



**Molecular and Functional Characterization of a
Novel Microtubule Associated Protein from
*Arabidopsis thaliana***

SKM Sehlabane-Abotseng

 **orcid.org 0000-0003-3747-3752**

Thesis accepted in fulfilment of the requirements for the
degree

Doctor of Philosophy in Biology
at the North-West University

Promoter: Prof O Ruzvidzo

Co-promoter: Dr TD Kawadza

Graduation ceremony: July 2020

Student number: 17001285

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DECLARATION

I, Selaelo Katlego Motlatso Sehlabane-Abotseng, declare that the thesis entitled "**Molecular and functional characterization of a novel microtubule associated protein from *Arabidopsis thaliana***" is my work and has not been submitted for any degree or examination at any other university or institution and that all sources of my information here have been acknowledged as indicated in the text and/or list of references.

Student: Selaelo Katlego Motlatso Sehlabane-Abotseng

Signature: **Date:**

Promoter: Prof. O Ruzvidzo

Signature: **Date:**

DEDICATION

This thesis is dedicated to God Almighty for his strength and guidance throughout this journey to successfully completing my PhD.

To my family; my husband, Molefhi Abotseng, undertaking our PhD's together was indeed a blessing. Thank you for the love, support and encouragement during this time of the study, you are amazing.

My parents; my father Lucas Sehlabane and mother Grace Sehlabane, I am a doctor today because of you, thank you very much for all that you have done for me, you have been my biggest cheerleaders throughout my life and I will forever be grateful for that. To my siblings; Mampeyane and Tshegofatso Malatja, and Mašoto and Peggy Sehlabane, I share this victory with you, thank you for your encouragement. To my in-laws; my mother Mmopiemang Abotseng and my brothers and sisters, you are the best, thank you very much for your support.

ACKNOWLEDGEMENTS

Many thanks to my supervisor, Professor Oziniel Ruzvidzo, for giving me an opportunity to work in his laboratory and under his esteemed guidance, I appreciate it.

The members of staff of the North-West University, Faculty of Natural and Agricultural Sciences, Department of Botany, in particular, Dr. Bridget Tshegofatso Dikobe and Dr. Dave Kawadza for your assistance whenever I needed it, I am thankful.

To the Plant Biotechnology Research Group; thank you for making lab work so much fun even when sometimes it was very stressful 😊

I would also like to acknowledge The National Research Foundation (NRF) and North West University Postgraduate bursaries for financially supporting me during my 3 years of the PhD study.

TO GOD BE THE GLORY

DEFINITION OF TERMS

Abiotic stress factors: Are negative impacts of non-living factors on the living crops and animals in a specific environment.

Adenosine triphosphate (ATP): An organic chemical that provides energy to drive many processes in living cells.

Adenylate cyclases (ACs): Enzymes capable of converting adenine 5'-triphosphate (ATP) to cyclic 3',5'-adenosine monophosphate (cAMP).

Annotation: The identification of regions of interest in sequence data.

Bioinformatics: Is an interdisciplinary field that develops and applies computational methods to analyse large collections of biological data.

Biotic stress factors: Are negative effects of living organisms in an environment arising from the interaction with non-living organisms.

Climate change: The periodic modification of earth's climate brought about as a result of changes in the atmosphere as well as interactions between the atmosphere and various other biological, chemical, geologic, and geographic factors within the earth system.

Cloning: The process of generating genetic information from one living thing and creating identical copies of it.

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

Complementary DNA (cDNA): DNA copy of mRNA molecule produced by reverse transcriptase.

Complementation test: A method of determining whether two independently isolated mutants with the same phenotype are in the same or different genes.

Cyclic guanosine monophosphate (cGMP): Cyclic nucleotide derived from guanosine triphosphate (GTP), it acts as a second messenger much like cyclic AMP.

Domain: A specific physical region or amino acid sequence in a protein which is associated with a particular function or corresponding segment of DNA.

Electrophoresis: An electric field applied across a gel matrix to separate large molecules such as DNA, RNA, and proteins by charge and size.

Endogenous: Developing or originating within an organism or part of an organism.

Enzyme immunoassay: An antibody based diagnostic technique used in molecular biology for the qualitative and quantitative detection of specific biological molecules.

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

Exon: An exon is a coding region of a gene that contains the information required to encode a protein.

Expression vector: A vector used for protein expression in which a specific gene is introduced into a cell.

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

Gene: A small sections of DNA within the genome that code for proteins.

Genomic DNA: The full complement of DNA contained in the genome of a cell or organism.

G-protein: Any of a class of cell membrane proteins that transmit signals from certain receptors to effector enzymes, enabling the cell to regulate its metabolism in response to hormones and other extracellular stimuli.

Guanylate cyclases (GCs): Enzymes capable of converting guanine 5'-triphosphate (ATP) to cyclic 3',5'-guanosine monophosphate (cAMP).

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.

Hybridization: A process in which single-stranded nucleic acids are allowed to interact to form hybrids with sufficiently similar complementary sequences.

