in environmental conditions that might reduce or adversely affect the normal growth and/or development of an organism. However, and most importantly, stress is not only an attribute of the stressor (the environmental component) but also that of the stressed (the biological component). Environmental stress is considered primarily to be a response to the physical features of the environment and extrinsic stresses that result from changes in abiotic factors, either naturally occurring or man-made, are regarded as the most important stress agents (Qureshi *et al.*, 2007). These stresses can either be biotic or abiotic; and are a particular threat to crop yields in regions that have already been impacted by climate change. The principal biotic stresses are normally those from pests, diseases, other plants and herbivores, while the world's most dominant abiotic stresses include, among others, drought, floods, high and low temperatures, strong light, high salinity, and low soil nutrient levels and also the man-induced pollutants (for example, heavy metals, ozone and acid rain). In line with this, it has been suggested that these stress factors reduce the average yields by >50% for most major crop plants. Growing in their native environment, plants often encounter a variety of these biotic and abiotic constraints that interfere with their normal growth and productivity and therefore, resulting in significant crop losses globally (Murphy, 2011; Wang *et al.*, 2003; Yang *et al.*, 2016).

Notably, plants have developed adaptive features against these stresses and have evolved to live in environments where they are more often exposed to different stress factors in combination. Being sessile, they have developed specific mechanisms that allow them to detect precise environmental changes and respond to complex stress conditions, thus minimizing damage while conserving valuable resources for growth and reproduction. Plants have been noted to activate specific and unique stress responses when subjected to a combination of multiple stresses (Rizhsky *et al.*, 2004). Drought and salt stress conditions among others, have been reported to retard plant growth and decrease crop productivity. Environmental stresses that affect plant growth and severe stresses always inflict severe damages. Plant responses to these stresses have been analyzed at the molecular and cellular levels as well as at the physiological level as these stresses are inducing various genes. Many genes regulating responses and resistance to various stresses have been precisely identified and these genes have various cellular functions in stress resistance and signal transduction. However, coordinated responses between roots and leaves at the whole plant level remains unknown (Bray, 1997; Hasegawa *et al.*, 2000; Ingram & Bartels, 1996; Shinozaki & Yamaguchi-Shinozaki, 1997).

Defence mechanisms can be extremely costly for the plant, therefore, plants have developed defence response systems which can be quickly activated in response to stressors and influencing the entire plant body. In actual terms, plants respond and adapt to environmental stresses through short-term responses to prevent severe damages and long-term adaptations to acquire stress tolerance at the whole plant level. This so-called systemic acquired resistance (SAR) is accomplished either by the transport of defence metabolites or through the production of new defence components (Heil & Ton, 2008; Mittler & Blumwald, 2015). SAR is acquired by a modification of the gene transcription patterns, leading to an overall increase in plant fitness to a broader spectrum of biota as well as environmental conditions (Gilroy *et al.*, 2014; Ryals *et al.*, 1996). Several signalling events, such as turgor changes, calcium oscillations and phytohormone production, mediate long-distance signalling to maintain stress adaptations. While changes in calcium levels and turgor pressure are rapidly triggered signals, hormones function as rather slow signals. They are first induced by stress conditions and then transported mainly through the vasculature at different speeds to respond to various environmental stresses (Choi *et al.*, 2017; Christmann *et al.*, 2013; Kudla *et al.*, 2018).

To this day, there is a very good amount of work to explain on the types of proteins that may be under- and/or over-expressed during a particular or integrative stress factor, their impacts on cellular metabolism and the location of such proteins. Therefore, and in line with this, various advances, through the specialized areas of plant biochemistry, plant physiology and plant biotechnology, have been made in attempting to understand the processes of gene expression, transcriptional regulation and signal transduction in plants, which are capable of increasing tolerance to both biotic and abiotic stress factors (Zhu *et al.*, 2010). As a result, molecular and genomic analyses have practically facilitated gene discovery and enabled the possibility of genetics to use several functional and/or regulatory genes to activate or repress specific or broadband pathways related to the maintenance of homeostasis and adaptation to stress responses (Abe *et al.*, 2003; Hussain *et al.*, 2010; Seki *et al.*, 2001; Seki *et al.*, 2007; Tran *et al.*, 2004; Trujillo *et al.*, 2009).

Extensive research has given rise to impressive achievements in genome and expressed sequence tag (EST) sequencing in the recent years, yielding a wealth of information for many model plants, such as *A. thaliana*, *Oryza sativa, Zea mays* and *Medicago truncatula* among others. However, genome sequence information alone is not sufficient to reveal the facts and details concerning developmental/regulatory biology, gene function and the biochemical kinetics of life. Comprehensive approaches, including quantitative and qualitative analysis of gene expression products are necessary at the proteomic, transcriptomic and metabolomic levels, to investigate these facts. Although transcriptome analysis with the use of microarray and serial analysis of gene expression technologies are potential tools, mRNA and protein levels cannot be correlated due to the inability of total mRNA to translate into protein, whereas proteomics provides a more direct assessment of the biochemical processes of monitoring the actual proteins performing the signalling, enzymatic, regulatory and structural functions encoded by the genome and transcriptome (Futcher *et al.*, 1999; Gygi *et al.*, 1999). Recent improvements in high-resolution two dimensional pulse-field polyacrylamide gel electrophoresis (PAGE), an increase in the number of sequences of protein and nucleotide sequences, increased capabilities for protein identification utilizing modern mass spectrometry methods, such as the matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry; and valuable bioinformatics tools, have made the large-scale profiling and identification of proteins a dynamic area of renewed plant research (Görg *et al.*, 1999; Yates III, 1998a; Yates III, 1998b; Zolla & Timperio, 2005).

The need to improve existing varieties and increase agricultural production remains a pressing issue, despite the great achievements of the past centuries, as it is associated with the continuous growth of the human population and the global climatic change accompanied by exposure of organisms to greater biotic and abiotic stresses. Intensive agriculture that was practiced over the past decades with only one aim to increase yield, without adherence to ecological or scientific principles, has led to pollution, loss of healthy soil, desertification, soil erosion, salinization and depletion of freshwater resources as well as agrobiodiversity. If we are to resume progress towards eliminating hunger, a sustainable model for intensive agriculture, combining the lessons deduced from history with the benefits of modern biotechnology, should be elaborated and practiced (Culliney, 2014). Plant scientists over the centuries have developed many technologies and various innovations aimed at improving and even boosting agricultural productivity. The most recent of all these is plant biotechnology, which facilitates the introduction of desirable traits into plant genomes of interest at the molecular level. Recently, plant biotechnologists have specifically focused onto plant enzymatic molecules known as adenylate cyclases (ACs) in an effort to elucidate how plants do respond and adapt to stressful factors, and for the possible eventual application of such knowledge into the current agricultural context.

1.2. EXPERIMENTAL PROPOSAL

1.2.1. Study Rationale

The linker histone-like (LHL) protein is a plant molecule responsible for binding to nucleosomes and linker DNA to stabilize condensed higher-order chromatin structures *in vitro* (Bednar *et al.*, 1998; Carruthers *et al.*, 1998) and chromatin de-compaction occurs *in vivo* when linker histones are genetically depleted from cells (Fan *et al.*, 2005; Lu *et al.*, 2009a). Altogether, the current paradigm holds that linker histones modulate nuclear processes such as transcription, replication, and DNA repair through their interactions with DNA and effects on chromatin condensation (Happel & Doenecke, 2009; Izzo *et al.*, 2008; Kowalski & Pałyga, 2012; Szerlong & Hansen, 2010). More so, it has become apparent that linker histones proteins also bind specifically to proteins (Szerlong *et al.*, 2015). Besides all this, the LHL protein has been bioinformatically annotated as a possible putative AC molecule in higher plant AC (Gehring, 2010a) but essentially, no study to date has practically and experimentally demonstrated the ability of this unique protein molecule to generate cAMP from ATP. In this regard, this study therefore, was set to fill up the gap through testing its probable AC activity and exploring for its possible functional roles in plants particularly in relation to plant stress response and adaptation mechanisms. The study focus was underpinned on the insight of a probable application of the derived knowledge into the South African agricultural context to increase crop yield and ultimately the status quo of food security in the country.

1.2.2. Aim of the Study

This study was aimed at determining the molecular AC activity of the AtLHL protein and its probable role in plant's stress response and adaptation mechanisms.

1.2.3. Research Specific Objectives

The specific objectives were set out to attempt and appropriately address the presented research questions:

1. To isolate the AC-containing gene fragment of the annotated LHL gene from *A. thaliana* (AtLHL-AC) and clone it into a stable and biologically viable heterologous prokaryotic expression system.

2. To optimize the expression strategies of the cloned AtLHL-AC gene fragment into a truncated recombinant AtLHL-AC protein.

3. To determine the endogenous AC activity of the expressed recombinant AtLHL-AC protein.

4. To determine the *in vivo* AC activity of the expressed recombinant AtLHL-AC protein.

5. To optimize the purification strategies of the expressed recombinant AtLHL-AC protein and determine its *in vitro* AC activity.

6. To further characterize the *in vitro* AC activity of the purified AtLHL-AC recombinant protein.

7. To elucidate through bioinformatics the probable molecular and/or functional properties of the LHL protein in Arabidopsis and other closely related plants.

1.2.4. Significance of the Research Study

The functional characterization of the annotated LHL gene from *A. thaliana* was expected to significantly contribute to the knowledge currently available by giving a better understanding of the possible mechanisms through which plants generally use to respond and adapt to stressful environmental conditions. Furthermore, the study would also provide a better scientific understanding of the genes responsible for stress response and adaptation mechanisms in plants for better and enhanced management of stressful conditions in plants. Additionally, the overall knowledge attained in the study could perhaps be used to address the issues of crop yields and thus food security in the country. Finally, the study would add more content to the already existing literature and scholarship on higher plant ACs to the body of plant biotechnology and scientific knowledge.

1.2.5. Ethical Clearance

Approval to perform this study was obtained from the School Research and Ethics Committee (SCREC) of the North-West University (Mafikeng Campus).

1.2.6. Thesis Design

The content of this study is presented in 6 Chapters, Chapter 1 being the general introduction and a review of the literature, followed by a preliminary bioinformatic analysis of the At3g18035 gene in Chapter 2. Chapter 3 covers details on partial expression of the recombinant AtLHL-AC protein and determination of its endogenous AC activity. Determination of the *in vivo* AC activity of the recombinant AtLHL-AC protein is articulated in Chapter 4, while chapter 5 entails the affinity purification and characterization of the AC activity of the recombinant AtLHL-AC protein *in vitro*. In Chapter 6, results obtained in all the previous chapters are briefly summarized upon to reflect on the initial problem statement. A reference list of all citations used in the document is finally included at the end of the thesis.

1.3. LITERATURE REVIEW

1.3.1. Second Messengers and Signal Transduction

Studies of cell signalling in higher plants are thriving, and according to some published reviews, this greatly signifies the importance of the field of cell signalling (Clark *et al.*, 2001). Understanding how whole organisms, tissues and cells perceive and process signals has given rise to the biological discipline of ‘signal transduction’ (Irving & Gehring, 2013). Signal transduction pathways are biological networks which all living organisms utilise to conduct their developmental programs, to obtain nutrients, to cope with their environment and to control their mechanisms (Kaplan *et al.*, 2007).

Plants are sessile living organisms that are constantly subjected to a number of abiotic and biotic stress factors in their natural habitats, and this forces them to acclimatize and respond flexibly to such aggressive conditions by switching their cell metabolism and physiological activities in response to environmental conditions (Lichtenthaler, 1996). In view of the above-given premises, it is outright that plants are sensitive organisms in need of really proactive biological mechanisms that will aid in their survival and also ensuring food security through the tolerance and continued adaptation to various stress factors. Signal generating and processing complexes and changes in concentrations of messenger molecules such as cyclic nucleotides, develop gradients that have critical roles in relaying messages within cells. Nucleic contents are densely packed, and in plant cells, this is compounded by the restricted nucleic space. To function in such crowded spaces, scaffold proteins have evolved to keep key enzymes in the correct place as well as to ensure ordered spatial and temporal and stimulus specific message generation. Hence throughout the nucleus, there are gradients of messenger molecules that influence signaling processes. However, it is only recently that it became apparent that specific complexes involving receptor molecules can generate multiple signal gradients and enriched microenvironments around the nucleic domains of the receptors that regulate downstream signaling (Irving *et al.*, 2018).

Correct spatial arrangements of individual enzymes is necessary for signals to be relayed through signal networks, to elicit cellular responses, because molecular crowding still occurs despite the limited copies of individual proteins present (Ellis, 2001; Luby-Phelps, 1999; Luby-Phelps, 2013). Several metabolic pathways or metabolons employ molecular channeling to efficiently deliver the product from the first enzyme to form the substrate for the next enzyme (Miles *et al.*, 1999; Møller, 2010; Srere, 2000; Sweetlove & Fernie, 2013; Winkel, 2004; Zhang *et al.*, 2017). Correct positioning of individual enzymes and scaffold proteins enhances signal cascades via molecular channeling. Positioning in this way creates subcellular microenvironments containing microcues of concentrated signaling molecules that in turn activate downstream points of signal cascades, and thereby highlighting the importance of spatial and temporal regulation of protein expression (Rohwer *et al.*, 1998; Wheeldon *et al.*, 2016).

A considerable body of literature exists on the biological functions and mechanisms of action of cyclic nucleotide signaling in lower and higher eukaryotes (Lemtiri-Chlieh *et al.*, 2011). In fact, cGMP and cAMP are accepted as key signaling molecules with key roles in many and diverse physiological response cascades and processes in prokaryotes and eukaryotes. However, acceptance that cyclic nucleotides have such a role in plants has been slow. This has been reported to be because, in plants, cellular cyclic nucleotide levels seemed generally lower than in animals or lower eukaryotes (Gehring, 2010a; Marondedze *et al.*, 2017; Newton *et al.*, 1999). An additional reason being also that the molecular evidence for mononucleotide cyclases in higher plants only came to light after publication of the first plant draft genome in 2000 (Ludidi & Gehring, 2003). Since then, the number of identified and experimentally tested mononucleotide cyclases has steadily been increasing and with indications that there might be more than 50 candidates in the *A. thaliana* proteome and that they come in many different domain organizations. Thus pointing to a diverse spectrum of biological functions for mononucleotide cyclases and their catalytic products (Meier *et al.*, 2007; Wong & Gehring, 2013).

cAMP has arguably become one of the most extensively studied second messengers in animals, lower eukaryotes and bacteria. For instance, in bacteria, cAMP positively regulates the *lac* operon. In an environment of low glucose, cAMP accumulates and binds to the allosteric site of the transcription activator protein (CRP). Once the CRP is activated, it binds to a *cis*-element upstream of the *lac* promoter and activates transcription (Meiklejohn & Gralla, 1985). cAMP signaling has also been reported to be important in the development of the slime mold *Dictyostelium discoideum* growing unicellularly but develops as a multicellular organism, thus resulting in cAMP dependent signals mediating cell sorting, pattern formation and morphogenetic changes (McMains *et al.*, 2008). In animals, cAMP can be readily incorporated into a hormonal cascade controlling various processes, including cardiac contractility and neurotransmitter release (Gancedo, 2013). The levels of cyclic nucleotides in plants have been reported to be lower than in animals, and this was the reason why the acceptance of cAMP as a second messenger in plant cells has then delayed (Gehring, 2010a; Newton & Smith, 2004). Another and possible more important reason for the reluctant acceptance of cAMP in higher plants is that, there was no genetic or molecular evidence of enzymes involved in its synthesis.

In plants, cAMP has been implicated in many processes, including cell cycle control in tobacco cells (Ehsan *et al.*, 1998), sodium ion transport via voltage independent channels (VICs) in *A. thaliana* roots (Maathuis & Sanders, 2001), activation of the phenylalanine ammonia lyase (PAL) enzyme in French beans (Bolwell, 1992) and phenyl propanoid pathway in Arabidopsis (Pietrowska-Borek and Nuc, 2013). cAMP is also involved in stress response (Choi & Xu, 2010; Thomas *et al.*, 2013), primarily via cyclic nucleotide-gated channels (CNGCs) (Zelman *et al.*, 2012). In addition, studies have implicated cAMP in activation of phytoalexin synthesis in the sweet potato (*Ipomoea batatas)* (Oguni *et al.*, 1976), early signaling events in the apoplastic oxidative burst (Bindschedler *et al.*, 2001), and also to be elevated at the infection site initiation in pathogen-related cytosolic Ca^{2+} signaling (Ma *et al.*, 2009).

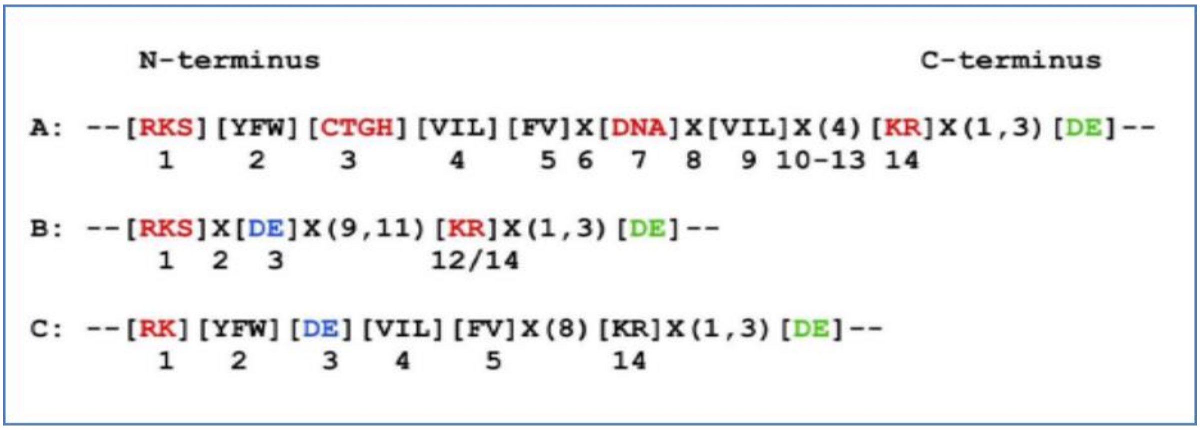
Moreover, production of elevated concentrations of cAMP by administration of elicitors has been demonstrated in *Phaseolus vulgaris*, a French bean (Bolwell, 1992), carrot (Kurosaki & Nishi, 1993), *Medicago sativa* or alfalfa exposed to the *Verticillium alboatrum* glycoprotein (Cooke *et al.*, 1994), *Cupressus lusitanica*, a Mexican cypress cell culture treated with yeast oligosaccharides (Zhao *et al.*, 2004), and Arabidopsis treated with *Verticillium dahliae* toxins, resulting in improved disease resistance of the plants (Jiang *et al.*, 2005). It is also worth to note that many signaling cascades generated in response to biotic stresses not only employ cNMPs, but also critically depend on CNGCs.

Typically, ACs are enzymes that catalyse the formation of the universal second messenger cAMP by converting the biological molecule ATP. These ACs naturally exist in two forms namely; the trans-membrane (membrane-bound) and the soluble (intracellular) forms. Trans-membrane ACs (tmACs) are mainly regulated by G-proteins as part of the G-protein coupled receptor pathways and are activated by forskolin and the fluoride ion while requiring magnesium as the co-factor for activity (Hess *et al.*, 2005; Seamon, 1984). Whereas, soluble ACs (sACs), on the other hand, are not associated with the plasma membrane and are mainly activated by the bicarbonate and calcium ions, are insensitive to forskolin and the fluoride ion, and generally, prefer manganese over magnesium as a co-factor of catalytic activity (Gomperts & Tatham, 2009; Rahman *et al.*, 2013; Steegborn, 2014). Moreover, in contrast to tmACs, sACs produce cAMP in various intracellular micro-domains, close to specific cAMP targets such as in the nuclei and mitochondria (Ladilov & Appukuttan, 2014). External factors such as the far-red and red light, temperature, exogenous phytohormones, as well as the specific triggering compounds of fungal and bacterial origin, exert a significant influence onto the activities of both plant tmACs and sACs (Lomovatskaya *et al.*, 2008).

Notably and to date, nine putative AC candidates in Arabidopsis genome have been bioinformatically identified with regards to higher plant ACs (Gehring, 2010a), but only few ACs of them have been experimentally tested. These include a *Zea mays* pollen signaling protein (PSiP) that participates in polarized pollen tube growth (Moutinho *et al.*, 2001a), an Arabidopsis pentatricopeptide repeat-containing protein (AtPPR), which is responsible for pathogen responses and gene expressions (Ruzvidzo *et al.*, 2013), a *Nicotiana benthamiana* protein (NbAC) with a role in tabtoxinine-β-lactam-induced cell deaths during wildfire diseases (Ito *et al.*, 2014b), a *Hippeastrum hybridum* protein (HpAC1) that is involved in stress signaling (Świeżawska *et al.*, 2014), two *A. thaliana* K^+-uptake permeases AtKUP5 and AtKUP7, both responsible for K^+ transportation (Al-Younis *et al.*, 2015; Al-Younis *et al.*, 2018), an Arabidopsis clathrin assembly protein (AtCAP) with a predicted role in actincytoskeletal remodeling during endocytic internalization (Chatukuta *et al.*, 2018) and, an *A. thaliana* leucine-rich repeat protein (LRR) which affects responses to pathogens (Bianchet *et al.*, 2018). One possible reason for the apparent elusiveness around cAMP is the evolutionary divergence of plant nucleotide cyclases was probably due to the fact that only key amino acid residues within the catalytic centers appear to be the only ones retained. Furthermore, in plants, many proteins exist as complex molecules consisting of primary domains and secondary moonlighting sites that may include ligand binding sites and catalytic centers (Irving *et al.*, 2018).

Due to the reason that these functional sites constitute only a small portion of a relatively large protein complex, they therefore, usually could not be searched for using BLAST. Key residues in canonical cyclases situated distantly from each other within a single protein chain or located on different protein chains may come together to form the catalytic pocket; and plant proteins harbour moonlighting sites containing key amino acids situated in much closer proximity (Xu *et al.*, 2018). Hence, rationally designed motifs containing only key residues of the catalytic centers have been implemented (Figure 1.1) and have since led to the discovery of a number of novel plant ACs as shown in Table 1.1 (Gehring, 2010a; Wong *et al.*, 2018). The presence of the center motif proving to be useful supporting the criteria for the identification of candidate ACs, it has shown not to be stringent enough to identify candidate ACs *ab initio*.