In vitro: A biological process that occurs outside the living organism, that is, in an artificial environment such as within a glassware.

In vivo: A biological process that occurs or made to occur within a living organism or natural setting.

Intron: An intron is a long stretch of noncoding DNA found between exons (or coding regions) in a gene.

Mass spectrometry: A biochemical method used to detect biological molecules according to their quantities and molecular weights.

Messenger ribonucleic acids (mRNAs): Convey genetic the information from DNA to the ribosomes that makes proteins.

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.

Plasmid DNA: A small cellular inclusion consisting of a ring of DNA that is not in a chromosome but is capable of autonomous replication.

Primers: Short synthetic nucleic acid sequences capable of forming base pairs with a complementary template RNA/DNA strand and facilitating its specific amplification.

Proteomes: A collection of cellular proteins whose expression levels are co-regulated by a single and/or specific signaling molecule.

Proteomics: A tool to integrate genome information and transcription activity with translation efficiency and enzyme activity.

Recombinant DNA: A molecule of DNA that has been modified to include genes from multiple sources, either through genetic recombination or through laboratory techniques.

Recombinant protein: A protein encoded by recombinant DNA that has been cloned in a system that supports expression of the gene and translation of messenger RNA.

Refolding: A process that restores the biological activity of an unfolded or misfolded protein.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR): A molecular method used to convert a short RNA segment into a DNA product termed copy DNA (cDNA) using an RNA-dependent DNA polymerase enzyme.

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

Sequencing: A process of determining the sequence of nucleotides within a DNA molecule.

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

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE): A technique used in molecular biology to separate different protein molecules according to their sizes and migration capacities in a polyacrylamide gel system subjected to a strong electrical field.

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

LIST OF ABBREVIATIONS

AC	:	Adenylate cyclase
ANOVA	:	Analysis of variance
AtKUP5	:	<i>Arabidopsis thaliana</i> K ⁺ -uptake permease 5
AtKUP7	:	<i>Arabidopsis thaliana</i> K ⁺ -uptake permease 7
ATP	:	Adenosine 5'-triphosphate
At-PPR	:	<i>Arabidopsis thaliana</i> pentatricopeptide
BLAST	:	Basic Local Alignment Searching Tool
cAMP	:	Cyclic 3',5'-adenosine monophosphate
CAP	:	Clathrin assembly protein
cGMP	:	Cyclic 3',5'-guanosine monophosphate
cGMP	:	Cyclic guanosine monophosphate
FAO	:	Food and Agriculture Organization
GC	:	Guanine-cytosine
GDP	:	Gross Domestic Product
GTP	:	Guanosine-5'-triphosphate
HpAC1	:	<i>Hippeastrum hybridum</i> adenylate cyclase 1

IPTG	:	Isopropyl- β ,D-thiogalactopyranoside
LB	:	Luria-Bertani
LRR	:	Leucine-rich repeat
MAP	:	Microtubule associated protein
MpCAPE	:	<i>Marchantia polymorpha</i> adenylate cyclase with phosphodiesterase
mRNA	:	Messenger ribonucleic acids
NbAC	:	<i>Nicotiana benthamiana</i> adenylate cyclase
Ni-NTA	:	Nickel-nitrilotriacetic acid
NO	:	Nitric oxide
OD	:	Optical density
PCR	:	Polymerase chain reaction
RLK	:	Receptor-like kinases
Rpm	:	Revolutions per minute
RT-PCR	:	Reverse transcriptase-polymerase chain reaction
sAC	:	Soluble adenylate cyclase
SDS-PAGE	:	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
ST	:	Sodium Tris-HCl

STAND	:	Signal transduction ATPases with numerous domains
TAIR	:	The Arabidopsis Information Resource
tmACs	:	Transmembrane adenylate cyclases
UN	:	United Nations
UV	:	Ultraviolet
YT	:	Yeast-tryptone

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OVERALL THESIS ABSTRACT

The question of whether cyclic 3',5'-adenosine monophosphate (cAMP) is an authentic second messenger molecule in higher plants has kept plant scientists busy for many decades, this being due to methodological challenges encountered in previous years, when techniques developed for its analysis in mammalian tissues were merely adopted for plant material. cAMP is a product of ATP hydrolysis catalyzed by the enzyme adenylate cyclase (AC). These two groups of molecules (cAMP and ACs) play an important role in mediating environmental stimuli to physiological responses in living cells. In this study, our work focused on an *Arabidopsis thaliana* microtubule associated protein (AtMTA) encoded by the At3g02930 gene, which was recently bioinformatically annotated as an AC candidate. In order to elucidate its possible functional roles in higher plants, a preliminary bioinformatic analysis of its encoding gene, the At3g02930, was conducted, where it was found that AtMTA is implicated in the photosynthetic processes of plants as well as the reciprocal meiotic recombination functions. Furthermore, to assess for its possible AC activity, total mRNA from 6 weeks old *A. thaliana* leaf material was extracted and used as a template in a specialized RT-PCR system for amplification of the At3g02930 gene fragment bearing the putative AC catalytic center (AtMTA-AC). The amplified gene fragment was then cloned into a pTrcHis-TOPO expression vector to form a pTrcHis-TOPO:AtMTA-AC recombinant construct, which was then used to transform competent *Escherichia coli* BL21 (DE3) pLysS cells. Protein expression then proceeded after induction of the transformed cell cultures with 1 mM IPTG. After expression, the resultant recombinant protein was then purified on an Ni-NTA affinity system under non-native denaturation conditions because the expressed recombinant product was found to be mostly expressed in its insoluble state. This was then followed by its refolding into its native and soluble form via a