Therefore, this research study has specifically focused on the AC-containing domain of the AtLHL protein that has bioinformatically been identified from the Arabidopsis genome through a search motif that consists of functionally assigned amino acids in the catalytic centres of annotated and/or experimentally tested nucleotide cyclases with extended 16 or 18 amino acid long AC motif that was designed based on the center motif having the C-terminal Mg^{2+}/Mn^{2+}- binding amino acid at positions 15 or 17 in Figure 1A-C, and the extended AC motif retrieved nine putative Arabidopsis AC candidates as shown in Table 1.1 (Gehring, 2010a).



```
     N-terminus                                   C-terminus

A: --[RKS][YFW][CTGH][VIL][FV]X[DNA]X[VIL]X(4)[KR]X(1,3)[DE]--
      1    2    3     4    5 6  7   8  9 10-13 14

B: --[RKS]X[DE]X(9,11)[KR]X(1,3)[DE]--
      1   2  3         12/14

C: --[RK][YFW][DE][VIL][FV]X(8)[KR]X(1,3)[DE]--
      1   2    3    4    5      14
```

Figure 1-1: Catalytic centre motifs of nucleotide cyclases. (A) Centre motif of experimentally tested GCs in plants. The residue (red) in position 1 does the hydrogen bonding with the guanine, the amino acid in position 3 confers substrate specificity and the residue in position 14 stabilizes the transition (GTP/cGMP). The Mg^{2+}/Mn^{2+}-binding site is C-terminal (green). In the derived motifs (B and C) specific for ACs, position 3 (blue) has been substituted to [DE] to allow for ATP binding. Adapted from (Gehring, 2010a).

Table 1-1: The thirteen bioinformatically identified *Arabidopsis thaliana* proteins containing the AC catalytic search motifs (adapted from Gehring, 2010).

ATG NUMBER	SEQUENCE	ANNOTATION
At1g25240	-KWEIFEDDFCFTCKDIKE-	Epsin N-terminal homology
At1g62590	-KFDVVISLGEKMQR--LE-	Pentatricopeptide (PPR) protein
At1g68110	-KWEIFEDDYRCFDR--KD	Clathrin assembly protein
At2g34780	-KFEIVRARNEELKK-EME-	Maternal effect embryo arrest 22
At3g02930	-KFEVVEAGIEAVQR--KE-	Chloroplast protein
At3g04220	-KYDVFPSFRGEDVR--KD-	TIR-NBS-LRR class
At3g18035*	-KFDIFQEKVKEIVKVLKD-	Linker histone-like protein – HNO4
At3g28223	-KWEIVSEISPACIKSGLD-	F-box protein
At4g39756	-KWDVVASSFMIERK--CE-	F-box protein

ATG denotes the assigned *A. thaliana* gene bank numbers for the thirteen putative AC proteins, followed by their amino acid sequences suspected to be their AC catalytic centers, and lastly, the names to which each protein was bioinformatically annotated.

*The AtLHL-AC protein that was cloned and functionally characterized in this study.

1.3.2. The LH-like Protein

Histones were first identified as fundamental components of the nucleus in the early days of molecular biology (Stedman & Stedman, 1951; Wilkins, 1956) and were described as circular structures responsible for compacting DNA (Finch & Klug, 1976; Kornberg & Thomas, 1974; Luger *et al.*, 1997). Later on, they were shown to be associated with the DNA periodicity and compaction (Busch, 2012; Kornberg & Thomas, 1974; Langridge *et al.*, 1957; Pardon *et al.*, 1974; Wilkins *et al.*, 1959), where DNA coils around an octamer of four core histone proteins (Luger *et al.*, 1997; Oudet *et al.*, 1975). The histone core with DNA is termed nucleosome and is known to be the first level of chromatin compaction. Vitally important, nuclear processes such as transcription, DNA replication and repair are accomplished within the chromatin environment and as a result, utterly depend on its structure and dynamics. The overall organization of chromatin at levels above the nucleosome arrays, i.e. “30-nm” chromatin fiber, chromatin loops, etc., has remained unclear. Nonetheless, significant evidence exists that LHs play an important role in building up the higher-order chromatin structure (Georgieva *et al.*, 2012; Hansen, 2002; McBryant *et al.*, 2010; Robinson & Rhodes, 2006; Sarg *et al.*, 2014; van Holde & Zlatanova, 2007).

Chromatin is a macromolecular complex made up of DNA and proteins that condense the genomic DNA in the nucleus of eukaryotic cells. The fundamental structural unit of chromatin is the nucleosome. This cylindrical, nanometric bead is made of 1.75 turns of DNA wrapped around the nucleosome core, an octamer that contains two copies each of the core histones H2A, H2B, H3, and H4 (Alberts *et al.*, 2002; Alberts *et al.*, 2007). In each chromosome, a single DNA molecule connects consecutive nucleosomes through linker DNAs, which determine the nucleosome repeat length (NRL), a crucial structural variable of chromatin defined by the sum of DNA base pairs (bp) of the nucleosomewound and linker DNA (Grigoryev, 2012; Perišić *et al.*, 2010; Van Holde, 1989). Physiological salt conditions and the presence of LHs H1/H5 compact this beads-on-a-string polymer of the nucleosomes and linker DNAs, leading to a thicker or interdigitated fiber organization that keeps hierarchically folding until they have shaped a chromosome in the nucleus (Grigoryev & Woodcock, 2012; Maeshima *et al.*, 2010; Misteli, 2007) as shown in Figure 1.2. The structure of chromatin is well characterized at the nucleosome level (Davey *et al.*, 2002; Luger *et al.*, 1997), and modern experimental techniques have made great improvements regarding visualizing chromatin at the chromosomal level (Dekker, 2008; Naumova *et al.*, 2013). Although the organization of chromatin at intermediate levels has remained controversial for more than three decades (Grigoryev & Woodcock, 2012; Quénet *et al.*, 2012), a combination of experiments, new imaging techniques, and computational models is producing significant progress (Schlick *et al.*, 2012; Song *et al.*, 2014).

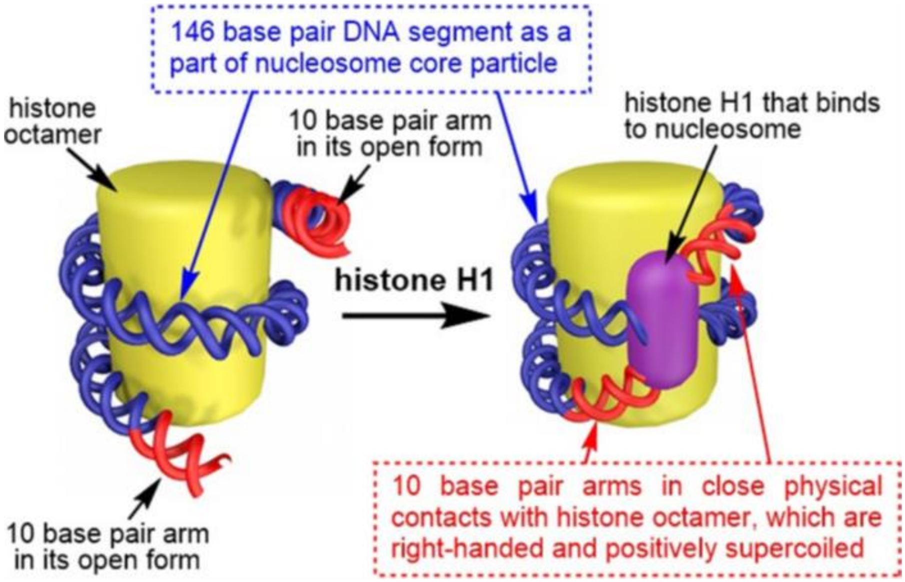


Figure 1-2: Illustration of the right-handed positive DNA supercoils in two arm DNA segments that are affiliated with the binding of histone H1 to a nucleosome (Luger *et al.*, 1997).

Previous studies of nucleosome recognition by LHs have been reported to focus mainly on how the globular domain of chicken H5 (GH5, $H5_{24-98}$) binds to the nucleosome (Allan *et al.*, 1980; Cui & Zhurkin, 2009; Fan & Roberts, 2006; Zhou *et al.*, 1998). Better still, either the full-length linker histone H5 or GH5 alone can protect the same native chromatin linker DNA from micrococcal nuclease digestion (Allan *et al.*, 1980; Puigdomènech *et al.*, 1983). Zhou and team (2015) combined X-ray crystallography and NMR to investigate the structural mechanism of nucleosome recognition by GH5 and found that it binds to the nucleosome on the dyad (Zhou *et al.*, 2015). Using this NMR, it was also shown that the globular domain of *Drosophila* LH H1 (GH1) binds to the nucleosome off the dyad (Zhou *et al.*, 2013). Later on, Zhou and team (2016) explored the determinant for the distinct binding modes of LHs and found that a small number of residues in the globular domain of LHs play critical roles in determining the nucleosome-binding modes (Zhou *et al.*, 2016).

1.3.3. LH Structure and Sub-types

LHs have a conserved tripartite structure consisting of a short flexible N-terminal tail, a central globular domain (~80 residue), and a long (~100 residue) intrinsically disordered highly basic C-terminal tail (Allan *et al.*, 1980). The short N-terminal tail of LHs contributes little to nucleosome binding (Allan *et al.*, 1980; Hendzel *et al.*, 2004; Syed *et al.*, 2010). The middle globular domain preferentially binds to the nucleosome core with one or two DNA linkers (Allan *et al.*, 1980; Singer & Singer, 1976; Zhou *et al.*, 2013). The long C-terminal tail interacts with linker DNA (Caterino & Hayes, 2010; Fang *et al.*, 2011; Lu & Hansen, 2004) and is important for higher-affinity binding of LHs to the nucleosome (Zhou *et al.*, 2013), folding of 30-nm chromatin fibers (Allan *et al.*, 1986), association of LHs with chromatin *in vivo* (Brown *et al.*, 2006; Hendzel *et al.*, 2004), and the stem structure formation of longer linker DNA *in vitro* (Bednar *et al.*, 1998; Syed *et al.*, 2010). Of note, the structured domain does not have a typical histone fold. Instead, it is organized in a winged-helix pattern with three helices (α1 to α3), three loops (L1-L3) and two short beta sheets (B1 and B2) in hair-pin (Cerf *et al.*, 1993; Ramakrishnan *et al.*, 1993a).

The LH family is composed of at least 15 species - the developmental stage and tissue specific isoforms (Lennox & Cohen, 1983; Lennox & Cohen, 1984; Lennox & Cohen, 1989). One important feature of the LH H1 family is its heterogeneity. Multiple H1 sub-types are expressed in organisms as diverse as plants or humans, with eleven of them, namely: H1.0, H1.1, H1.2, H1.3, H1.4, H1.5, H1t, H1T2, H100, H1LS1 and H1X found in mammals (Parseghian *et al.*, 1994). In humans, the histone H1 family includes 11 different members with 7 somatic sub-types (H1.1 to H1.5, H1.0 and H1X), 3 testis-specific sub-types (H1t, H1T2 and H1LS1) and 1 oocyte-specific sub-type (H100) (Izzo *et al.*, 2008; Parseghian & Hamkalo, 2001). Seven of the mammalian variants, H1. (1–5), H1.0 and H1X, were detected in somatic cells (Franke *et al.*, 1998; Parseghian *et al.*, 1994). Although, H1.1 is specific for the spleen, thymus and testis, H1.0 is characteristic for the terminally differentiated cells. A specific sub-type, characteristic for the avian and amphibian erythrocytes, is the LH H5 (Morris, 1976). Distinct LH embryonic sub-types B4, H100 and dBigH1 in amphibians, mouse and Drosophila respectively, were also identified and functionally characterized (Dimitrov *et al.*, 1993; Pérez-Montero *et al.*, 2013; Tanaka *et al.*, 2003). A particular case is Hh01p which is an LH reported in *Saccharomyces cerevisiae* with two globular domains (Patterton *et al.*, 1998). *In vivo*, the deposition of LHs to nucleosome is assisted by dedicated chaperones. Individual somatic H1 sub-types differ in their expression patterns during development and differentiation (Khochbin &

Wolffe, 1994), their ability to bind to and condense chromatin substrates (Talasz *et al.*, 1998) and their phosphorylation pattern during cell cycle (Talasz *et al.*, 1996).

1.3.4. LH Functions and other Roles

The most obvious functions of LHs are those involved in chromatin architecture and gene expression. LHs have various posttranslational modifications (Harshman *et al.*, 2013) and interact with non-histone proteins (Kalashnikova *et al.*, 2013). The first posttranslational modification (PTM) of LH was reported in 1972 (Balhorn *et al.*, 1972). Since then, many studies have shown that LHs can have methylation, acetylation, ADP ribosylation, ubiquitination, formylation, and PARylation PTMs (Bonet-Costa *et al.*, 2012; Deterding *et al.*, 2008; Garcia *et al.*, 2004; Jiang *et al.*, 2007; Kim *et al.*, 2005; Lu *et al.*, 2009b; Poirier *et al.*, 1982; Sarg *et al.*, 2015; Snijders *et al.*, 2008; Villar-Garea & Imhof, 2008; Wiśniewski *et al.*, 2007a; Wiśniewski *et al.*, 2007b). Izzo and Schneider in 2016, extensively reviewed human and mouse H1 PTMs and reported that H1 phosphorylation can have opposing effects on chromatin condensation (Izzo & Schneider, 2016). Horn *et al*. in (2002) suggested that H1 phosphorylation may regulate ATP-dependent chromatin remodeling enzymes and thus impacting on chromatin compaction (Horn *et al.*, 2002). Furthermore, high H1 phosphorylation levels are linked with DNA repair (Chubb & Rea, 2010), apoptosis (Kim *et al.*, 2012), cellular aging (Happel *et al.*, 2008), and cancer events (Harshman *et al.*, 2014). H1 methylation has also been associated with heterochromatin organization (Fischle *et al.*, 2003) and cell-cycle-regulated chromatin binding (Bonet-Costa *et al.*, 2012).

As a chromatin factor, LHs play important roles in regulating important cellular functions, including gene expression (Fan *et al.*, 2005; Shen *et al.*, 1996), mitotic chromosome architecture and segregation (Maresca *et al.*, 2005), muscle differentiation (Lee *et al.*, 2004), embryonic stem cell differentiation (Zhang *et al.*, 2012), genetic activity of heterochromatin (Lu *et al.*, 2013), and cell pluripotency (Christophorou *et al.*, 2014). However, there are intriguing hints that LHs also may have important DNA-independent functions. A handful of studies have linked microtubules to LHs. This is particularly interesting for plants, because, unlike animals, plants lack microtubule organizing centers. In spite of this, microtubules still play complex roles in determining cell shape and growth (Ehrhardt & Shaw, 2006). Hence an ongoing research is aimed at understanding how microtubules are organized without this centralized structure. Previous work has shown that sea urchin flagella microtubules were stabilized by histone H1 (Multigner *et al.*, 1992). In plants, altering LH content in cultured tobacco cells has resulted in cells with abnormal microtubule organization (Calikowski *et al.*, 2000). Subsequently, two studies have suggested that LHs are responsible for nucleating microtubules in tobacco cell culture (Hotta *et al.*, 2007; Nakayama *et al.*, 2008). These data suggest that LH variants may have distinct functions because of their different nucleosome interaction and chromatin compaction mechanisms (Öztürk *et al.*, 2018).

1.3.5. Effect of LHs on Gene Expression

The reported data shows the difficulty to evaluate the exact impact and role of LH in gene expression whereas in some lower eukaryotes, a complete depletion of LH has shown to have no effect on the organism’s viability (Patterton *et al.*, 1998; Shen *et al.*, 1996). Fan and colleagues (2001, 2003 and 2004) have demonstrated this complexity by knock-out experiments. It was reported that the single deletion of selected LH variants, such as H1.2, H1.3 and H1.4; did not have any significant effect on the phenotype, suggesting at least limited redundancy and interchangeability of some somatic LH sub-types in the chromatin functioning. However, the simultaneous depletion of three variants were reported to be lethal (Fan *et al.*, 2003; Fan *et al.*, 2001; Fan & Skoultchi, 2004). LH binding can result both in a suppressing or activating effect on transcription. (Shen & Gorovsky, 1996) observed that an H1 knockout in *Tetrahymena thermophila* had no effect on the amount of mature RNAs produced by genes transcribed by Pol I and Pol III and most genes transcribed by Pol II.

However, H1 was found to be required for the normal basal repression of a gene (*ngoA*) in growing cells but not required for its activated expression in starved cells. This type of effect was opposite for a gene (*CyP*), where H1 was required for its activated expression in starved cells but not for its repression in growing cells. A study in mouse embryonic stem cells showed that 50% of the H1 depletion was caused by the dramatic chromatin structure changes, but the expression of only a small number of genes was affected, mostly on the X chromosome (Fan *et al.*, 2005). As these genes were regulated by DNA methylation, the results indicated that LHs can participate in the epigenetic regulation of gene expression by contributing to the maintenance or establishment of specific DNA methylation patterns. This hypothesis was supported by finding that LH promotes epigenetic silencing by regulating both DNA methylation and histone H3 methylation through mediating reversible changes in DNA methylation and gene expression at the H19 and Gtl2 loci (Yang *et al.*, 2013). Similarly, H1-mediated gene silencing through H3 methylation was observed in Drosophila, where silencing of repetitive sequences was linked to the H1 presence. Depleting the LH H1 *in vivo* have resulted in strong activation of these elements. Data revealed that H1 physically interacts and recruits an H3 lysine 9 methyl-transferase Su (var) 3–9, which leads to massive H3K9 methylation specific for the heterochromatin (Lu *et al.*, 2013).

1.3.6. LH Mobility

In both the naturally isolated and/or experimentally reconstituted (using either purified or recombinant components) nucleosomal templates, LHs are found stably and specifically bound to the entry and exit of nucleosomal DNA and the dyad of the nucleosome. In contrast, LHs are highly mobile *in vivo*. Notably, the recovery half time after photo-bleaching of core histones is tens of minutes to several hours (Gautier *et al.*, 2004; Kimura & Cook, 2001) and the recovery half time after photo bleaching for different LHs is only 30– 50 s (Misteli *et al.*, 2000). This apparent discrepancy between the *in vitro* and *in vivo* mobility of LHs remains still an enigma, although data from *Tetrahymena* revealed that the *in vivo* dynamics of H1 binding is energy-dependent (Dou *et al.*, 2002). Evidently, a study has reported that depletion of ATP resulted in marked decrease of LHs mobility. To date, there is no data available indicating the existence of such system in higher eukaryotes. One could however expect that the *in vivo* higher LH mobility should be essential for different nuclear processes, and indeed, available data showed that embryonic stem cells in which the dynamic exchange of H1 was restricted, displayed differentiation arrest (Meshorer *et al.*, 2006).

It is worthwhile to note that nearly all of the above-mentioned molecular functions of LHs, which among others, include involvement in chromatin architecture, gene expression, posttranslational modifications (Harshman *et al.*, 2013) and interactions with non-histone proteins (Kalashnikova *et al.*, 2013) are essentially mediated by cAMP as a second messenger. Now considering that the LHL protein in Arabidopsis (AtLHL) has recently been annotated to harbor a probable AC catalytic center (Figure 1.1) (Gehring, 2010), it might be possible that the sole cAMP reported to be key in its functions might essentially be generated by this same molecule. Based on this notion, the AtLHL protein was therefore herein targeted to attempt and confirm its probable AC activity followed by a detailed characterization of such a function and particularly with regard to the key cellular processes of growth, development and plant response to the various environmental stress factors.

CHAPTER 2

PRELIMINARY BIOINFORMATIC ANALYSIS OF AT3G18035

ABSTRACT

Typical literature review of textbooks and journals somehow, fail to deliver comprehensive information that provides a clear picture of a gene and its protein product; and its involvement in both cellular systems and biological processes. At3g18035 is a gene that encodes the LHL protein in *A. thaliana* and has been annotated as a possible AC protein in higher plants. A justification for attempting to understand the possible functional and physiological properties of this gene was then initiated. In this regard, various bioinformatic programs and computer-based tools were used to mine out the LHL protein data, and such were: TAIR, NCBI, GenevestigatoR, ARAMEMNON, KEGG, PSORT II, SUBA3, PSIPRED, ATTED-II, ThaleMine, GeneMANIA and STRING. The preliminary study showed that the annotated LHL is actually a nucleosomal protein that is chiefly involved in selective and non-covalent binding of nucleosomal double-stranded DNA and proteins as well the associated regulation of DNA transcription, templating and recombination; chromatin silencing; and nucleosomal positioning, condensation and assembly.

2.1. INTRODUCTION

Typical literature review of textbooks and journals somehow fail to deliver comprehensive information that provides a good picture of a gene and its protein product; and its involvement in both cellular systems and biological processes. In that regard, other systems such as bioinformatics and molecular systems may then have to be employed. Bioinformatics is defined as the study of research, development or application of computational tools and approaches for expanding the use of biological, medical, behavioral or health data, including those to acquire, store, organize, analyze and visualize such data (Hogeweg, 2011; Luscombe *et al.*, 2001). Virtually, the term has evolved from a reference to the study of information processes in biological systems to refer to the creation and use of biological databases. In this regard, an assessment of the relationships among members of a large biological data set can therefore be easily made through the use of algorithms, computational techniques and statistical measures to manage the information contained in databases (Elloumi & Zomaya, 2013; Fayyad *et al.*, 1996).

A major challenge in biology today is the large-scale determination of gene function (Boyes *et al.*, 2001). First, the establishment of standards and controlled vocabularies facilitates the integration of experimental data into a computational framework, thereby allowing structured and systematic processing of information (Ashburner *et al.*, 2000; Brazma *et al.*, 2001). Second, the structured databases and data querying tools provide means to assign putative functional information to genes. The complete sequencing of the Arabidopsis genome achieved in the year 2000, has enabled us to extract information and monitor gene expression of this flowering plant on a genome-scale level using microarrays (Grennan, 2006). To date, quite few tools have been developed for biologists to query large current information available on various biological databases. The Yeast

Microarray Global Viewer (yMGV) is a database providing online tools for the analysis of transcriptional expression profiles of yeast genes among 82 different datasets (Lelandais *et al*., 2004). In the plant community, NASCArrays provides a repository for Arabidopsis microarray data and some simple ‘‘gene-centric’’ data mining tools (Craigon *et al.*, 2004).

In other words, the *in situ* synthesis of high-density oligonucleotides on glass slides has become a powerful tool to rapidly integrate the sequence knowledge into expression profiling platforms, such as the ATH1 full genome array developed by Affymetrix and The Institute for Genomic Research (TIGR), which represents approximately 23,750 genes from Arabidopsis (Redman *et al.*, 2004). The availability of a full-genome array and the complete technical environment provided by the Affymetrix system led to a wide use of the GeneChip technology in the plant community. Thousands of arrays have since been processed, of which a significant number are publicly available through services and repositories such as the Nottingham Arabidopsis Stock Centre Transcriptomics Service (Craigon *et al.*, 2004), ArrayExpress at the European Bioinformatics Institute (Brazma *et al.*, 2003), or Gene Expression Omnibus (GEO) at the National Center for Biotechnology Information (NCBI) (Edgar *et al.*, 2002).

The exploitation of large-scale gene expression datasets, mainly from *Saccharomyces cerevisiae* and *E. coli*, has already led to the discovery of global structures governing metabolic and regulatory networks (Ihmels *et al.*, 2004; Lee *et al.*, 2002; Ravasz *et al.*, 2002). Multiple-genome comparisons have also yielded interesting observations on the modularity and connectivity distributions of gene expression data (Bergmann *et al.*, 2003). For this reason, the tool of bioinformatics analysis was hereby used to mine out published data on the At3g18035 gene and the protein it encodes to provide an insight and opportunity about their current probable biological functions and/or roles in plants.

2.2. AT3G18035 GENE DATABASE ANNOTATIONS

Universal Protein Knowledge Base (UniProtKB) describes the ordered locus Q9LSK7_ARATH (Yang *et al.*, 2014), as a gene from the eudicot *A. thaliana* (L) Heynh., commonly known as the mouse-ear cress or the thale cress (Berardini *et al.*, 2004). The At3g18035 gene is listed on various sequence databases, whereby its accession number in Araport is AT3G18035 (Cheng *et al.*, 2017), in TAIR, it is At3g18035 (Berardini *et al*, 2015), and in NCBI accession it is NP_188431.3 (Salanoubat *et al.*, 2000). This gene has other names in other databases, such as AT3G18035 at KEGG (Kanehisa & Goto, 2000). At EnsemblPlants, the gene is known as At3G18035.1 and 821328 at GeneID (Charbonnel *et al.*, 2018; Gaudet *et al.*, 2011). It is also known as 821328 at Entrez (Yang *et al.*, 2014) while at Genevisible, it is known as 258141_at (Grennan, 2006). This gene is also named AT3G18035 in eggNOG (Huerta-Cepas *et al.*, 2015).

2.3. AT3G18035 GENE LOCUS DETAILS AND OVERLAPPING FEATURES

The At3g18035 is given a gene symbol of HON4, and sits on chromosome 3 and coordinates 6168963 to 6172005 on the reverse strand that is 3043 bases pairs (bp) in length (Kotliński *et al.*, 2017). The At3G18035 has 14 gene scripts in 7 species of the gene family HOMO007371 and 2 gene scripts in 2 species of the ORTHO041711 sub-family (Charbonnel *et al.*, 2018; Rigola *et al.*, 2006). On TAIR, the At3g18035 is given the At3G18035.1 code with its protein product given a length of 480 amino acids (aa), a molecular weight of 51524.2 Da, and an isoelectric point of 10.59. The genome features that overlap coordinates of this gene are as follows: 2 CDS sequences of 1443 bp in length, 6 exons, 2 five prime UTR's, 19 gene flanking regions, 4 introns, 3 mRNAs, 4 three prime UTR's, 2 transposable elements, 8 transposable element insertion and 2 transposon fragments with one gene model (Berardini *et al.*, 2004; Rosen *et al.*, 2014).

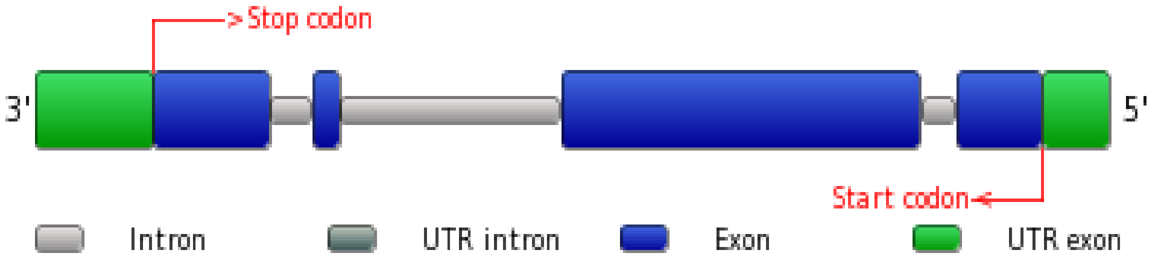


Figure 2-1: Gene structure of At3g18035 as presented by the Plaza v2.5 (adapted from Van Bel *et al*. (2012)).

2.4. AT3G18035 COMPUTATONAL DESCRIPTION AND EXPRESSION PROFILE

Genevestigator also shows data of expression of the At3g18035 gene in 111 tissues and results in the top 10 tissues have been reported to be as follows: cotyledon and leaf guard cell, embryo, peripheral endosperm, guard cell protoplast, leaf primordial, non-micropylar endosperm, shoot vascular tissue and bundle sheath cell, suspensor, ovule and leaf protoplast (Hruz *et al.*, 2008). AceView describes this gene to be expressed at very high levels of 8.0 times the average gene in its release. The sequence of this gene is defined by 291 GenBank accessions from 283 cDNA clones, some from aerial, ovule, flower, leaf and roots, above ground organ, flowers and buds, developing seed, hypocotyl, adult vegetative tissue, inflorescence meristem, mixture of silica and flower, and green siliques (Thierry-Mieg & Thierry-Mieg, 2006).

Plant ontology has shown that the protein is well expressed in leaf apex, shoot system, carpel, petal, root, plant embryo, leaf lamina, petiole, cotyledon, guard cell, hypocotyl, stem, inflorescence meristem, pollen, stamen, shoot apex, cauline leaf, vascular leaf, sepal, seed, collective leaf structure, flower and flower pedicel (Rosen *et al.*, 2014). HON4 has also shown expression patterns in 23 plant structures and also during 13 growth and developmental stages of the Arabidopsis plant. Namely: Lp.02 at two leaves visible stage, Lp.04 four leaves visible stage, Lp.06 six leaves visible stage, Lp.08 eight leaves visible stage, Lp.10 ten leaves visible stage, Lp.12 twelve leaves visible stage, flowering stage, mature plant embryo stage, petal differentiation and expansion stage, plant embryo bilateral stage, plant embryo cotyledonary stage, plant embryo globular stage and vascular leaf senescent stage (Obulareddy *et al.*, 2013; Zhao *et al.*, 2008).

The gene has 2301 blast hits to 2184 proteins in 377 species: 0 in Archeae, 351 in Bacteria, 868 in Metazoa, 344 in Fungi, 528 in Plants, 17 in Viruses and 193 in other Eukaryotes, respectively (Millar *et al.*, 2014). The gene At3g18035 contains 4 InterPro domains; namely, Winged helix-turn-helix transcription repressor DNA-binding (InterPro:IPR011991), A.T hook-like (InterPro:IPR020478), AT DNA-binding motif (InterPro:IPR017956) and Histone H1/H5 (InterPro:IPR005818) (Hooper *et al.*, 2017). ATTED-II describes microarray co-expressional network around the HON4 Alias with a score of </=20 as is shown in the Figure 2.2 below (Obayashi *et al.*, 2018). The HON4 protein has been reported to be located sub-cellularly in the nucleus (Fakih *et al.*, 2016; Wu *et al.*, 2006) and in the plasma membrane (Mitra *et al.*, 2009).

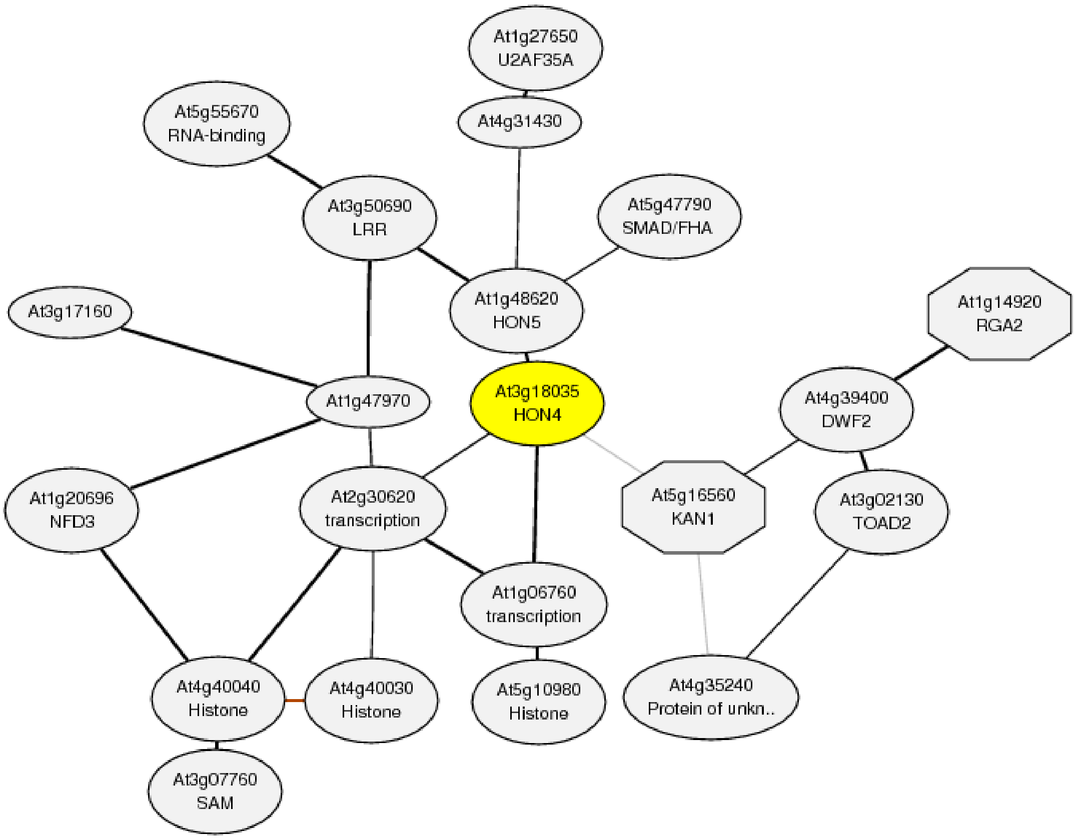


Figure 2-2: Co-expressed gene network around At3g18035 adapted from the ATTED-II Ver. 9.2 (Obayashi *et al.*, 2018).

2.5. AT3G18035 PROTEIN STRUCTURE

The LHL protein is briefly described as a winged-helix DNA-binding transcription factor (TF) family protein in ARAPORT (Salanoubat *et al.*, 2000).

2.5.1. The linker histone H1/H5 domain

The central role of the histone proteins is for both chromatin organization (as structural units of the nucleosome) and gene regulation (as dynamic components that have a direct impact on DNA transcription and replication). Of what is known, is that the eukaryotic

DNA wraps around a histone octamer to form a nucleosome - the first order of compaction of eukaryotic chromatin. The core histone octamer is composed of a central H3-H4 tetramer and two flanking H2A-H2B dimer, and each of the core histones contains a common structural motif, called the histone fold, which facilitates the interactions between the individual core histones. In addition to the core histones, LH exists and is normally called H1 (or H5 in avian species) (Isono, 1970).

Notably, the protein encoded by the At3g18035 gene belongs to the H1 and H5 family, and has a crystal structure of the globular domain of histone H5, which is implicated in nucleosome binding. H1 has been reported to be an essential component of the chromatin structure. H1 links nucleosomes into higher order structures, and H5 performs the very same function as H1, whereby it replaces H1 in some cell types. The structure of GH5, the globular domain of the H5, is known and its fold is similar to the DNA-binding domain of the catabolite gene activator protein, CAP, thus providing a possible model for the binding of the GH5 to DNA (Ramakrishnan *et al.*, 1993b; Zarbock *et al.*, 1986). LHs that do not contain the histone fold motif, have been shown to be critical to the higher-order chromatin compaction, and this is because they bind to the inter-nucleosomal DNA and facilitate interactions between individual nucleosomes.

Additionally, H1 variants have been shown to be involved in the regulation of the developmental genes. A tripartite structure is a common feature of this protein family in which a globular (H15) domain of about 80 aa is flanked by two less structured N- and C-terminal tails. The H15 domain is also characterized by high sequence homology among the family of LHs. The highly conserved H15 domain is essential for the binding of H1 and H5 to the nucleosome and consists of the C terminus. A short three-residue stretch has been shown to exist between the helices I and II that are in the beta-strand conformation.

Together with the C-terminal beta-hairpin, the strand forms the third strand of an antiparallel beta-sheet (Cerf *et al.*, 1993; Mariño-Ramírez *et al.*, 2006).

LH also contains a winged helix Storkhead-box1 domain and it is the conserved N-terminal winged helix domain of Storkhead-box1 protein which is likely to be a DNA binding domain. It has been reported that the full-length LH protein controls polyploidization of extravillus trophoblast and is implicated in pre-eclamsia (van Dijk *et al.*, 2005). An SSDB motif search at the KEGG site also confirmed the existence of the two above mentioned domains. Surprisingly, the protein domains have been reported to have no relationship at all, but various protein approaches, matching the LH H1/H5 domain and the H15 domain have experimentally proved the two to be involved in protein-protein interactions (Kasinsky *et al.*, 2001).

2.6. PROTEIN ONTOLOGY AND FUNCTION OF THE AT3G180350 GENE

In Tagname, At3g18035 has been described to be associated with a series of processes such as biological processes, cellular component, molecular function, plant structure and finally, growth and developmental stages. Under cellular component, this gene has been reported to be located in the nucleus (Kaundal *et al.*, 2010), nucleolus (Gaudet *et al.*, 2011) and nucleosome (Schlueter *et al.*, 2005). The molecular functions include interacting selectively and non-covalently with double-stranded DNA, double-stranded DNA binding, nucleosomal DNA binding, protein binding and DNA binding. Whilst biological processes include regulation of transcription, DNA-templating, negative regulation of DNA recombination, negative regulation of chromatin silencing, nucleosome positioning, chromosome condensation and nucleosome assembly (Gaudet *et al.*, 2011).

Notably, all these listed processes are either directly or indirectly linked to the second messenger, cAMP, which controls and/or coordinates them.

Surprisingly and despite the fact that the LHL protein is centrally involved in key cellular processes that are chiefly linked to cAMP and yet the same LHL protein has recently been annotated to harbour a possible functional AC center, no experimental work to date, has ever showed the exact source of such a critical and essential second messenger molecule (cAMP). In this regard therefore, this reported study was thus set to attempt and demonstrate the possible AC activity of this putative protein (LHL), followed by elucidation of its probable function in Arabidopsis and other closely related higher plants.

2.7 CONCLUSION

The outcome from the preliminary bioinformatic expressional analysis study of the At3g18035 or HON4 strongly suggests that the protein is expressed mostly in the nucleus as compared to its expression in the plasma membrane. In addition, the analysis also revealed that the protein is mainly involved in both the selective and non-covalent binding of nucleosomal double-stranded DNA and proteins as well the associated regulation of DNA transcription, templating and recombination; chromatin silencing; and nucleosomal positioning, condensation and assembly (Gaudet *et al.*, 2011) – processes that are essentially regulated by the second messenger, cAMP.

CHAPTER 3

MOLECULAR CLONING, PARTIAL EXPRESSION AND ENDOGEASSAYING OF THE LINKER HISTONE-LIKE RECOMBINANT PROTEIN FROM *Arabidopsis thaliana*

ABSTRACT

Adenylate cyclases (ACs) catalyze the formation of the second messenger cAMP from ATP. The existence and possible functional roles of ACs in living systems have been well studied and at times, involving several controversies and critical debates with cAMP having been firmly established as a vital second messenger signaling molecule in higher plants. Here, we report a putative linker histone like (AtLHL) protein encoded by the At3g18035 gene in *A. thaliana* that has been bioinformatically annotated as a probable AC molecule based on a rational search strategy developed against conserved and functionally assigned residues in the catalytic centre of annotated AC-specific search motifs, supported by computational assessments of protein models. We thus report the molecular cloning and partial expression of an AC-containing fragment domain of the AtLHL protein molecule (AtLHL-AC) in chemically competent *E. cloni* EXPRESS BL21 (DE3) pLysS DUOs cells followed by a molecular demonstration of its ability to induce the generation of endogenous cAMP in these recombinant prokaryotic expression systems.

3.1. INTRODUCTION

The functional analysis of a gene always comprehends better the physiological processes in living systems (Turcan *et al.*, 2011). Since a small percentage of annotated genes in databases exists, and have experimental proof for their annotations, then the greater importance is being linked to the assignment of functions to the rest of the individual genes in genomes. Furthermore, reports have shown an advanced drive towards a reduction of reliance onto the nucleotide and amino acid sequence similarities as a means of defining gene functions, thus resulting in increased use of experimental means and pure proteins to the end (Farrokhi *et al.*, 2009). Although the use of experimental measures to purify proteins is most desirable, the purification process of specific proteins from extracts of biological materials has shown to be subjective to many difficulties. Such among others are, the protein may be available in somewhat very low concentrations; secondly, the proteins may have multiple variants; and lastly, the proteins may be found in multiple post-translationally modified forms (Smith, 2005). Evidently, isolation and expression of the targeted genes in heterologous organisms can technically be employed to overcome all these above mentioned difficulties, and resulting in targeted functional analysis (Frommer & Ninnemann, 1995).

The development of methods through which cDNAs from almost any source can be expressed in suitable host organisms has been facilitated by the relative ease with which cDNAs can now be cloned. This then enables the generation of sufficient levels of the targeted proteins for meaningful functional analysis (Seki *et al.*, 2002). Thus the heterologous production of proteins involves principally, the identification and isolation of a target gene, the identification of a vector together with its subsequent construction into a recombinant system; and the identification and transformation of a suitable host expression system for the recombinant gene, resulting in eventual over-expression of the desired protein molecule (Rai & Padh, 2001). *Escherichia coli (E. coli)* is the most practically and widely used bacterial system available among host organisms for the heterologous expression of proteins (Makino *et al.*, 2011). It is considered a convenient host for the heterologous expression of foreign proteins because it has the ability to express labelled proteins, has relatively high levels of the heterologous gene expression systems, lacks the post-translational modifications, has a fast growth rate and an ease of transformation with exogenous DNA molecules (Pope & Kent, 1996). This system has proved to be compatible with almost all commercially available inducible cloning vectors; and its biochemical and genetic information is extensively available (Lueking *et al.*, 2000).

Generally, recombinant proteins expressed in *E. coli* can comprise up to 50% of its total cellular protein (Francis and Page, 2010). Among the different strains of *E. coli*, the BL21 cells have shown to be highly suitable for the heterologous expression of recombinant proteins and these cells are deficient in the Lon protease, which normally degrades many foreign proteins, and the outer membrane protease (OmpT), which normally degrades extracellular proteins (Grodberg & Dunn, 1988). Additionally, their hsdSB mutation always prevents plasmid loss by disruption of the DNA methylation and degradation processes (Rosano & Ceccarelli, 2014).

In this chapter therefore, we report the isolation, cloning and recombinant expression strategies of an At3g18035 gene fragment domain from *Arabidopsis thaliana* into the *E. coli* recombinant system followed by determination of its probable endogenous catalytic activity in this heterologous prokaryotic system. The At3g18035 gene is one of the 14 putative Arabidopsis molecules that recently, were annotated bioinformatically to harbour a probable adenylate cyclase (AC) center responsible for generation of the essential second messenger, cyclic adenosine monophosphate (cAMP) (Gehring, 2010c). To date, only 5 of these 14 putative molecules have been experimentally confirmed as functional

ACs and these being the AtPPR, the AtKUP7, the AtKUP5, the AtClAP and the AtLRRAC1 (Al-Younis *et al.*, 2015; Al-Younis *et al.*, 2018; Bianchet *et al.*, 2018; Chatukuta *et al.*, 2018; Ruzvidzo *et al.*, 2013). More so, 2 additional molecules harbouring the same AC catalytic center have also been identified in *Zea mays* (Moutinho *et al.*, 2001a) and in *Nicotiana benthamiana* (Ito *et al.*, 2014a). Hence in an effort to perpetuate the continued identification of such important molecules in Arabidopsis specifically and higher plants in general, the At3g18035 gene was herein targeted and its studied truncated version termed AtLHL-AC.

3.2. MATERIAL AND METHODS

3.2.1. Plant Re-generations and Growth Conditions

3.2.1.1. Seed surface sterilisation

About 100 mg of the *A. thaliana* ecotype Columbia seeds were transferred into a sterile 1.5 mL Eppendorf tube, then briefly washed by adding 500 µL of 70% ethanol and vortexed on a vortex mixer (VX-200 Vortex Mixer, Labnet International Inc., New Jersey, USA) for 30 seconds. The seeds were left to settle through gravity and subsequently removed from the washing ethanol. The seeds were then further washed repeatedly with filter sterile distilled water for five times. The seeds were then submerged into a 500 µL sterilization buffer comprising 0.1% (w/v) sodium dodecyl sulphate (SDS) (Merck chemicals (Pty) Ltd, Darmstadt, Germany) and 50% (v/v) commercial bleach, and vortexed for 30 seconds. The buffer was removed while the seeds were further washed in 5 rounds of sterile distilled water, then finally suspended in 500 µL of filter sterile distilled water.

3.2.1.2. Seed vernalization

Seed sterilisation was followed by seed vernalisation, a process that eliminates seed dormancy and improves the rate of germination. Furthermore, vernalisation ensures uniform germination. This was achieved by introducing the sterilized seeds to a cold temperature of 4°C for 1 up to 3 days while they were still submerged in water (Gupta *et al.*, 2011) and in preparation for germination.

3.2.1.3. Seed germination

About 10 stratified *A. thaliana* seeds were allowed to germinate and grow onto the sterilised solid Murashige and Skoog (MS) medium [0.43% w/v of organic salts, 3% (w/v) sucrose and 0.8% w/v tissue culture grade agar] supplemented with 1 mL/L of Gamborg's vitamins (Sigma-Aldrich Corp., Missouri) at pH 5.7 for 2 weeks in a GC 300TL growth chamber (Lab Companion Systems, Seoul, Korea) on petri dishes that were sealed with parafilm. The plates were incubated under sterile growth room conditions for 14 days in a GC 300TL growth chamber (Lab Companion Systems, Seoul, Korea) under adjusted greenhouse tissue culture conditions (average day/night temperature: 23°C; day/night periods: 16 hours day and 8 hours night with the light for the day times set at 10,000 lux). After the 14 days, the seedlings were transplanted onto commercially obtained 2:2:1 potting soil mix composed of 40% (v/v) peat-based soil, 40% (v/v) leaf-derived humus and 20% (v/v) vermiculite respectively, and watered with sterile distilled water containing Gaucho as pesticide to systematically protect them from fungal attack, then covered with a plastic cling wrap to retain moisture. The germinated seedlings were allowed to successively grow under greenhouse conditions for a further 6 weeks from which all the *A. thaliana* plants were harvested for isolation of the targeted and intended AtLHL gene.

3.2.2. Extraction of Total Messenger RNA from *A. thaliana* Plants

Total mRNA was extracted using a commercial RNeasy Plant Mini kit and in accordance with the manufacturer's instructions (Catalog# 74904; Qiagen, South Africa), whereby the 6 weeks old *A. thaliana* plant materials were harvested in whole and then snap-frozen in liquid nitrogen (Afrox Industrial Gases, Klerksdorp, South Africa) (to protect the RNA from degrading), followed by grinding thoroughly with cooled (-80°C) sterile pestle and mortar to form a fine tissue powder. The ground tissue material was transferred into a cooled 1.5 mL Eppendorf tube and allowed the liquid nitrogen to evaporate. 500 µL of Plant Lysis solution was added onto the ground material in the 1.5 mL Eppendorf tube and vortexed for 30 seconds vigorously at high speed. The mixture was then incubated for 3 minutes at 56ºC to increase the disruption of the tissue material, then centrifuged for 5 minutes at 16,300*g* in an LSE High Speed Microcentrifuge (Corning Inc., Amsterdam, Netherlands). The supernatant was pipetted out into a sterile Eppendorf tube and mixed up with 250 µL of 96% (v/v) ethanol through immediate pipetting. The mixture was then transferred into a purification spin column inserted into a 2 mL collection tube and centrifuged for 1 minute at 12,000*g*. The flow-through was discarded while the spin column and its collection tube were re-assembled.

The purification column was washed three times (3x); firstly, by adding 700 µL of Wash buffer 1 and centrifuging for 2 minutes at 12,000*g*. The flow through was discarded while the collection tube was replaced with a new sterile collection tube. A second wash with 500 µL of Wash buffer 2 was carried out through centrifugation for 1 minute at 12,000*g*.

The flow through was discarded while the wash step was repeated for the second time. The purification spin column that was washed 3 times was then placed into a sterile 1.5 mL RNAse-free microcentrifuge tube then eventually, mRNA was eluted by adding 50 µL of nuclease-free water directly onto the column membrane and centrifuging for 1 minute at 12,000*g*. The purification column was discarded while the eluted mRNA was kept. The concentration of the eluted total mRNA was then quantified using a 2000 Nanodrop Spectrophotometer (ThermoScientific Inc., Massachusetts, USA), followed by resolution on a 1.0% (w/v) agarose gel using a Multi-Sub Mini Electrophoresis System (MS7) (Biocom Ltd, Bridge of Weir, UK) for 40 minutes at 80 V. To ascertain its purity and integrity. The resolved mRNA was visualized under ultraviolet (UV) illumination using a UV-2000 Trans-illuminator (Bio-Rad Laboratories, California, USA) and its resultant image captured by a Complete ChemiDoc Imaging System (Bio-Rad Laboratories Inc., California, USA). The collection microfuge tube with the eluted mRNA was finally stored at -80°C for further use.

3.2.3. Isolation and Amplification of the AtLHL Gene Fragment

3.2.3.1. Designing of sequence-specific primers and their acquisition

Sequence-specific primers targeting the 540 bp AtLHL-AC gene fragment were manually designed based on the AtLHL gene sequence as described previously (Gehring, 2010b) and as is shown in Figure 3.1 below. This was achieved by retrieving the genomic sequence of the At3g18035 gene encoding the putative AtLHL protein from The Arabidopsis Information Resource (TAIR) (http://www.arabidopsis.org). The manually designed sequences were then sent to Inqaba Biotechnological Sciences (Pretoria, South Africa) for chemical synthesis.

MDPSLGDPHHPPQFTPFPHFPTSNHHPLGPNPYNNHVVFQPQPQTQTQIPQPQMFQLSPHVSMP
HPPYSEMICAAIAALNEPDGSSKMAISRYIERCYTGLTSAHAALLTHHLKTLKTSGVLSMVKKS
YKIAGSSTPPASVAVAAAAAAQGLDVPRSEILHSSNNDPMASGSASQPLKRGRGRPPKPKPESQ
PQPLQQLPPTNQVQANGQPIWEQQQVQSPVPVPTPVTESAKRGPGRPRKNGSAAPATAPIVQAS
VMAGIMKRRGRPPGRRAAGRQRKPKSVSSTASVYPYVANGARRR**GRPRRVVDPS**SIVSVAPVGG
ENVAAVAPGMKRGRGRPPKIGGVISRLIMKPKRGRGRPVGRPRKIGTSVTTGTQDSGELKK**KFD**
IFQEKVKEIVKVLKDGVTSENQAVVQAIKDLEALTVTETVEPQVMEEVQPEETAAPQTEAQQTE
AAETQGGQEEGQEREGETQTQT**EAEAMQEALF**

Forward primer: 5′-GGA AGG CCT AGG AGA GTT GTT GAC CCT AGC-3′
Reverse primer: 5′-GAA CAG AGC TTC TTG CAT TGC CTC TGC TTC-3′

Figure 3-1: A complete nucleotide sequence of the At3g18035 showing its annotated AC catalytic centre highlighted in red, bolded and underlined. The priming sites for the truncated AtLHL-AC gene fragment that was cloned, partially expressed and characterized in this study are highlighted in blue and bolded while sequences for the forward and reverse primers used to isolate and amplify this truncated gene fragment are highlighted in green.

3.2.3.2. Preparation and amplification of the AtLHL-AC gene fragment

Using mRNA that was extracted in section 3.3.3 and sequence specific primers that were synthesized previously in 3.3.3.1 respectively, a PCR master system was prepared and ran following the 1-Step RT-PCR Reddy Mix RT-PCR Kit instructions (Thermo Scientific Inc., Massachusetts, USA) on a C1000 thermo-cycling system (Bio-Rad Laboratories Inc., California, USA). Components of the reaction master mix are shown in Table 3.1 below while the necessary thermocycling conditions are shown in Table 3.2 below.

Table 3-1: Components of the RT-PCR reaction mix for the targeted amplification of the LHL-AC gene fragment.

Composition	Volume (µL)	Final Concentration
Verso Enzyme Mix	1	
1-Step PCR Master Mix	25	1 X
Forward Primer (10 µM)	1	200 nM
Reverse Primer (10 µM)	1	200 nM
RT Enhancer	2.5	-
Water (PCR Grade)	19.5	-
Template (RNA)	1	1 ng
Total Volume	50	-

Table 3.2: The Verso 1-step RT-PCR thermal cycling conditions used for detection of the targeted LHL-AC gene fragment.

STEP	TEMPERATURE	TIME	NUM OF CYCLE
cDNA Synthesis	50ºC	15 min	1 cycle
Thermo-Start Activation	95ºC	15 min	1 cycle
Denaturing	95ºC	20 sec	↕ 45 cycles
Annealing	520ºC	30 sec	
Extension	72ºC	1 min	
Final Extension	72ºC	5 min	1 cycle

3.2.3.3. Agarose gel electrophoresis of the amplified AtLHL-AC gene fragment

The amplified AtLHL-AC gene fragment was detected by ethidium stained agarose gel electrophoresis (AGE), whereby a 1% (w/v) agarose gel was prepared by weighing 1 g of agarose powder and mixing it with 100 mL of 1X TBE buffer (Crystal TBE, Bioline, United Kingdom); pH 7.0. The agarose powder with the TBE buffer were melted in a microwave and mixed thoroughly by shaking. After cooling, 5 µL of 0.5 µg/mL ethidium bromide was added to the mixture and poured onto the casting tray with combs inserted and left to set at room temperature for a maximum of 35 minutes. Using a pipette tip for each sample, 10 µL of the amplicon sample were mixed with 3 µL of the loading dye and loaded into the wells of the set gel. The sample was then resolved alongside a 100 bp (0.1 µg/µL) Gene-Ruler™ DNA ladder (Catalog# SM1143-Fermentas International Inc., Burlington, Canada). Electrophoresis was performed for 1 hour at 80 volts and 300 mA current in 1X TBE. The gel was visualized by using a 2000 UV trans-illuminator system (Bio-Rad Laboratories Inc., California, USA) and resultant images captured with a Chemi Doc™ Imaging System (Bio-Rad Laboratories Inc., California, USA).

3.2.4. Molecular Cloning of the Amplified AtLHL-AC Gene Fragment

The amplified AtLHL-AC gene fragment in section 3.3.3.3 above was ligated into a commercially-acquired pTrcHis2-TOPO® expression vector (Invitrogen, Carlsbad, USA) according to the manufacturer’s instructions.

3.2.4.1. Addition of the 3'-adenine overhangs to the AtLHL-AC gene fragment

Single A-overhangs were added to the 3′ ends of the blunt-ended AtLHL-AC gene fragment, whereby 1 µL of the *Taq* polymerase was added to 40 µL of the RT-PCR product and the mixture incubated for 10 minutes at 72ºC on a C1000 Touch Thermocycling system (Bio-Rad Laboratories, California, USA). The resultant reaction was then placed on ice before being used in the pTrcHis2-TOPO expression cloning system.

3.2.4.2. Ligation of the AtLHL-AC gene fragment into the pTrcHis2-TOPO expression vector

A total of 4 µL of the adenylated AtLHL-AC gene fragment were added to 1 µL in a new Eppendorf tube of the ice-cold pTrcHis2-TOPO expression vector and the mixture mixed gently by tapping on the tube. The mixture was then incubated at room temperature for 5 minutes. The resultant ligation mixture was then used for the transformation of the One Shot TOPO10 chemically competent *E. coli* expression cells (Invitrogen Corp., Carlsbad, USA).

3.2.4.3. Transformation of the One Shot TOPO10 chemically competent *E. coli* cells with the pTrcHis2-TOPO:AtLHL-AC expression construct

In accordance with the manufacturer’s protocol, 2 µL of the pTrcHis2-TOPO:AtLHL-AC fusion construct were added to an ice-cold vial containing 40 µL of the One Shot TOPO10 chemically competent *E. coli* cells (Invitrogen Corp., New York, USA). The mixture was gently mixed through tapping the tube and incubated on ice for 30 minutes. The mixture was then heat-shocked for 1 minute at 42ºC on an AccuBlock Digital Dry Bath (Labnet International Inc., New Jersey, USA). The Eppendorf tube was immediately put on ice for 30 minutes. Subsequently, 250 µL of Super Optimal broth (SOC) (Lucigen Inc, Wisconsin, USA) broth that was thawed at room temperature was added to the mixture. The culture was then horizontally shaken in an SI-600 Bench-top Shaking Incubator (Lab Companion GMI Inc., Minnesota, USA) for 1 hour at 37ºC at 225 rpm. This step was undertaken so as to allow cells to express β-lactamase enzyme, which during and later on in the selection process, detoxifies ampicillin. The shaken cultures were then harvested through centrifugation for 5 minutes at 12,000*g*. Around 150 µL of the SOC broth were pipetted out and discarded as waste while the remaining broth was used to resuspend the pellet. About 20 µL aliquots of the concentrated transformation culture were spread-plated onto pre-warmed selective LB agar plates [1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) sodium chloride, 0.5% (w/v) glucose, 1.5% (w/v) agar, 50 µg/mL ampicillin, pH: 7.0] and incubated overnight at 37ºC in an Incubat 2001651 Table-top Incubator (JP Selecta SA., Barcelona, Spain). The plates were visually analysed for the growth of colonies in the next morning.

3.2.4.4. Screening of positive clones

Single colonies of the One Shot TOPO10 chemically competent *E. coli* cells harbouring the designed pTrcHis2-TOPO:AtLHL-AC expression construct were screened for successful cloning, whereby both the successful ligation of the AtHLH-AC fragment into the pTrcHis2-TOPO vector as well as its correct orientation in an acceptable reading frame in the vector were assessed.

3.2.4.4.1. Plasmid extraction

Several single colonies of the One Shot TOPO10 chemically competent *E. coli* cells harbouring the generated pTrcHis2-TOPO:AtLHL-AC expression fused constructs were aseptically picked from the selective LB plates and each used to individually inoculate 10 mL selective LB broth media [1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1.0% (w/v) sodium chloride, pH: 7.0 supplemented with 0.5% (w/v) glucose and 50 µg/mL ampicillin]. The cultures were incubated overnight at 37ºC in a shaking incubator at 225 rpm. Cell cultures from the overnight were then harvested by centrifugation for 20 minutes at 4,000*g* using a TD3 800B Low Speed Table Top Centrifuge (Xiangyi Laboratory Instrument Development Ltd., Hunan, China). The supernatant was discarded while plasmid extraction was then carried out from the pelleted cells following the manufacturer’s instructions using the Thermo Scientific GeneJET Plasmid Miniprep Kit (Catalogue # K0503; ThermoScientific Inc., Massachusetts, USA).

Briefly, the pelleted cells were re-suspended in 250 µL of the Resuspension Solution supplemented with RNase and the bacteria were completely mixed by vortexing until no cell clumps remained. 250 µL of the Lysis Solution was then added to the cell suspension and the mixture was gently mixed by inverting the tube 8 times or until the culture became slightly clear. 350 µL of the Neutralization Solution was added to the cell suspension and mixed immediately and thorough inversion of the tube 8 times. The cell suspension was subsequently clarified by centrifuging for 5 minutes at 16 300*g* and the supernatant transferred into the supplied Gene Jet spin column and further centrifuged for 1 minute at 16,300*g*. The flow-through was discarded. 500 µL of the Wash Solution (diluted with 96% ethanol prior its first use) was then added to the spin column and centrifuged for 1 minute at 16,300*g*. The flow-through was discarded. The wash step was repeated one more time, with an additional 1-minute centrifugation to remove any residual wash solution and/or chemical elements. The washed spin column was immediately transferred into a sterile 1.5 mL microfuge tube and 50 µL of pre-warmed (70ºC) sterile water was directly added onto the spin column membrane followed by incubation at room temperature for 2 minutes. The assembled spin column was then centrifuged for 2 minutes at 16,300*g* and the plasmid was eluted. The eluted plasmid was collected and then kept in a 1.5 mL microfuge tube while the used spin column was dissembled and discarded. The DNA concentration of the eluted plasmid (fused construct) was then determined by a 2000 Nanodrop Spectrophotometer (Thermo Scientific Inc., Massachusetts, USA).

3.2.4.4.2. Analysis of the positive constructs

Positive clones were confirmed using convectional PCR on the C1000 Thermo-cycler system and in accordance with the standard MyTaq Mix PCR protocol (Catalogue # BIO-25041; Bioline, London, UK). The PCR process was used to check successful ligation of the LHL-AC gene fragment insert into the pTrcHis2-TOPO expression vector and also to confirm if such a ligation was in the correct orientation. Successful ligation was confirmed by re-amplifying the fused gene fragment in the construct using both of its own primers (forward and reverse primers), while correct orientation was verified by re-amplifying the fused gene fragment in the construct with one of its own primer (forward) and one of the vector primer (reverse). The reaction mixtures for these two PCR processes are respectively shown in Tables 3.3 and 3.4 below and their associated thermal cycling conditions are shown in Table 3.5.

Table 3.3: Components of a PCR reaction mixture to confirm the successful ligation of the AtLHL-AC gene insert into the pTrcHis2-TOPO expression vector.

Component	Volume µL	Final Concentration
Plasmid DNA Template	2	2 ng
Insert Forward Primer	1	200 nM
Insert Reverse Primer	1	200 nM
MyTaq Reaction Mix	25	1X
Grade Water (sdH_2O)	21	
Final total	50	

Table 3.4: Components of a PCR reaction mixture to confirm the correct orientation of the AtLHL-AC gene insert into the pTrcHis2-TOPO plasmid expression vector.

Component	Volume µL	Final Concentration
Plasmid DNA Template	2	2 ng
Insert Forward Primer	1	200 nM
Vector’s Reverse Primer	1	200 nM
MyTaq Reaction Mix	25	1X
Grade Water (sdH_2O)	21	
Final total	50	

Table 3.5: The reaction thermal cycling conditions for the step by step assessment episode of the successful cloning of the AtLHL-AC gene insert into the pTrcHis2-TOPO expression vector.

Step	Temperature (ºC)	Time	Cycles
Initial Denaturation	95	1 minute	1
Denaturation	95	15 seconds	35
Annealing	47	15 seconds	
Extension	72	10 seconds	

3.2.4.4.3. Agarose gel electrophoresis of the amplified AtLHL-AC gene fragment from the confirmed clones

All products from performed PCR of Tables 3.3 to 3.5 were resolved on an ethidium bromide stained 1% agarose gel and images captured as already been outlined in section 3.3.3.4. In this regard, a double amplification of the targeted AtLHL-AC gene insert in both reaction samples of Tables 3.3 and 3.4 respectively, would correspondingly confirm a successful cloning (both ligation and correct orientation) of the AtLHL-AC gene insert into the pTrcHis2-TOPO™ plasmid expression vector. In addition, all the successfully confirmed clones were further sent to Inqaba Biotechnological Sciences (Pretoria, RSA) for validation of their insert in-frame orientations within the pTrcHis2-TOPO expression vector through sequencing.

3.2.4.5. Transformation of the *E. cloni* EXPRESS BL21 (DE3) pLysS DUOs chemically competent cells with the pTrcHis2-TOPO:AtLHL-AC fusion expression construct

Following confirmation of the successful cloning the AtLHL-AC gene fragment into the pTrcHis2-TOPO expression vector, the resultant expression construct (pTrcHis2-TOPO:AtLHL-AC fusion construct) in section 3.3.4.3 above was used to transform chemically competent *E. cloni* EXPRESS BL21 (DE3) pLysS DUOs cells in accordance with the manufacturer’s protocol and instructions (Catalogue # MA019; Lucigen Corp., Wisconsin, USA), and in preparation for the subsequent expression of the desired recombinant AtLHL-AC fused expression product.

Briefly, a total of 1 µL (200 ng) of the ice-cold pTrcHis2-TOPO:AtLHL-AC expression construct was added to 100 µL of ice-cold chemically competent *E. cloni* BL21 (DE3) pLysS DUOs cells. The contents were gently mixed followed by incubation on ice for 30 minutes. The mixture was heat-shocked at 42ºC on a heating for 90 seconds block and immediately re-incubated on ice for 5 minutes. After that, 250 µL of the room temperature SOC broth was aseptically added to the mixture and incubated for 90 minutes at 37ºC at 225 rpm. This step was undertaken so as to allow cells to express β-lactamase enzyme, which during and later on in the selection process detoxifies ampicillin. After incubation, the cell contents were sedimented through centrifugation at 12,000*g* for 5 minutes. About 150 µL of the supernatant was pipetted out and discarded as waste while the remaining small volume was used to re-suspended the pellet cells. About 20 µL of the cell culture were spread-plate pre-warmed selective LB agar plates [1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) sodium chloride, 0.5% (w/v) glucose, 1.5% (w/v) agar, 100 µg/mL ampicillin, and 34 µg/mL chloramphenicol, pH: 7.0 and the plates incubated overnight at

37ºC in an Incubat 2001651 Table-top Incubator (JP Selecta SA., Barcelona, Spain). The following morning, plates were visually analysed for the growth of colonies.

3.2.5. Partial Expression and Endogenous Activity Assaying of the Recombinant AtLHL-AC Protein

3.2.5.1. Partial expression of the AtLHL-AC protein

An *E. cloni* EXPRESS BL21 (DE3) pLysS cell colony harboring the pTrcHis2-TOPO:AtLHL-AC expression construct was used to inoculate 10 mL of LB media supplemented with 0.5% (w/v) glucose, 34 µg/mL chloramphenicol and 100 µg/mL ampicillin in a 50 mL falcon tube. The falcon tube was incubated overnight at 37ºC while shaking at 225 rpm. On the following day, 1 mL of the overnight culture was then use to inoculate fresh 20 mL LB broth media containing 100 µg/mL ampicillin, 34 µg/mL chloramphenicol and 0.5% (w/v) glucose. The culture was incubated at 37ºC while shaking at 225 rpm up until an optical density (OD_{600}) of 0.6 was reached and as was measured by the Hekios Spectrophotometer (Merck, Gauteng, RSA). Immediately, the culture was placed on ice and then split into two tubes each of 10 mL. One culture was induced to express the desired AtLHL-AC recombinant protein by adding 1 mM of isopropyl-β,D-thiogalactopyranoside (IPTG) (Sigma-Aldrich Corp., Missouri, USA) while the other culture was left uninduced and as control. The individual split cultures were then shaken in an incubator for 3 hours at 225 rpm at 37ºC. After the 3 hours, 500 µL of each cell culture was collected separately to be analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) for protein expression, while the rest of the cells (bulk) were separately centrifuged at 16,300*g* for 5 minutes and stored at -20ºC for further analysis.

3.2.5.2. Determination of the endogenous AC activity of the recombinant AtLHL-AC protein

The bulk cell cultures harvested from section 3.2.5.1 above were each re-suspended in 1 mL Lysis Buffer 1 (Amersham Healthcare, New Jersey, USA) supplemented with 2 mM 3-isobutyl-1methylxanthine (IBMX). The resuspended cells cultures were then subsequently lysed through shaking on an orbital shaker at 200 rpm for 1 hour at room temperature. The generated crude lysate was centrifuged for 5 minutes at 16,300*g* into a clear supernatant. 200 µL of this cleared supernatant was thoroughly mixed with 200 µL of Lysis Buffer 2 (Amersham Healthcare, New Jersey, USA) and the resultant solution was acetylated by the addition of the acetylating reagent (2:1 triethylamine: acetic anhydride (v/v)) at a volumetric ratio of 1:20 acetylating reagent: sample (v/v). The mixture was then vortexed to mix thoroughly for 2 seconds at high speed. The endogenous cAMP content from each of the generated lysates was measured with a cAMP-linked enzyme immunoassaying kit (Catalog # CA201), following the acetylation version of its protocol and in accordance with the manufacturer’s instructions (Sigma-Aldrich Corp., Missouri, USA). The measurements were obtained using a Microplate Reader (Labtech International Limited, East Sussex, UK) at 405 nm.

3.2.6. Statistical Analysis of the Enzyme-immunoassay Data

The results of the immunoassaying system obtained in section 3.2.5.2 above were all based on the means of three replicates whereby outcomes of each assay were sequentially subjected to analysis of variance (ANOVA) (Super-Anova, Statsgraphics Version 7, 1993, Statsgraphics Corporation, USA). Wherever the ANOVA revealed some significant differences between outcomes, the means (n = 3) were then separated by the post hoc Student Newman Kuehls (SNK), multiple range test ($p \leq 0.05$).

3.3. RESULTS

3.3.1. Generation of Arabidopsis Plants

Wild-type *A. thaliana* ecotype Col. seeds were surface-sterilized, cold-stratified and germinated on solidified MS basal media in a growth chamber (Figure 3.2A). Further, the germinated seedlings were transplanted onto planting potting mix (Figure 3.2B). Then the 6-week old whole plant material was harvested for the subsequent isolation of total RNA.

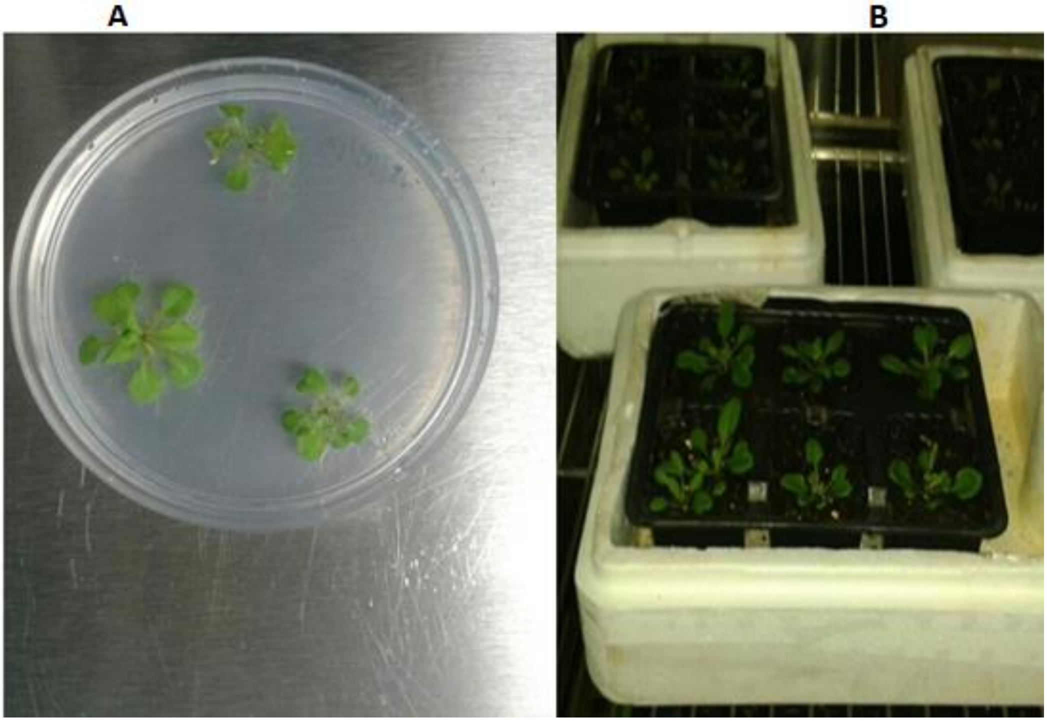


Figure 3.2: Generation of the *A. thaliana* plants. (**A**) The newly germinated Arabidopsis seedlings on Murashige and Skoog growth medium under monitored growth chamber conditions, and (**B**) the six week-old *A. thaliana* ecotype Columbia plants on potting soil.

3.3.2. Amplification of the AtLHL-AC Gene Fragment

Total RNA was extracted from the 6-week old *A. thaliana* leaf tissue material followed by synthesis of copy DNA (cDNA) for the whole AtLHL gene using a specialized 1-Step RT-PCR system. Together with the sequence-specific primers, the generated cDNA was used to amplify the targeted AtLHL-AC gene fragment in a similar specialized RT-PCR system. The successfully isolated AtLHL-AC gene fragment is shown below in Figure 3.3.

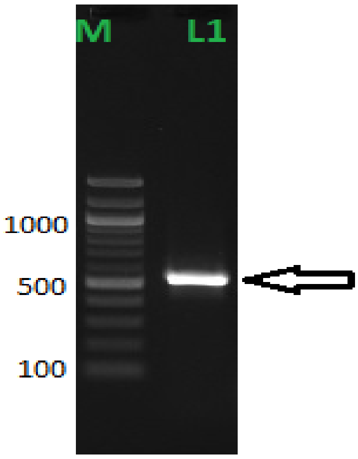


Figure 3.3: Representative 1% agarose gel of the amplified AtLHL-AC gene fragment from *A. thaliana*. Lane 1 (M) is the 100 bp GeneRuler ladder and lane 2 (L1) is the 540 bp AtLHL-AC gene fragment. The arrow marks the resultant and successfully isolated At LHL-AC gene fragment.

3.3.3. Confirmation of the Successful Cloning of the AtLHL-AC Gene Fragment

After successfully isolating the targeted AtLHL-AC gene fragment from the Arabidopsis plant, this gene fragment was then ligated onto a pTrcHis2-TOPO expression vector to yield a pTrcHis2-TOPO:AtLHL-AC fusion construct. The resultant pTrcHis2-TOPO:AtLHL-AC fusion construct was then assessed to confirm whether the ligated AtLHL-AC gene fragment was cloned successfully. In this regard, two aspects were tested; firstly, if the AtLHL-AC gene fragment was successfully ligated into the pTrchis2TOPO expression vector and secondly, if such a successful ligation was in the correct orientation. As is shown in Figure 3.4 below, the AtLHL-AC gene fragment was successfully cloned into the pTrchis2TOPO expression vector.

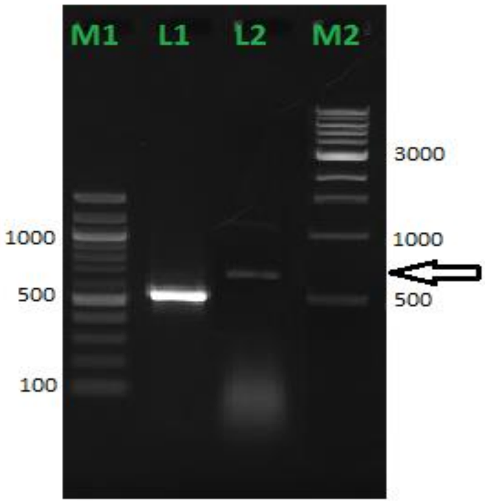


Figure 3.4: Representative 1% agarose gel to confirm the successful designing of the pTrcHis2-TOPO:AtLHL-AC expression construct. Lane 1 (M1) is the 100 bp GeneRuler ladder, lane 2 (L1) is the 540 bp AtLHL-AC gene fragment amplified with its own self primers, lane 3 (L2) is the 671 bp AtLHL-AC gene fragment amplified with its own forward primer and the pTrcHis2-TOPO vector reverse primer and lane 4 (M2) represents the 1 kb Gene-Ruler ladder. The arrow marks the two respective AtLHL-AC gene fragments amplified and resolved in this analysis.

3.3.4. Partial Expression of the Recombinant AtLHL-AC Protein

In order to facilitate partial expression of the targeted and desired recombinant AtLHL-AC protein, some chemically competent *E. cloni* BL21 (DE3) pLysS DUOs cells were transformed with the correctly confirmed pTrcHis2-TOPO:AtLHL-AC fusion construct followed by the inducing protein expression with 1 mM IPTG. As is shown in Figure 3.5, the desired recombinant AtLHL-AC protein of approximately 22.13 kDa was successfully expressed.

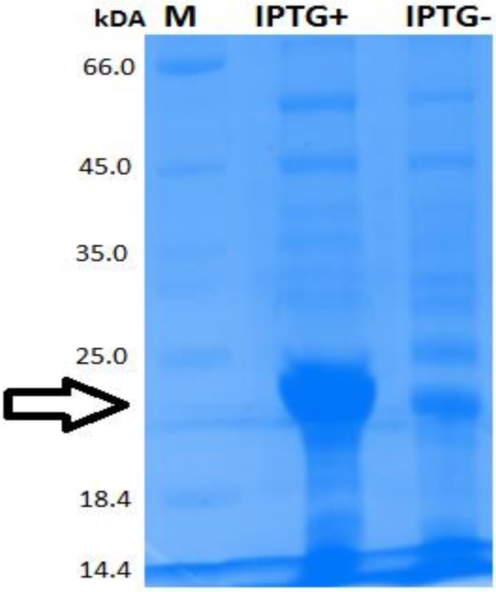


Figure 3.5: Partial expression of the recombinant AtLHL-AC protein. An SDS-PAGE of protein fractions expressed in chemically competent *E. cloni* EXPRESS BL21 (DE3) plysS cells transformed with the pTrcHis2-TOPO:AtLHL-AC fusion construct. In lane 1 (M) is the unstained molecular weight marker, in lane 2 (IPTG+) is the un-induced control cell culture and in lane 3 (IPTG-) is the bacterial cell culture treated with 1 mM IPTG to induce partial expression of the targeted and desired recombinant AtLHL-AC protein. The arrow marks the partially expressed recombinant AtLHL-AC protein.

3.3.5. Determination of the Endogenous AC Activity of the Recombinant AtLHL-AC Protein

The ability of AtLHL-AC protein to possibly generate or induce the generation of cAMP from ATP within the transformed *E. cloni* EXPRESS BL21 (DE3) pLysS DUOs cells was assessed and determined by the cAMP-linked enzyme immunoassaying system as is shown below in Figure 3.6.

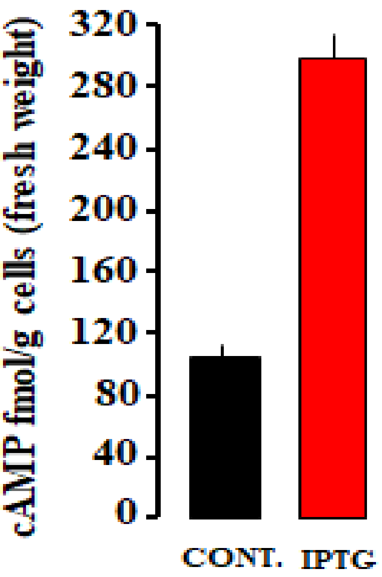


Figure 3.6: Determination of the endogenous AC activity of the recombinant AtLHL-AC protein. Cyclic AMP levels generated by the un-induced (CONT) and induced (IPTG) *E. cloni* EXPRESS BL21 (DE3) pLysS DUOs cells harbouring the AtLHL-AC gene fragment. All cAMP levels were determined by the cAMP-linked enzyme immunoassaying system based on the acetylation protocol and error bars represent the standard errors of the mean readings (n = 3).

3.4. DISCUSSION

In this study, to achieve a sterile clean and non-contaminated plant regeneration process, first, the seeds surfaces were sterilized rigorously followed by vernalization - a process that eliminates seed dormancy and improves germination rate (as well as uniform germination). The seedlings were then subsequently germinated as is shown in Figure 3.2A and further grown in soil as is shown in Figure 3.2B. Eventually, the generated plants were used for the isolation of mRNA, which later was used to isolate the targeted AtLHL-AC gene fragment.

With the information available on the bioinformatic annotation of the At3g18035 gene as a possible AC molecule (Gehring, 2010a), attempts were thus herein made to isolate and possibly further characterize its fragment domain harbouring the AC catalytic center (AtLHL-AC). Ideally, the specific nucleotide sequence of the At3g18035 gene (Figure 3.1) was retrieved from the TAIR database and sequence specific primers for the targeted AtLHL-AC gene fragment were then manually designed as is shown in Figure 3.1. Those sequence-specific primers were then sent over for synthesis and subsequent supply by the Inqaba Biotechnological Sciences (Pretoria, RSA). Those acquired primers were then later used to amplify and isolate the targeted AtLHL-AC gene fragment from the *A. thaliana* ecotype Columbia plants grown under controlled and monitored greenhouse conditions in a growth chamber (Figure 3.2). Specifically, the fully grown plants in Figure 3-2B were used to isolate total mRNA, which together with the sequence-specific primers and in a specialized RT-PCR system, were then used to firstly, generate cDNA and secondly, amplifying the expected 540 bp size of the AtLHL gene fragment (Figure 3.3).

In line with this, plant tissues have been reported to present numerous hurdles for protein extraction because: their rigid cellulosic wall must be sheared off first for the cell contents to be released; the presence of contaminating compounds may cause proteins to degrade, modify and/or loose activity; and finally, the low levels of the targeted protein can exist as part of highly complex protein mixtures (Fido *et al.*, 2004). As an alternative, genes that encode the targeted and/or desired plant proteins can be extracted and expressed in either bacterial, fungal or animal cell systems, but however, the genomic DNA isolation from eukaryotic organisms for protein expression may still result in non-functional protein products because of the presence of introns. Notably, this dilemma can only be practically overcome by expressing the desired plant genes in fungal and/or animal cells, which in their eukaryotic nature, are able to process the required post-transcriptional modifications required for targeted recombinant proteins to function (Clark and Pazdernik, 2011).

However, eukaryotic systems better still present a substantial challenge in terms of the complexity of their physiological systems, which in turn makes the aspect of protein processing still a big problem. However, this challenge can still be further overcomed by cloning the cDNA of the plant desired gene in a bacterial system because this type of DNA lacks introns and consists solely of the uninterrupted coding sequences. Furthermore, the bacterial system can also be controllably induced to over-express the desired recombinant plant protein in desired amounts (Clark and Pazdernik, 2011).

In this study, the isolated AtLHL-AC gene fragment was ligated onto a pTrcHis2-TOPO plasmid vector that utilizes the TA cloning system, which has been recognized as the simplest and most efficient method of cloning PCR products. Poly-A tailing was done by first adding the single 3′-adenine overhangs facilitated by the enzyme *Taq* polymerase to the ends of the PCR product to make it become complementary to the singly 5′-thymine overhangs on both ends of the expression vector. The adenylated PCR complimentary to the linearized vector allows for a direct and highly efficient cloning system of these two (Zhou & Gomez-Sanchez, 2000). However, the TA cloning system still has a specific and inherent drawback of a possible non-directional cloning because both ends of the vector are complementary to either end of the insert (Chen & Janes, 2002). Therefore, in order to check and validate the successful cloning of the AtLHL-AC gene fragment in the pTrcHis2-TOPO expression vector, the pTrcHis2-TOPO:AtLHL-AC was used as a template to re-amplify the AtLHL-AC gene fragment. The process involved use of both AtLHL-AC primers and then one of the AtLHL-AC primer and one of the pTrcHis2-TOPO primers. Any successful (double) amplification here would mean successful cloning.

In this regard and when resolved on a 1% agarose gel, the amplification of 540 bp DNA fragment (Figure 3.3) confirmed that the AtLHL-AC gene fragment had been successfully ligated onto the pTrcHis2-TOPO after a re-amplification with both of its own primers. In addition, an amplification of a 671 bp DNA fragment in Figure 3.4 after using a forward AtLHL-AC primer plus a reverse pTrcHis2-TOPO vector primer also confirmed that the ligated AtLHL-AC gene fragment was orientated correctly within the plasmid vector. Additionally, the correct cloning system of the AtLHL-AC into the pTrcHis2-TOPO was also further confirmed and fully validated by the nucleotide specific sequencing approach (results not included).

As described before, the expression of functional proteins in heterologous hosts has remained the cornerstone of modern biotechnology, and the heterologous protein expression process transfers the targeted DNA fragments other than the original source to hosts for subsequent synthesis of such encoded and desired protein products (Gustafsson *et al.*, 2004). This is specifically helpful in the study of plant proteins, because protein isolation from plants can at times be very slow-moving, costly and lengthy. Therefore, foreign hosts provide simpler and effective system for studies on functional and structural properties of specific proteins and an elucidation of their biological and functional roles in complex physiological and cellular processes (Yesilirmak & Sayers, 2009).

BL21 cells, among other known *E. coli* host strains, are the most commonly used strains for recombinant expression and affinity purification of most foreign proteins (Paliy & Gunasekera, 2007). This is because *E. coli* has been the most commonly used host for the high throughput yield expression of most recombinant proteins by exploiting the high promoter specificities and transcriptional activities of its associated bacteriophage T7 RNA polymerase (Robichon *et al.*, 2011). But in this study, the *E. cloni* EXPRESS BL21 (DE3) pLysS DUOs cells were used as the expression host strain because of it being evidently reported to have the same DE3 chromosomal genotype as is the *E. coli* BL21 strain. It also carries a chloramphenicol-resistant pLysS plasmid, which encodes for the T7 lysozyme (a natural inhibitor of the T7 RNA polymerase), and it produces a small amount of the T7 lysozyme in order to suppress the basal expression of the T7 RNA polymerase prior to induction with IPTG. Furthermore, as a lysogen of the λDE virus, the *E. cloni* EXPRESS BL21 (DE3) pLysS DUOs T7 RNA polymerase gene is under the control of an IPTG-inducible *lac* UV5 promoter and the strain is also deficient in the lon and ompT proteases (Miroux & Walker, 1996).

In our case, the successfully designed and correctly confirmed pTrcHis2-TOPO:AtLHL-AC fused construct was used to then transform the chemically competent *E. cloni* EXPRESS BL21 (DE3) pLysS cells followed by a partial expression of the desired AtLHL-AC recombinant protein through induction of the the transformed cells with 1 mM IPTG at an OD_{600} of 0.5. The induced recombinant protein was then resolved by SDS-PAGE and as expected, a recombinant C-terminus His-tagged fusion protein product of around 22.13 kDa was obtained as is shown in Figure 3.5.

This success in expressing the desired and intended recombinant AtLHL-AC protein in the chemically competent *E. cloni* EXPRESS BL21 (DE3) pLysS cells, prompted our interest to quickly assess and determine the probable ability of this partially expressed recombinant to generate cAMP from ATP within these bacterial systems using a cAMP-linked enzyme immunoassaying system. As is depicted in Figure 3.6, cells induced with 1 mM IPTG had a cAMP generation increase of greater than 3.5-fold as compared to the un-induced cells confirming indeed the ability of AtlHL-AC protein to generate cAMP from using up ATP endogenously.

3.5. CONCLUSION

Findings from this section propose that AtLHL-AC recombinant protein could either be a *bona fide* AC molecule capable of directly converting ATP to cAMP or perhaps it is just another functional signalling plant molecule capable of stimulating the functional activities of resident ACs (*E. coli* ACs in this case) to produce cAMP.

3.6. RECOMMENDATIONS

This chapter points out the necessity for further experimental work on both the *in vitro* and *in vivo* AC activities of the recombinant AtLHL-AC protein, which will provide a clearer picture and understanding as to whether or not this very same recombinant protein is indeed a *bona fide* higher plant AC and/or functional signaling plant molecule.

CHAPTER 4

DETERMINATION OF THE *IN VIVO* ADENYLATE CYCLASE ACTIVITY OF THE RECOMBINANT LHL-AC PROTEIN

ABSTRACT

Adenylyl cyclases (ACs) are enzymes that generate cyclic adenosine monophosphate (cAMP) from adenosine monophosphate (ATP), which is involved in various physiological and developmental processes in different organisms. The significant functional roles played by ACs in nearly all living organisms and for some time, the existence and/or functional involvements of these molecules in higher plants had remained relatively elusive and highly debatable. Following the annotation of the linker histone like protein from *A. thaliana* (AtLHL) as a possible putative AC molecule, we hereby detail the cloning of its AC-containing fragment domain (AtLHL-AC) into an *Escherichia coli* SP850 mutant strain deficient in AC function and show that this truncated version of the AtLHL is capable of rescuing the mutant strain. In other words, the results revealed that the AtLHL functions as an AC that produces cAMP, thus complementing the mutant strain to behave as if it was a wild type strain capable of fermenting lactose.

4.1. INTRODUCTION

The biological science of ACs and their role in the production of cAMP from ATP has been extensively studied in animals, microbes and lower eukaryotes. However, the presence and specific functional roles of these particular molecules in higher plant signal transduction systems have for a while, remained a bit obscure and a huge subject of controversy (Gehring, 2010a; Lomovatskaya *et al.*, 2008; Talke *et al.*, 2003). With the understanding that AC's are enzymes capable of converting the biological molecule ATP to cAMP after stimulation, the ACs, already functioning at a basal level in the absence of agonistic elements, catalyze the generation of cAMP, to be released and utilized intracellularly. ACs are important molecules involved in the signal transduction pathways of all living organisms as they respond to various environmental stimuli and cues. More so, cAMP is an important signaling molecule in bacterial species, where it is involved in the regulation of gene expression and responds to a variety of environmental stimuli and cues (Lory *et al.*, 2004). Furthermore, given that several molecules with guanylate cyclase (GC) activity and different structural domains of nucleotide cyclases have been experimentally confirmed in *A. thaliana*, it is therefore not likely that a single AC or just a few could account for all the observed and known cAMP-dependent processes in higher plants (Gehring, 2010a).

From previous studies, various reasons have been given with regard to ACs and cAMP playing pivotal roles in the cellular and physiological processes of *E. coli*. cAMP acts as a second messenger necessary, specifically for the synthesis of many inducible enzymes in *E. coli* (Kuo *et al.*, 2003; Pastan & Perlman, 1970). The cAMP forms a complex with the catabolite activator protein (CAP) and the formed cAMP-CAP complex then binds to the promoter region of the regulated operon. The DNA-bound cAMP-CAP complex in turn, physically interacts with RNA polymerase and increases its affinity for the *lac* promoter, thus activating the transcriptional process of the *lac* operon (De Gunzburg, 1985). In the presence of lactose, the lactose repressor (*lac* repressor) generally falls off the *lac* operator site, allowing RNA polymerase to bind to the operator and leads to transcription of the β-galactosidase, lactose permease and transacetylase genes of the operon. Ideally, this process is carried out by a standard level of the β-galactosidase, which basically converts some basal lactose to the inducer of the *lac* operon, allolactose (Hames & Hooper, 2005).

While GCs have never been reported in *E. coli*, only a single AC naturally exists in this prokaryotic and is encoded for by the *cya*A gene. In 1983, Yang and team reported the first isolation of this one and only enzyme (Côté *et al.*, 2010; Yang & Epstein, 1983). Later on, it was found that the *Bordetella pertussis cya*A encodes for an AC toxin (Gross *et al.*, 1992). Naturally, the wild type *E. coli* strain produces a deep red colour on MacConkey lactose agar and as a result of its ability to ferment lactose while on the other hand, the SP850 strain, due to its *cya*A mutation, cannot utilize lactose and therefore, produces colourless colonies on the same growth medium. Apparently, when this mutant strain is transformed with any foreign gene capable of encoding for a functional AC activity, it also produces a deep red colour on the MacConkey lactose agar, which is indicative of a complementation of its AC deletion (Cotta *et al.*, 1998), leading to the production of the most needed cAMP, which in turn, then restores the normal utilization of lactose as a carbon source (Perlman & Pastan, 1969).

The SP850 mutant strain was reported to not being able to ferment carbon sources such as lactose, maltose, galactose, arabinose and glycerol, and has shown to grow very slowly on glucose, fructose and galactose sugars (Perlman & Pastan, 1969). The mutant is actually deficient in the *lacZ* expression because it lacks the AC needed for the cAMP production, and therefore, cannot activate the catabolite activator protein, meaning that its cAMP receptor protein-dependent promoter is presumably inactive (Dole *et al.*, 2004). The mutant is a vibrant host for the recombinant expression systems of foreign proteins, especially when it is in its exponential phase (Grossman *et al.*, 1998). It has also been shown to be resistant to kanamycin (Cotta *et al.*, 1998) and pH-sensitive during growth (Ahmad & Newman, 1988).

In the current modern science system, the *cya*A SP850 mutant strain has provided a very useful platform to test for the possible AC activities of foreign recombinant proteins through a complementation phenomenon of the mutation. So, complementation test is literally used to determine whether two recessive mutations that produce the same phenotype are located on the same gene in genetic analysis (Hanoune & Defer, 2001). Hence, through this same test, the *in vivo* AC activities of unknown recombinant protein molecules can then be pre-screened and the promising candidates subsequently evaluated further through some specific *in vitro* spectroscopic and/or immunoassaying systems. Previously, a recombinant cloning of the *Prevotella ruminicola* D31d protein into the *E. coli cya*A SP850 mutant cells has shown to have resulted in the growth of deep-red colony cells when grown on MacConkey lactose agar, thus indicating lactose fermentation and complementation of the AC-deficiency in the mutant cells (Cotta *et al.*, 1998). Another previous study has also demonstrated the ability of a PSiP protein that is involved in polarized growth of pollen tubes in *Zea mays* to rescue the very same SP850 *cya*A mutant host (Moutinho *et al.*, 2001a).

Furthermore, an AC-containing fragment domain of a pentatricopeptide protein from *A. thaliana* (AtPPR-AC), cloned into the SP850 mutant, was also reported to have influenced the mutant cells to exhibit a magenta coloured phenotype on MacConkey lactose agar as a result of complementation (Ruzvidzo *et al.*, 2013). Additionally, another plant AC, the HpAC1 from *Hippeastrum hybridum*, explicitly exhibited the ability to compensate for AC deficiency in the SP850 mutant (Świeżawska *et al.*, 2014). In 2015, Al-Younis and team investigated on whether the AtKUP7 AC was capable of rescuing the *E. coli cya*A mutant.

Using this mutant strain, the AtKUP7 recombinant was cloned and expressed and as a result of complementation, the mutant strain was rescued (Al-Younis *et al.*, 2015). In 2016, Kasahara and team also employed the very same principle to examine whether an MpCAPE recombinant from a lower plant (fern) could also rescue the SP850 mutant host and as a result of complementation, the mutant host was successfully rescued (Kasahara *et al.*, 2016). In 2018, Al-Younis and team also demonstrated the very same element when their recombinant AC protein, AtKUP5, increased cAMP levels in the cloned *cya*A *E. coli* cells (Al-Younis *et al.*, 2018). Again, yet another recent study has described the recombinant cloning of an Arabidopsis AC-containing fragment domain (AtCAP-AC) into the *cya*A SP850 cells followed by its ability to generate cAMP *in vivo* (Chatukuta *et al.*, 2018). Finally, a more recent study by Bianchert, also demonstrated the ability of a recombinant AtLRRAC1 protein to complement the SP850 mutant host via complementation (Bianchet *et al.*, 2018).

In this chapter and following the annotation of the Arabidopsis LHL protein (AtLHL), (encoded by the At3g1805 gene) as a putative AC molecule, we report the recombinant cloning of its AC-containing fragment domain (AtLHL-AC) into an *E. coli* SP850 mutant strain deficient and show its ability to rescue the mutant strain in metabolizing lactose.

4.2. MATERIALS AND METHODS

Complementation testing was undertaken to check and determine the ability of the AtLHL-AC recombinant protein to generate cAMP from ATP within a mutant *E. coli* strain (the *cyaA* SP850) (Coli Genetic Stock Center, Yale University, Connecticut, USA) known to be deficient in AC activity.

4.2.1. Isolation and Purification of the pTrcHis2-TOPO:AtLHL-AC Expression Construct from the *E. cloni* EXPRESS BL21 (DE3) PLysS DUOs Cells

The pTrcHis2-TOPO:AtLHL-AC fusion expression construct that was confirmed to be correctly and properly designed in section 3.2.4.3 of Chapter 3 and maintained in the chemically competent *E. cloni* EXPRESS BL21 (DE3) pLysS DUOs expression cells (section 3.2.4.5, Chapter 3) was extracted using the GeneJET Plasmid Miniprep Kit according to the manufacturer's instructions (Catalogue # K0503; Thermo Scientific Inc., New York, USA) and as previously outlined in section 3.2.4.4.1 of Chapter 3. The resultant pTrcHis2-TOPO:AtLHL-AC fusion expression construct was then used to transform chemically competent mutant SP850 host cells for undertaking the intended complementation testing.

4.2.2. Preparation of Competent Complementation Host Cells and their Transformation with the pTrcHis2-TOPO:AtLHL-AC Expression Construct

4.2.2.1. Preparation of the competent *E. coli cya*A SP850 cells

About 10 mL of the selective LB broth containing 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1.0% (w/v) sodium chloride, 0.5% (w/v) glucose, 15 µg/mL kanamycin; pH: 7.0 were inoculated with a colony of the *E. coli cya*A SP850 cells followed by incubation overnight at 37ºC at 200 rpm. The next morning, 20 mL of pre-warmed fresh LB broth supplemented with 15 µg/mL kanamycin were inoculated with 1 mL of the overnight culture and incubated at 37ºC shaking at 200 rpm up until an OD_{600} of 0.5 was reached. Cells were then harvested by centrifugation at 4,000*g* for 5 minutes at 4ºC. The supernatant was discarded while the harvested cells were re-suspended in 15 mL of an ice-cold Transformation Buffer 1 made-up of 30 mM potassium acetate, 50 mM manganese chloride, 100 mM rubidium chloride, 10 mM calcium chloride, 15% (v/v) sterile glycerol, pH: 5.8 and was incubated on ice for 90 minutes. The cells were harvested by centrifugation at 4,000*g* for 5 minutes at 4ºC. After centrifugation, the supernatant was discarded while the harvested cells were re-suspended in 4 mL of ice-cold Transformation Buffer 2 containing 10 mM MOPS [3-(N-morpholino) propane sulfonic acid], 75 mM calcium chloride, 10 mM rubidium chloride, 15% (v/v) glycerol of pH: 6.8. The re-suspended cells were then aliquoted into 100 µL portions and kept on ice for further use in the subsequent steps.

4.2.2.2. Transformation of the competent *E. coli cya*A SP850 cells

About 10 µL of the pTrcHis2-TOPO:AtLHL-AC fusion expression construct were added into an ice-cold 1.5 mL microfuge tube containing 100 µL of the chemically competent SP850 *E. coli* mutant cells (Shah & Peterkofsky, 1991). The mixture was gently mixed through swirling and then incubated on ice for 20 minutes. The mixture was then heat-shocked through incubation at 42ºC for 90 seconds on a dry bath heating block and brought back for incubation on ice for 5 minutes. The transformed mixture was supplemented with 500 µL of the SOC medium and incubated at 37ºC for 90 minutes in an orbital shaker at 200 rpm. This step was undertaken so as to allow cells to express the β-lactamase enzyme, which during and later on in the selection process, detoxifies ampicillin. After incubation, 100 µL aliquots of the transformation mixture were then used to spread-plate LB agar plates supplemented with 15 µg/mL kanamycin and 100 µg/mL ampicillin. The inoculated plates were incubated at 37ºC overnight for the generated cell colonies to be used in the subsequent step.

4.2.2.3. Cell culturing and phenotypic analysis

A MacConkey agar plate containing 2% (w/v) peptone, 1% (w/v) lactose, 0.5% (w/v) bile salts, 0.0075% (w/v) neutral red, 1.5% (w/v) agar, 15 µg/ml kanamycin and 0.1 mM IPTG for transgene induction was prepared and divided into three segments using a permanent marker. Segment 3 was streaked with *cyaA* cells transformed with the pTrcHis2-TOPO:AtLHL-AC fusion expression construct, segment 2 with the non-transformed *cyaA* mutant cells, and segment 1 left un-streaked. The plate was then incubated at 37ºC for 40 hours and the segments later on visually analysed for their different phenotypic characteristics (MacConkey, 1905).

4.3. RESULTS

The *in vivo* AC activity of the cloned recombinant AtLHL-AC gene fragment was investigated via a complementation test using the *E. coli cya*A SP850 mutant host strain cells (Coli Genetic Stock Center, Yale University, Connecticut, USA) whose AC gene (*cya*A) has been knocked out and thus preventing it from fermenting lactose (Shah & Peterkofsky, 1991). Herein, the mutant cells were transformed with the pTrcHis2TOPO fusion expression vector harbouring the AtLHL-AC gene fragment (pTrcHis2-TOPO:AtLHL-AC) followed by analysing the ability of the AtLHL-AC to generate cAMP on a differential and selective MacConkey agar media as is shown in Figure 4.1 below.

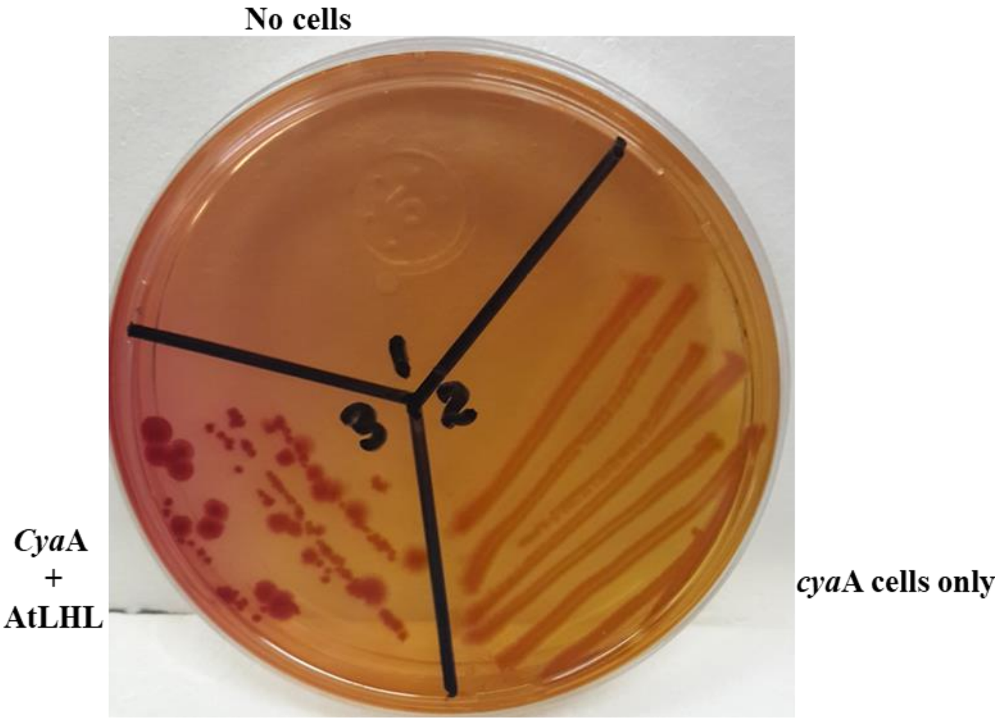


Figure 4.1: Functional Characterization of the AtLHL-AC by Complementation Testing. Segment 1 of the plate contains no cells, segment 2 contains the non-transformed *cya*A mutant cells and segment 3 contains the mutant cells transformed with the pTrcHis2-TOPO:AtLHL-AC fusion construct. Cells in segment 2 are non-lactose fermenters and therefore, produce white or yellow colonies. Cells in segment 3 have a deep-red purple phenotype; a characteristic signifying their acquired ability to ferment lactose. The fermentation ability was acquired from the cloned AtLHL-AC protein, which in this case, generated the most required cAMP for this process of lactose metabolism.

4.4. DISCUSSIONS

Ideally, the *E coli cya*A SP850 mutant strain is reported to have been created through a deletion of this same gene from the SP793 strain into the CA8000 strain through a P1 background transduction system (Shah & Peterkofsky, 1991). Essentially and in all wild type *E. coli* cells capable of generating cAMP, when the cAMP is bound to the transcriptional catabolite gene-activator protein (CGAP), it acts as a pleiotropic regulator for the expression of genes involved in the catabolism of carbohydrates such as lactose and maltose. Hence any *E. coli* strain lacking the AC gene is systematically unable to ferment sugars – but this is a condition that can be practically reversed through expression of putative AC protein candidates within this mutant (Moutinho *et al.*, 2001b) as was done herein in our present study. This test was undertaken using the SP850 *cya*A host that is systematically deficient in endogenous AC activity and therefore, cannot ferment lactose (Moutinho *et al.*, 2001a) and a putative Arabidopsis linker histone-like protein candidate (AtLHL-AC) that recently has been annotated as a probable AC molecule. When grown on MacConkey agar, the SP850 *cya*A host strain normally produces white/yellowish colonies as compared to the magenta red/deep purple colonies produced by its wild-type counterpart (Moutinho *et al.*, 2001a; Ruzvidzo *et al.*, 2013; Świeżawska *et al.*, 2014).

In this scenario and when the AtLHL-AC transgene was used to transform and get expressed within the the SP850 *cya*A host strain, the host cells turned deep-red or purple in colour on MacConkey agar (Figure 4.1) - a characteristic signifying their acquired ability to now ferment lactose as a result of the availability of the most needed cAMP, generated by the AtLHL-AC recombinant. This outcome is not unusual since various studies have also previously shown similar outcomes using bacterial and other related plant protein AC candidates. Firstly, a recombinant cloning of the *Prevotella ruminicola* D31d protein into the *E. coli cya*A SP850 mutant cells has shown to have resulted in the growth of deep-red colony cells on MacConkey lactose agar, thus indicating lactose fermentation and complementation of the AC deletion in the mutant host cells (Cotta *et al.*, 1998). Secondly, Moutinho *et al*. (2010) has demonstrated the ability of a PSiP protein that is involved in the polarized growth of pollen tubes in *Zea mays* to rescue the very same SP850 *cya*A mutant host. Thirdly, Charania *et al*. (2009) functionally demonstrated the AC activities of the *cya*A*, cya*B and *cya*C genes from *Shewanella oneidensis* MR-1, using a complementation system of the SP850 mutant. Fourthly, Ruzvidzo *et al*. (2013) by cloning the AC fragment domain of a pentatricopeptide protein from *A. thaliana* (AtPPR-AC) into the SP850 mutant, have reported a phenotypic display of a magenta colour of the recombinant cells on MacConkey lactose agar. Again, another plant AC, the HpAC1 from *Hippeastrum hybridum*, explicitly exhibited an ability to rescue the AC deficiency in SP850 cells through complementation (Świeżawska *et al.*, 2014). In 2015 and 2018, Al-Younis and team also demonstrated a rescuing of the *E. coli* SP850 strain by two Arabidopsis recombinant proteins, the AtKUP7 and AtKUP5, as a result of complementation (Al-Younis *et al.*, 2015). In 2016, Kasahara and team employed the very same principle and demonstrated a successful complementation of the SP850 cells with the MpCAPE recombinant from a lower plant (Kasahara *et al.*, 2016). In 2018, Chatukuta *et al*. reported that the recombinant fragment AtClAP from Arabidopsis has a similar capacity to rescue AC deficiency in *E. coli* (Chatukuta *et al*., 2018). Lastly and recently, Bianchert and colleagues (2018) demonstrated the ability of an Arabidopsis AtLRRAC1 recombinant to rescue the SP850 host cells as a result of complementation (Bianchet *et al.*, 2018).

4.5. CONCLUSION

The outcomes of the complementation test in this chapter validly established the Arabidopsis LHL protein (AtLHL) as a biologically functional higher plant AC molecule, capable of generating the signalling molecule, cAMP from ATP. In this regard, the AtLHL then becomes the tenth ever AC candidate to be identified and confirmed in plants.

4.6. RECOMMENDATIONS

Not underestimating the establishment made herein that the Arabidopsis LHL protein is a *bona fide* higher plant AC molecule, its molecular properties and possible physiological roles in plants still have to be fully studied and elucidated. Therefore, further experimental work on this protein involving *in vitro*, *in silico* and/or *in planta* assays needs to be undertaken.

CHAPTER 5

AFFINITY PURIFICATION OF THE RECOMBINANT ATLHL-AC PROTEIN AND CHARACTERISATION OF ITS ACTIVITY

ABSTRACT

Cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) are molecules synthesized by adenylate cyclases (ACs) and guanylate cyclases (GCs) respectively, and have been recognized as important second messengers, controlling many cellular functions in animals nearly half a century ago. This cyclic nucleotide monophosphate (cNMPs) signaling system is universal and its operation in plants has been a matter of debate until recently. The demonstration of potential AC activities in higher plants has been conducted with the histochemical and biochemical methods for several years, although the very same conventional methods have been severely criticized on the basis of their strong reliance onto either intact cells and/or crude cellular extracts. With the information available on the linker histone protein from Arabidopsis (AtLHL) that is a putative AC and encoded for by the At3g18035 gene, we report the affinity purification of its AC-harbouring fragment domain (AtLHL-AC) followed by a concerted functional characterization of its AC activity *in vitro*.

5.1. INTRODUCTION

Despite the fact that cAMP and cGMP have been recognized nearly half a century ago as important second messengers controlling many cellular functions in animals and synthesized by ACs and GCs respectively, the notion that the cNMPs signaling system is universal and operates in plants has been a matter of huge debate and severe controversy up until recently. Both the existence and physiological functions of cNMPs in higher plants have been questioned for a long time, mainly due to the fact that the cAMP and cGMP levels in plants appeared to be very low compared (usually in the nanomolar range based on fresh weight) to those found in animals and lower eukaryotes (concentrations in the nanomolar to micromolar range) and often below the detection limits of most of the analytical methods available in the past (Gehring & Turek, 2017).

The intracellular concentration of cAMP is firmly regulated by the activities of AC and its degradation enzyme, phosphodiesterase (PDE) (Conti & Beavo, 2007; Danchin, 1993). Genes encoding ACs have been isolated from most species of various organisms and the physiological functions of cAMP have also been well characterized (Botsford & Harman, 1992; D'Souza & Heitman, 2001; Montminy, 1997). For example, cAMP has been reported to bind to a receptor protein called the cAMP receptor protein (CRP) and the formed cAMP-CRP complex then regulates the transcriptional activation of catabolite-sensitive operons in *E. coli* (Ullmann, 1983). In mammals, increased cAMP levels in response to the actions of hormones such as glucagon and adrenaline have also been reported to promote phosphorylation of several intracellular enzymes through activation of protein kinase A, thus resulting in the enhancement of enzyme activities in glycogen and lipid metabolisms (Carmen & Víctor, 2006; Cohen, 1983). cAMP also plays crucial roles in learning, memory and olfactory sensation by regulating gene expression and channel activity (Hanoune & Defer, 2001; Zagotta & Siegelbaum, 1996).

Generally, ACs in mammals are classified into nine trans-membrane enzymes (tmACs) and one soluble enzyme (sAC) (Steegborn, 2014). The sAC appears evolutionarily distinct from the tmACs and more closely related to Cyanobacterial ACs, and playing central roles in sperm capacitation (Buck *et al.*, 1999; Buffone *et al.*, 2014; Chen *et al.*, 2000). Therefore much effort has been put into attempting to isolate ACs from plants because of their crucial functions in other organisms (Assmann, 1995; Gehring, 2010a; Newton *et al.*, 1999).

Studies have reported the discovery of components of cAMP-dependent signaling pathways as well as cAMP-interacting proteins (Donaldson *et al*., 2016) and cAMP-dependent kinases (protein kinase A; PKA) (Assmann, 1995; Gehring, 2010a), thus further supporting the role of cAMP in plant signaling cascades. Invariably, the question of how a single messenger, like cAMP or cGMP, is capable of triggering highly specific responses to different developmental and/or environmental stimuli still persisted. It therefore, seemed obvious that saturating a cell with either cAMP or cGMP could not be the answer. One solution relies on strict compartmentalization of the messengers and the combination and/or integration of several messengers, e.g., cAMP/cGMP with the cytoplasmic calcium ions and/or pH.

A recent review has highlighted the interplay of calcium ion signatures with cGMP in plant–microbe interactions (Yang *et al*., 2017). Specific response signatures and cooperation between messengers arise through a spatial clustering of stimulus-dependent cyclases and their downstream signaling components and/or through the specific binding of the cyclic nucleotides to effector molecules such as kinases (Isner & Maathuis, 2018; Kwezi *et al.*, 2011; Wheeler *et al.*, 2017) or channel subunits (Hoshi, 1995; Zelman *et al*., 2012). A study by Irving and others (2018), where calcium ion influxes flood intracellular compartments, highlights the need for transient and controlled levels of signaling molecules to generate appropriate responses to environmental and developmental stimuli within defined cellular compartments (Irving *et al*., 2018; Yuan *et al*., 2017). When Ca^{2+} is produced under environmental stress, it rapidly transmits external information from sensing tissues to appropriate target tissues (Konrad *et al.*, 2018; Kudla *et al.*, 2018).

Extracellular Ca^{2+} is transmitted into cells through plasma membrane proteins and Ca^{2+} channels. REDUCED HYPEROSMOLARITY-INDUCED Ca^{2+} INCREASE1 (OSCA1) has been identified as a plasma membrane protein mediating the rapid osmotic stress-induced Ca^{2+} accumulation and stomatal closure in guard cells. This therefore, indicated that the rapid Ca^{2+} influx mediates short-term responses of stomatal closure before the synthesis and/or transport of ABA into guard cells under stress conditions (Yuan *et al*., 2014).

A number of studies, to date, have been conducted, which successfully identified and characterised GCs in plants. The AtGC1 protein from *A. thaliana* has been shown to possess a magnesium-dependent GC activity *in vitro*, which is not dependent on nitrous oxide and also has a slight AC activity that was partly dependent on both magnesium and manganese as co-factors of activity (Ludidi & Gehring, 2003). The AtBRI1 protein from *A. thaliana* has also been shown to possess an *in vitro* GC activity (Kwezi *et al*., 2007). In addition, the PSKR1 protein from Arabidopsis was identified as a functional GC, which enables a cGMP-dependent signalling and when characterised, was found to be able to generate cGMP in a non-specific preference for either magnesium or manganese as the crucial co-factor of activity (Kwezi *et al*., 2011). Furthermore, studies have shown that the *in vitro* GC activity of another protein, the AtNOGC1 from *A. thaliana*, has a highly specific preference for Mn^{2+} to Mg^{2+} as a co-factor, has a substrate specificity for GTP and not ATP and binding to nitric oxide (NO) with a higher affinity than it binds to oxygen, thus it is dependent on NO and its expression is elevated during the NO starvations (Mulaudzi *et al*., 2011).

The available literature, together with amino acid sequence analyses and biochemical studies, indicate that magnesium or manganese ions are necessary for the ATP hydrolysis (Carricarte *et al.*, 1988; Lomovatskaya *et al.*, 2007; Lusini *et al.*, 1991; Mulaudzi *et al.*, 2011; Świeżawska *et al.*, 2014). Meanwhile, of the nine Arabidopsis proteins that previously had been bioinformatically annotated as putative ACs, the linker histone-like (LHL) protein encoded by the At3g18035 gene has since been implicated in signal transduction processes linked to stress response (De Vos & Jander, 2009; De Vos *et al.*, 2005; Schmid *et al.*, 2005) via cAMP mediations (Gehring, 2010a). Therefore, different signaling systems function in cell-to-cell and organ-to-organ communication as well as in local cellular responses. Thus further analyses are warranted to identify novel signaling mediators and ultimately, understanding how plants integrate different mobile signals through cell-to-cell and/or long-distance communication in order to robustly adapt to varied environmental stresses. In this chapter therefore, we unfold steps taken in purifying, refolding and biochemically characterizing the recombinant truncated version of AtLHL protein harbouring the AC-catalytic center (AtLHL-AC protein). Importantly, these experimental parameters have been proven effective in characterising novel plant ACs i.e., AtKUP5, AtCAP, AtKUP7 and AtPPR cyclases in plant signal transduction systems (Al-Younis *et al.*, 2015; Al-Younis *et al.*, 2018; Chatukuta *et al.*, 2018; Ruzvidzo *et al.*, 2013).

5.2. MATERIALS AND METHODS

5.2.1. Over-expression and Purification of the Recombinant AtLHL-AC Protein

5.2.1.1. Large scale expression of the recombinant protein

For a large scale expression of the recombinant AtLHL-AC protein, 200 µL from the glycerol stocks from the expression profiles in Chapter 3 were to inoculate 20 mL of the LB broth media supplemented with 0.4% glucose, 100 µg/mL of ampicillin and 34 µg/mL chloramphenicol. The inoculated culture was then grown overnight at 37ºC in a Labcon shaking incubator at 225 rpm. The next morning and in a 500 mL Erlenmeyer flask, 200 mL of the LB broth media containing 100 µg/mL ampicillin, 34 µg/mL chloramphenicol and 0.4% glucose were inoculated with 2 mL of the overnight culture and incubated at 37ºC with agitation at 225 rpm. At an OD_{600} of 0.6, the culture was induced 1 mM IPTG (Sigma-Aldrich Corp., Missouri) and then allowed to grow for a further 2 hours. After the 2 hours, the culture was centrifuged at 16,300*g* for 10 minutes, supernatant discarded while the pellet was stored at -20°C for further analysis.

5.2.1.2. Determination of the solubility or insolubility nature of the recombinant AtLHL-AC protein

After expression, the resultant recombinant AtLHL-AC protein was first assessed under native non-denaturing conditions to check if it had been produced as a soluble or insoluble product. In this regard, 10 mL of the induced *E. cloni* EXPRESS BL21 (DE3) pLysS DUOs cells were centrifuged at 9,200*g* for 5 minutes, where the supernatant was discarded while the harvested cells were kept as a pellet. The pelleted cells were then re-suspended in 2.5 mL sterile Tris-buffered saline (TBS) solution (50 mM Tris-HCl, 150 mM NaCl, pH:

7.5) that was supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.5 µg/mL lysozyme (Catalogue # 62970; Sigma-Aldrich Inc., Missouri, USA). For 1 hour, the mixture was incubated on ice and then periodically mixed at medium speed for 10 minutes through vortexing with 1 minute intervals of incubation on ice. Through centrifugation at 9,200*g* for 2 minutes, the mixture was separated into a pellet and supernatant, which were then kept separately as the insoluble and soluble fractions of the cellular component respectively. The presence and relative amounts of the expressed recombinant AtLHL-AC within each of these two fractions were then determined and ascertained by SDS-PAGE.

5.2.1.3. Affinity purification of the recombinant AtLHL-AC protein under non-native denaturing conditions

From the analysis undertaken above, the targeted recombinant AtLHL-AC protein was found to be wholly expressed in its an insoluble nature, therefore, the recombinant protein was then purified under non-native denaturing conditions using a HIS-Select nickel-nitrilotriacetic acid (Ni-NTA) affinity matrix and in accordance with the manufacturer's instructions (Catalogue # P6611; Sigma-Aldrich Inc., Missouri, USA). Briefly, the insoluble fraction obtained in section 5.2.1.2. above was resuspended in 5 mL of Lysis Buffer comprised of 8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl; pH: 8.0, 500 mM NaCl, 20 mM β-mercaptoethanol, 10 mM imidazole and 7.5% (v/v) glycerol at a ratio of 5 mL buffer to 1 g of the pellet weight. The resuspended cell pellet component was then solubilized by vigorous vortexing for 1 hour at full speed into a crude lysate. The crude lysate was then centrifuged for 30 minutes at 10,000*g* at room temperature to yield a cleared lysate through its separation from the overall cell debris. The yielded cleared lysate was then collected and stored for further downstream processing.

5.2.1.4. Equilibration of the His-Select Ni-NTA affinity matrix

An aliquot of about 2 mL of the 50% (v/v) HIS-Select Ni-NTA affinity bead solution were collected in a sterile 15 mL falcon tube and then allowed to settle down at room temperature for about 10 minutes. The beads were then washed three times with 2 mL sterile distilled water, to remove all the residual storage ethanol. The bead solution was then equilibrated with 2 mL of Lysis Buffer by mixing the two and allowing the mixture to settle down at room temperature for 5 minutes completely removing and discarding the buffer and keeping the equilibrated beads.

5.2.1.5. Binding of the recombinant AtLHL-AC protein onto the HIS-Select Ni-NTA affinity matrix

The 5 mL of the cleared lysate generated previously in section 5.2.1.4 above were added into 2 mL of the equilibrated beads and allowed to mix gently mix for 1 hour at room temperature on an Adjustable Rotator Revolver (Labnet International Inc., New Jersey, USA) at 30 rpm. During this phase, the recombinant AtLHL-AC protein was then bound onto the HIS-Select Ni-NTA affinity beads while the rest of the other bacterial proteins were left un-bound. The mixture was then centrifuged for 10 minutes at 5,000*g* and 50 µL of the supernatant (the flow-through) then collected for later analysis by SDS-PAGE while the rest of it was discarded.

5.2.1.5.1. Washing of the bound HIS-Select Ni-NTA affinity matrix

The bound beads were then washed three times to remove all the unbound bacterial proteins after the recombinant AtLHL-AC protein was bound onto the equilibrated HIS-Select Ni-NTA affinity matrix. Briefly, about 5 mL of Wash Buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl; pH: 8.0, 500 mM NaCl, 20 mM β-mercaptoethanol, 7.5% (v/v) glycerol, and 10 mM imidazole) were added to the bound affinity beads and mixed on a bench revolver at 30 rpm for 5 minutes. The suspension was then centrifuged for 1 minute at 5,000*g* before the supernatant (wash 1) was removed and its aliquot collected for analysed by SDS-PAGE. The washing step was repeated three more times (wash 2 and 3) and 50 µL aliquot of the washed bound beads also collected and saved for analysis by SDS-PAGE. Additionally, both washes 2 and 3 (20 µL aliquots) were also saved for analysis by SDS-PAGE.

5.2.1.6. Chemical refolding of the bound, purified and denatured recombinant AtLHL-AC protein

The following refolding procedures were sequentially undertaken to convert the bound purified recombinant AtLHL-AC protein from its denatured and non-functional state into its native and functional form.

5.2.1.6.1. Preparation of the refolding column

The washed HIS-Select Ni-NTA affinity beads carrying the bound denatured and purified recombinant AtLHL-AC protein were re-suspended in 10 column volumes (cv) of Gradient Buffer or Buffer A (8 M urea, 200 mM NaCl, 50 mM Tris-HCl; pH: 8.0, and 20 mM β-mercaptoethanol) and the suspension slurry then loaded into an empty XK16 column (Bio-Rad Laboratories Inc., California, USA) with the column tap closed at the bottom. Following that, then beads were then allowed to sediment by draining out 8 cv of the buffer through the tap and the loaded column then connected to the BioLogic DuoFlow Chromatography System (Bio-Rad Laboratories Inc., California, USA) to initiate the gradient refolding process.

5.2.1.6.2. The refolding gradient system

A linear refolding gradient system was created and run on the BioLogic DuoFlow chromatography system following parameters listed in Table 5.1 below. In this process, the 8 M urea gradient buffer was slowly and linearly diluted to 0 M urea concentration with a Refolding Buffer or Buffer B (200 mM NaCl, 50 mM Tris-HCl; pH: 8.0, 500 mM glucose, 0.05% (w/v) polyethylglycol, 4 mM reduced glutathione, 0.04 mM oxidized glutathione, 100 mM non-detergent sulfobateine, and 0.5 mM PMSF). The ultimate goal in this process was to convert the purified AtLHL-AC recombinant protein into a native and biologically active molecule from its denatured and non-functional state.

Table 5.1: Conditions for the refolding process of the recombinant AtLHL-AC protein using the BioLogic DuoFlow chromatography system.

Variable	**Value**
Column volume	1.00 ml
Flow rate	0.50 ml/min
Column pressure limit	2.80 MPa
Average tome for UV	1.00 sec
System pump for automatic pressure and flow regulation	Normal
Starting concentration of Buffer B (Refolding buffer)	0.00%
Target concentration of Buffer A (Lysis buffer)	100.00%
Equilibrate column with HIS-Select Ni-NTA Affinity Matrix	2.00 cv
Gradient length	600 min
Clean after gradient with wash buffer	10.00 ml
Elute protein with elution buffer	1.00 ml

5.2.1.7. Chemical elution of the refolded recombinant AtLHL-AC protein

The fully refolded bound purified AtLHL-AC recombinant protein was then eluted from the HIS-Select Ni-NTA affinity beads by adding 2 mL of Elution Buffer (200 mM NaCl, 50 mM Tris-Cl of pH: 8.0, 250 mM imidazole, 0.5 mM PMSF, and 20% (v/v) glycerol) by first allowing the mixture to settle for 20 minutes and then eluting using a process directly linked to the DuoFlow system. The resultant supernatant containing the wholly eluted AtLHL-AC protein was collected and stored at 4ºC for further downstream use and also analysis by SDS-PAGE.

5.2.1.7.1. Concentration and desalting of the recombinant AtLHL-AC protein

The 2 mL eluted recombinant AtLHL-AC protein sample was both concentrated and de-salted by transferring it into a Spin-XUF concentrating and de-salting device with a molecular weight cut off point of 3.0 kDa (Corning Corp., New York, USA) followed by centrifuging at 2,540*g* (Hermle Labortechnik, Wehingen, Germany) for 5 hours at 4ºC. The concentrated and de-salted protein fraction was then transferred into a new sterile Eppendorf tube and concentration of the protein determined with a 2000 Nanodrop spectrophotometer (Thermo Scientific Inc., Massachusettes, USA). Finally, a small fraction (20 µL) of the eluted protein fraction was put aside for analysis by SDS-PAGE while the rest of the sample eluent was stored at -20ºC for further downstream use of characterizing the AC activity.

5.2.1.8. Characterization of the AC activity of the recombinant AtLHL-AC protein

The enzymatic activity of the purified refolded AtLHL-AC recombinant protein was characterized *in vitro* by assessing the ability of this recombinant to convert ATP into cAMP in the presence of various molecular and ionic components known to affect ACs (GTP, Mg^{2+}, Mn^{2+}, Ca^{2+}, HCO_{3-}, and F^{-}).

5.2.1.8.1. Preparation of samples and enzyme immunoassaying

Some 200 µL reaction mixes were prepared in sterile 1.5 ml microfuge tubes as detailed in Table 5.2 below. Each tube contained 50 mM of Tris-HCl; pH: 8.0 and 2 mM IBMX, 10 µg of the recombinant protein, 5 mM $MgCl_2$ or $MnCl_2$ and 1 mM ATP and/or GTP (Al-Younis *et al.*, 2015; Kwezi *et al.*, 2007). Each reaction mix was then left to incubate at room temperature for 20 minutes and the reaction system stopped by adding 1 mM EDTA to chelate out all the divalent metal ions, thus removing the co-factors necessary for the enzymatic action of the recombinant AtLHL-AC. In addition, the samples were boiled for

5 minutes to inactivate the protein. The boiled samples containing the inactivated protein were then centrifuged for 5 minutes at 16,300*g* in order to clarify them. Their cAMP contents were then acetylated by adding the acetylating reagent (2:1), triethylamine:acetic anhydride (v/v) at a volumetric ratio of 1:20 acetylating reagent:sample. The acetylation reaction solutions were then mixed vigorously through vortexing for 2 seconds at high speed and the resulting supernatant assayed for cAMP content using the cAMP-linked enzyme immunoassaying kit (Catalog # CA201) following the acetylation version of its protocol and as specifically described by the manufacturer's manual (Sigma-Aldrich Corp., Missouri, USA) (Al-Younis *et al.*, 2015; Kwezi *et al.*, 2007). Readings were taken in triplicates using the Microplate Reader machine (Labtech, International Limited East Sussex, UK) at a wavelength of 405 nm.

Table 5.2: Reaction compositions for characterization of the AC activity of the recombinant AtLHL-AC protein.

	TUBE 1	**TUBE 2**	**TUBE 3**	**TUBE 4**	**TUBE 5**	**TUBE 6**	**TUBE 7**	**TUBE 8**	**TUBE 9**
Tris	50mM	50mM	50mM	50mM	50mM	50mM	50mM	50mM	50mM
$MnCl_2$	5mM	5mM		5mM	5mM	5mM	5mM	5mM	5mM
Protein		25µg	25µg	25µg	25µg	25µg	25µg	25µg	25µg
ATP	1mM	1mM	1mM	1mM	1mM	1mM		1mM	1mM
$MgCl_2$			5mM						
$CaCl_2$				250µM					
GTP						1mM	1mM		
$NaHCO_3$					50mM				
NaF + $AlCl_3$									10mM+ 30µM

5.2.1.9. Statistical analysis of enzyme immunoassaying data

All results of the enzyme immunoassaying system were based on means of three replicates, whereby all outcomes of each assay were subjected to a one-way analysis of variance (ANOVA) (Super-Anova, Statsgraphics Version 7, 1993, Statsgraphics Corporation, USA). Wherever the ANOVA revealed some significant differences between outcomes, then the means ($n = 2$) were separated with a *post hoc* Student Newman-Kuehls (SNK), multiple range test ($p \leq 0.05$).

5.3. RESULTS

5.3.1. Determination of the Solubility or Insolubility Nature of the Recombinant AtLHL-AC Protein

The resultant recombinant AtLHL-AC protein was first assessed under native non-denaturing conditions to check if it had been produced as a soluble or insoluble product, during its expression. As is shown below in Figure 5.1, the recombinant AtLHL-AC was mostly recovered in the insoluble component of the cell content and meaning that it was in this case, an insoluble product.

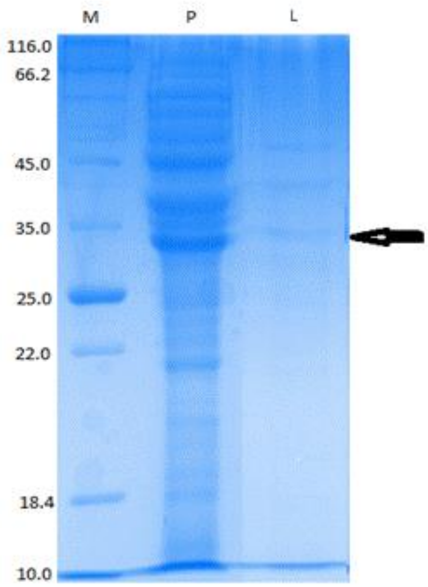


Figure 5.1: Determination of the solubility/insolubility status of the recombinant AtLHL-AC protein. The presentation is a 12% SDS-PAGE gel showing the presence and relative quantities of the recombinant AtLHL-AC protein in either the soluble or insoluble component of the cell content after expression. M is the molecular weight marker, P is the insoluble component of the cell content while L is the soluble component of the cell content. The arrow is marking the AtLHL-AC recombinant protein, which was wholly expressed as an insoluble product of the cell.

5.3.2. Purification of the Recombinant AtLHL-AC Protein

After finding out that the AtHLH-AC protein was mostly expressed in an insoluble form, the protein was therefore, purified off the rest of the other bacterial proteins using an Ni-NTA affinity matrix system (Sigma-Aldrich Inc., Missouri, USA) under non-native denaturing conditions. As is presented in Figure 5.2 below, the recombinant AtLHL-AC protein was successfully purified.

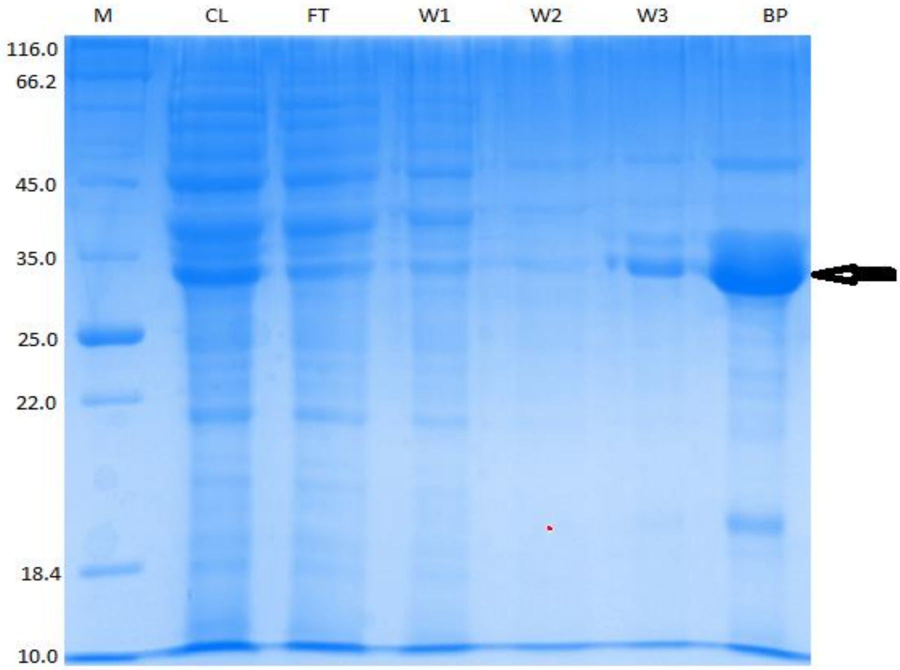


Figure 5.2: Affinity Purification of the recombinant AtLHL-AC Protein. A representative SDS PAGE of the recombinant AtLHL-AC protein fractions collected at different stages of its purification process using the Ni-NTA affinity matrix system (Sigma-Aldrich Inc., Missouri, USA). M represents the unstained low molecular weight marker (Fermenters Int., Burlington, Canada); CL represents the cleared cellular lysate generated by solubilizing the insoluble cellular protein fraction in 8 M urea before its passage through the Ni-NTA affinity matrix; FT represents the flow-through of the cleared lysate after it had been passed through the Ni-NTA matrix; W1 is the first wash of the bound AtLHL-AC recombinant protein onto the Ni-NTA affinity matrix with the wash buffer; W2 and W3 are the second and third washes respectively; and BP is the purified and bound AtLHL-AC recombinant protein. The arrow marks the resultant and purified AtLHL-AC recombinant protein product.

5.3.3. Chemical Refolding and Elution of the Recombinant AtLHL-AC Protein

After the purification process of the recombinant AtLHL-AC protein under non-native denaturing conditions, it was necessary for the denatured and non-functional recombinant protein to be facilitated to regain back its native form and thus biological activity. This was achieved by using a linear refolding gradient system controlled by the DuoFlow BioLogic system (Bio-Rad Laboratories Inc., California, USA) while the recombinant protein was still bound onto the HIS-Select Ni-NTA affinity matrix. In this regard, the 8 M urea buffer into which the recombinant AtLHL-AC was originally resuspended into was now diluted over a 600-minute time period with a non-denaturing buffer to make a final concentration of 0 M urea. In subsequency to that, the refolded and purified recombinant AtLHL-AC protein was eluted from the HIS-Select Ni-NTA affinity matrix system, desalted and concentrated (Figure 5.3 below) in preparation for the enzyme immunoassaying.

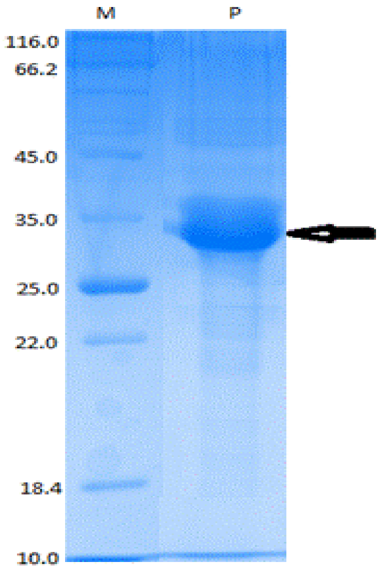


Figure 5.3: Refolding and chemical elution of the purified of the recombinant AtLHL-AC protein. The presentation is a 12% SDS-PAGE of the eluted refolded version of the recombinant AtLHL-AC protein, where M is the molecular weight marker and P the recombinant P protein. The arrow marks the eluted, de-salted and concetrated AtLHL-AC protein fraction.

5.3.4. Functional Characterisation of the Recombinant AtLHL-AC Protein

After successfully refolding and eluting the purified version of the recombinant AtHLH-AC protein, the AC activity of the protein was then further functionally characterized *in vitro* by assessing its catalytic responses to the various chemical ions and molecules components commonly known to influence and/or affect AC activity. Figure 5.4 below shows the observed *in vitro* catalytic responses of this recombinant to such various chemical and molecular components (Mg^{2+}, Mn^{2+}, ATP, GTP, Ca^{2+}, CO_3^{2-}, and F^-).

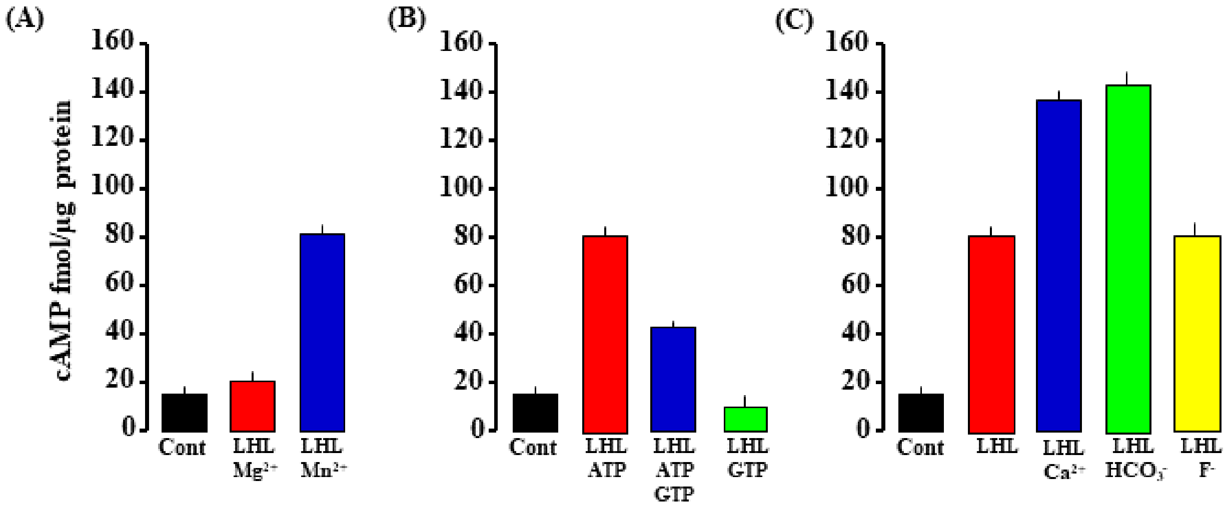


Figure 5.4: Molecular characterisation of the AC activity of the recombinant AtLHL-AC protein. (**A**) Cyclic AMP levels generated with the purified recombinant AtLHL-AC protein in the presence of magnesium or manganese ions as co-factors of activity, (**B**) cAMP levels generated with the purified recombinant AtLHL-AC in the presence of either ATP and/or GTP as possible sole substrates, and (**C**) cAMP levels generated with the purified recombinant AtLHL-AC protein in the presence of calcium, bicarbonate or fluoride ions as functional modulators. All the assays were undertaken in sets of threes (triplicates) and the error bars represent the standard errors of the triplicate means (n = 3) as analyzed by ANOVA.

5.4. DISCUSSIONS

The heterologous expression of recombinant proteins in various prokaryotic systems such as *Escherichia coli* usually results in aggregation of the recombinant protein into insoluble cellular products termed inclusion bodies (Rudolph & Lilie, 1996). This has been reported to be probably a result of the host cells failing to tolerate the high expression rates of the foreign proteins that are normally induced in expression mediums and the intrinsic posttranslational requirements normally necessary for most recombinant proteins of the eukaryotic origin (Rogl *et al.*, 1998). Generally, purification of recombinant proteins that are over-expressed in *E. coli* systems is always necessary so that all contaminating and toxic impurities are purged away (Tranken *et al*., 2000). Several purification systems are now available and commonly practised of affinity purification is one of them, which was successfully used in this study to purify the over-expressed recombinant AtLHL-AC protein. This system takes advantage of the C-terminus negative 6x-His tag that is always associated with most recombinant proteins including AtLHL-AC, which when passed through a positively charged affinity platform like the Ni-NTA matrix, the recombinants will be easily and selectively purified.

Therefore, and after ascertaining in section 5.2.1 that the recombinant AtLHL-AC protein was wholly expressed in a form of inclusion bodies (Figure 1), the AtLHL-AC was then purified under non-native denaturing condition, whereby 8 M urea was used as the denaturant of choice. Purification was undertaken and achieved using the Ni-NTA affinity system that has a 6xNi positive charging compatible with the C-terminus 6xHis negative charging of the expressed fusion AtLHL-AC protein. More so, the Ni-NTA affinity system is also very highly stable and compatible with the non-native denaturing conditions (Hochuli *et al.*, 1987; Rogl *et al.*, 1998) that were used in this study. Ideally, recombinant proteins over-expressed in *E. coli* systems need to be thoroughly purified to remove all contaminating and toxic impurities. This is by far a typical fast and simple procedure which facilitates the association between the protein-fused tag (e.g., polyhistidine (His) (Profinity IMAC resin), glutathione-S-transferase (GST), (Profinia™ GST resin), and Profinity eXact resin) and its corresponding affinity matrix (e.g., antibody/antigen, enzyme/substrate, and enzyme/inhibitor interactions), and thus allowing for the other protein contaminants even under the strongly and harsh denaturing conditions required to solubilise the inclusion bodies to be easily washed off (Crowe *et al*., 1994). As is shown in Figure 5.2, the recombinant AtLHL-AC protein was successfully purified using this approach at its expected molecular weight size of approximately 22.13 kDa.

After purification, the purified and denatured recombinant AtLHL-AC protein thereof, needed to be reverted back into its native, soluble and functional state using a well-known and designed refolding system that involved a gradual dilution of a high concentration of chaotropic agent or denaturant (6-8 M urea or GnCl) into a low or no (2-0 M) concentration (Singh & Panda, 2005). After refolding, the recombinant AtLHL-AC protein was then eluted off the Ni-NTA column, a sign indicating its successful refolding into a soluble native molecule (Figure 5.3).

After successful refolding chemical elution, the resultant recombinant AtLHL-AC protein was then assessed to establish if it had regained its functional activity during the refolding process by testing for its ability to generate cAMP from ATP *in vitro*. In actual fact, such an *in vitro* activity was assessed in the presence of several molecular and ionic components commonly known to influence AC activity. As is shown in Figure 5.4A, the recombinant AtLHL-AC showed a significant *in vitro* AC active that was more dependent onto Mn^{2+} than Mg^{2+} metal ions as co-factors of activity. Evidently, several studies have previously reported magnesium as a necessary co-factor for most nucleotide cyclases activities (Bradham, 1977; Buck *et al.*, 1999; Field *et al.*, 1988; Kasahara *et al.*, 1997; Londos & Preston, 1977), and furthermore, it was also found that the Mn^{2+} ion could also successfully substitute Mg^{2+} ion as the co-factor activator of a turkey erythrocyte sAC (Steer & Levitzki, 1975). Therefore, the *in vitro* activity of the recombinant AtLHL-AC was tested with regard to either of the two metal ions and the obtained results showed that the Mg^{2+} ion had no apparent effect onto the enzymatic activity of the recombinant protein while the Mn^{2+} ion had a significant effect of a factor of approximately 6-fold (Figure 5.4A).

Thus this finding on its own shows that even though both the Mg^{2+} and Mn^{2+} metal ions can be able to activate other known nucleotide cyclase in various organisms (Hurley, 1999; Steer & Levitzki, 1975), the recombinant AtLHL-AC protein has shown to be solely dependent onto the Mn^{2+} ion as a sole co-factor for enzymatic activity. This thus establishes that the AC activity of AtLHL-AC has a sole specific preference for Mn^{2+} metal ion as a co-factor much like the AC activities of the AtPPR-AC (Chatukuta *et al*., 2018), AtKUP7 (Al-Younis *et al*., 2015), AtClAP (Chatukuta *et al.*, 2018), AtKUP5 (Al-Younis *et al.*, 2018) and AtLRRAC1 (Bianchet *et al.*, 2018), and the GC activities of the AtBRI1-GC (Kwezi *et al.*, 2007; Wheeler *et al.*, 2017), AtPEPR1-GC (Qi *et al*., 2010) and AtNOGC1 (Mulaudzi *et al*., 2011).

Notably, the plant nucleotide cyclases (ACs and GCs) have shown consistently lower activities than their animal counterpart. This is presumably due to a more intricate regulatory role afforded to such AC and GC molecules that may enable plant cells to rapidly switch from one cyclic mononucleotide-dependent signalling network to another in localized cellular micro-environments (Irving *et al.*, 2018; Kwezi *et al.*, 2018; Muleya *et al.*, 2014; Wong *et al.*, 2015).

Furthermore, the recombinant AtLHL-AC protein was further characterized with regards to the structural analogs ATP and/or GTP as possible substrates of catalytic activity. As is shown in Figure 5.4B, it was deduced that ATP alone could increase the levels of cAMP production with a factor of approximately 5-folds compared to the control, whereas GTP alone, drastically decreased the cAMP production by almost 20% but the presence of GTP in an ATP reaction (ATP/GTP) system had an apparent effect of decreasing the cAMP production with a factor of up to 3-fold (Figure 5.4B). Therefore and evidently from this outcome, it is conceivable to conclude that even though the GTP could have had been structurally competing with ATP for the AtLHL-AC binding site, ATP remained the preferred substrate for the AtLHL-AC protein's catalysis.

Additionally, the catalytic activity of the recombinant AtLHL-AC protein was also further characterized in response to various molecular or ionic components commonly known to influence AC activity. With calcium as an additive modulator, it was found that this molecule could significantly increase the catalytic activity of the AtLHL-AC protein when ATP was a sole substrate and Mn^{2+} metal ion the co-factor of activity (Figure 5.4C), and this outcome was consistent with one of the previous findings, which reported sACs to be typically stimulated by calcium (Jaiswal & Conti, 2003; Zippin *et al*., 2013). Concurringly, previous studies on the effect of calcium onto the activity of sACs further suggested that the Ca^{2+} ion appears to affect the enzymes in a bi-phasic manner, suggesting stimulatory at low concentrations and inhibitory at high concentrations (Potter *et al*., 1980). Moreover, the glioma sAC was found to be stimulated by less than or equal to 1 mM Ca^{2+} but inhibited as the concentration increased to approximately 100 mM (Brostrom *et al*., 1976). Our findings therefore suggested that the recombinant AtLHL-AC protein is significantly stimulated by a 0.1 mM Ca^{2+} concentration, and thus concurrently presenting it as a sAC. Interestingly, as all tmACs are mediated by the second messenger cAMP through control mechanisms that are regulated by GTP-binding proteins while all sACs are mediated by the second messenger, cAMP via control mechanisms that are regulated by calmodulin, a calcium-binding protein (Kamenetsky *et al*., 2006).

When the activity of the recombinant AtLHL-AC protein was also further characterised with respect to the bicarbonate ion, the results suggested that the bicarbonate ion could significantly increase the AtLHL-AC activity by a factor of about 30% using ATP as a sole substrate and Mn^{2+} metal ion the co-factor of activity (Figure 5.4C). Previously, it has been reported that the membrane-compatimentalized (soluble) AC (or sAC) of the fluid transporting tissue was stimulated by 10 mM bicarbonate and this activation was solely dose-dependent up to a concentration of around 100 mM (Mittag *et al*., 1993). Therefore, sACs are able to systematically synthesise cAMP in response to bicarbonate ion (Steegborn *et al*., 2005), with the ion itself being able to activate the soluble mammalian AC up to 6-folds (Garty & Salomon, 1987), as well as activating the sperm sAC with a similar margin (Okamura *et al*., 1985). Furthermore, it has also been reported that bicarbonate stimulates the activities of mammalian sAC and a Cyanobacterial AC15 both *in vivo* and *in vitro* in a pH-independent mode (Chen *et al*., 2000).

Interestingly, it has further been reported that the sACs that contain the amino acid threonine in their active sites respond to bicarbonate, whereas those that contain a corresponding aspartate amino acid are bicarbonate-insensitive (Cann *et al*., 2003). So, the catalytic centre of our recombinant AtLHL-AC protein, however, does not contain threonine, thus providing a potential evolutionary difference between plant sACs and the other general sACs in that they are stimulated by bicarbonate in the absence of an active site threonine. Bicarbonate signalling through cAMP synthesis is a mechanism where various organisms can possibly respond to the ever carbon-increasing environment due to the essential process of respiration in their cells (Cann *et al*., 2003).

Finally, the AtLHL-AC protein was characterised with respect to its modulation effects by the fluoride ion and the results indicated that ion has no significant effect upon the catalytic activity of the protein (Figure 5.4C). This is consistent with some previous studies reporting that sAC from rat testis are fluoride-insensitive (Braun *et al*., 1977) another study one later on, showing that a sAC from mouse testis could not be stimulated with fluorine (Adamo *et al*., 1980). The outcome of this study consistently displayed that, as a sAC, the recombinant AtLHL-AC protein is not sensitive to fluorine.

5.5. CONCLUSION

Outcome of this chapter evidently established that the recombinant AtLHL-AC protein is indeed a sAC, whose activity is positively modulated by the Mn^{2+}, Ca^{2+} and bicarbonate ions while on the other hand, it has been shown to be non-reactive to the Mg^{2+} and fluoride ions. Therefore, we can certainly infer that the AtLHL-AC protein from *A. thaliana* is a *bona fide* sAC, whose physiological and biochemical activities could be mediated by cAMP via pathways that are solely dependent on calmodulin.

5.6. RECOMMENDATION

Approaches such as liquid chromatography, tandem mass spectrometry (LCMS/MS), cultural and mutational studies and/or bioinformatic analysis of the AtLHL-AC protein across the whole Arabidopsis genome could be undertaken to provide at least a better insight into establishing the typical signalling pathways in which this protein is involved, which will completely elucidate its exact biochemical, physiological and functional roles in Arabidopsis and other closely related higher plants.

CHAPTER 6

6.1. OVERALL DISCUSSION

Food security always depends heavily on the development of crop plants with increased resistance to both biotic and abiotic environmental stress factors (Atkinson & Urwin, 2012). In line with this, various advances, through the specialized areas of plant biochemistry, plant physiology and plant biotechnology, have been implemented in attempting to understand the processes of gene expression, transcriptional regulation and signal transduction in plants, which are capable of increasing tolerance to both the biotic and abiotic stress factors (Zhu *et al.*, 2010). As a result of all this, molecular and genomic analyses have now practically facilitated gene discovery (Abe *et al.*, 2003; Seki *et al.*, 2001; Seki *et al.*, 2007; Tran *et al.*, 2004) and enabled the possibility of genetics to use several functional and/or regulatory genes to activate or repress specific or broadband pathways related to the maintenance of homeostasis and adaptation to stress responses (Hussain *et al.*, 2010; Trujillo *et al.*, 2009). This study was aimed at determining the molecular/functional activity of a linker histone-like (LHL) protein from *Arabidopsis thaliana* (AtLHL-AC) and its probable mechanisms of action particularly in plant's stress responses and adaptation processes.

Firstly, a preliminary bioinformatic analysis of the AtLHL protein was conducted (Chapter 2). This analysis revealed that the AtLHL protein is mostly expressed in the nucleus, where it is chiefly involved in both the selective and non-covalent binding of nucleosomal double-stranded DNA and proteins. The protein is also closely associated with the regulation of DNA transcription, DNA templating and recombination; chromatin silencing;

and nucleosomal positioning, condensation and assembly (Gaudet *et al.*, 2011) of which all these processes that are essentially regulated by the second messenger, cAMP. Secondly and following that, Chapter 3 then entailed the cloning and partial expression processes of the AC-containing fragment domain of this putative AtLHL protein (AtLHL-AC) followed by assessment of its inherent endogenous AC activity in chemically competent *Escherichia cloni* BL21 (DE3) pLysS DUOs prokaryotic system. The results obtained in this chapter showed that the AtLHL protein could either be a *bona fide* AC capable of generating cAMP from ATP by itself or simply just yet another functional plant signaling molecule capable of stimulating other resident ACs (*E. coli* ACs in this case) to produce their own cAMP.

Thirdly, the *bona fide* AC activity of the AtLHL-AC was then confirmed in Chapter 4 through a molecular cloning and subsequent recombinant expression of the truncated AtLHL-AC cDNA in a mutant *E. coli* SP850 *cya*A strain that lacks the activity of the AC and therefore, unable to ferment lactose. Here, the truncated AtLHL-AC recombinant protein demonstrated its ability to rescue the mutant *E. coli* strain into a wild type status capable of fermenting lactose. This process is termed complementation testing (Charania *et al.*, 2009; Cotta *et al.*, 1998) and is a convenient and quicker way of assessing and determining the AC activities of unknown recombinant proteins.

We then purified and functionally characterized the AC activity of the recombinant AtLHL-AC protein *in vitro* in Chapter 5. Findings of this chapter then conceivably established that the recombinant AtLHL-AC protein is indeed a sAC, whose activity is positively modulated by the Mn^{2+}, Ca^{2+} and $HCO3^-$ ions while on the other hand, it is non-reactive to the Mg^{2+} and F^- ions. Therefore, in the same chapter, we could certainly infer that the AtLHL-AC protein from *A. thaliana* is a *bona fide* sAC, whose functional activities are mediated by cAMP via a calmodulin intermediate protein.

6.2. OVERALL CONCLUSION

From all the findings gathered in this study, the AtLHL is indeed established as a functional sAC with specific roles in gene regulatory mechanisms in the *A. thaliana* and other closely related higher plants. Furthermore, our findings have positively indicated a likelihood that all the currently known and/or reported cAMP-dependent signalling processes in plants may possibly be mediated by more than one AC molecules since our studied AtLHL protein is the sixth such AC molecule to be experimentally identified and functionally confirmed in *A. thaliana* and the ninth one to be identified in higher plants among the currently existing ones. Such higher plant ACs include the *Zea mays* pollen signalling protein that participates in polarized pollen tube growth (Moutinho *et al.*, 2001a), a *Nicotiana benthamiana* adenylyl cyclase protein with a role in tabtoxinine-β-lactam-induced cell death (Ito *et al.*, 2014b), a *Hippeastrum hybridum* adenylyl cyclase protein that is involved in stress signalling (Świeżawska *et al.*, 2014), an Arabidopsis pentatricopeptide repeat-containing protein which is responsible for pathogen responses and gene expressions (Ruzvidzo *et al.*, 2013), two *A. thaliana* K^+-uptake permeases AtKUP5 and AtKUP7, both responsible for K^+ transportation (Al-Younis *et al.*, 2015; Al-Younis *et al.*, 2018), an Arabidopsis clathrin assembly protein with a predicted role in actin cytoskeletal remodelling during endocytic internalization (Chatukuta *et al.*, 2018), and, an *A. thaliana* leucine-rich repeat protein (LRR) which, affects responses to pathogens (Bianchet *et al.*, 2018).

6.3. OVERALL RECOMMENDATIONS

Since the AtLHL protein was bioinformatically identified together with the other putative Arabidopsis candidates as probable functional ACs, it is therefore strongly recommended that such outstanding and functionally uncharacterized putative candidates be also functionally characterized in the same way the AtLHL was characterized in this study. Thus taping into a better understanding of their exact physiological, biological and expressional roles in plants, and particularly in critical cellular processes such as growth, development and responses to stressful environmental factors would possibly, provide the basis for putting in place strategies and measures that may enable plants (crops) to withstand the currently threatening environmental extremes brought about by climate change. Ideally, this could also be an ultimate and valuable investment towards the possible eradication and/or sustainable alleviation of hunger and poverty on this troubled planet. Further assays on the AtLHL protein are strongly recommended that should involve testing its activities in living plant tissues (*in planta*), followed by some detailed bioinformatics correlational expression analyses in tandem with the other known plant signaling-related genes. And also studies involving AtLHL knockouts or knockdown are also strongly recommended so that more and detailed information on the gene can be obtained at a whole plant level.

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