linear gradient on the same purification system. The resultant protein product was then tested for its possible endogenous, *in vitro* and *in vivo* AC activities followed by a further characterization of such an activity. The endogenous and *in-vitro* assays were determined via a cAMP-specific enzyme immunoassay system, whereby it was shown indisputably, that the AtMTA-AC protein has the ability to induce the generation of endogenous cAMP in a prokaryotic expression system while at the same time, capable of generating cAMP as a pure product. On the other hand, the *in vivo* activity was tested and determined via a complementation testing, where it was strongly confirmed that this recombinant protein is indeed a *bona fide* functional AC molecule capable of generating cAMP from ATP, and rescuing a non-lactose mutant *E. coli* strain into a lactose fermenting host. Results from all the three tests showed that this putative protein has some inherent endogenous, *in vitro* and *in vivo* AC activities and therefore, confirming it as a higher plant AC with a possible cAMP-mediated signaling function. Lastly, a detailed overview of the bioinformatic expressional profile of the At3g02930 gene was conducted, where the gene was found to be directly linked to three other genes on the microarray network which are the: At5g52280 and At1g64330 genes, which function as myosin heavy chain-related proteins and At3g53350 that functions as a repressor of primer (ROP) interactive partner 4. Thus since co-expressed genes are generally expected to be involved in related and/or similar cellular functions, then the AtMTA-AC gene may potentially be a myosin heavy chain-related protein or a ROP interactive partner 4 with essential roles in growth and development. The gene could also response to various environmental stress factors.

ORGANIZATION OF CHAPTERS IN THESIS

This thesis has been written and presented in a divided block format consisting of seven chapters.

Chapter One is an introductory chapter for the study in which an insight into the functionality and characterization aspects of the microtubule associated protein (MTA) encoded by the gene At3g02930 from *Arabidopsis thaliana* is provided. This chapter also includes the research aims, research objectives and significances of the study of this doctoral thesis. It also consists of a critical review of literature that is relevant to the current study, whereby the literature review details the *A. thaliana* plant as a choice plant for the study. Additionally, this chapter further highlights a better understanding of the functionality aspects of the adenylate cyclase (AC) enzymes and their role in the conversion of ATP into cAMP, which is of vital significance to both scientists and agriculturists as this can be strategically applied into the improvement of crop yields and the subsequent eradication of hunger and poverty. Furthermore, the chapter details the presence of microtubule associated proteins in higher plants, cross-talking systems between signalling pathways and lastly, it is concluded by providing the bioinformatic identification of the AC catalytic centre of the At3g02930 gene.

Chapter Two consists of a presentation of a preliminary bioinformatic analysis of the At3g02930 gene. The focus of this chapter is to discuss bioinformatic data available on several biological databases about the AtMTA encoded by the gene At3g02930 from *A. thaliana*. Thus, the chapter defines the different database annotations of the AtMTA gene, its locus detail, its expression profile, its subcellular localization and function, its protein structure and model information, its protein orthologues, homology and gene clustering and finally and a brief discussion of the conclusions of the chapter.

Chapter Three is divided into two main sections, that is, the DNA work which consists of the isolation and molecular cloning of the AtMTA-AC gene fragment into a stable heterologous prokaryotic expression system and the expression process of the targeted protein product proceeded by the determination of the endogenous activity.

Chapter Four describes the determination of the *in vivo* AC activity of the recombinant AtMTA-AC protein via a complementation system, whereby the recombinant protein was shown to successfully rescue the mutant *E. coli* SP850 strain to metabolize lactose.

Chapter Five describes the affinity purification of the recombinant AtMTA-AC protein on a Ni-NTA matrix followed by a concerted characterization of its AC activity. The chapter then eludes on the findings, whereby the AtMTA protein was established as a soluble AC (sAC) modulated by molecular components such as calcium, the carbonate and manganese ions via a cAMP-linked calmodulin system.

Chapter Six provides a detailed overview of the bioinformatic expressional analysis of the At3g02930 gene, whereby the aspects of expression profile, anatomical expression, developmental expression, co-expression and stimulus-specific expression of the AtMAP protein is detailed. The chapter closes by inferring the probable functional role of this novel protein in Arabidopsis and other closely related plants

Chapter Seven consists of an overall summary of the major findings of the whole study, its associated conclusions and recommendations for new directions in research advances and proposed possible future studies.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

1.1.1 Background

This research project focused on the molecular characterization of a signalling molecule, the microtubule associated protein (MTA) encoded by the gene At3g02930 from *Arabidopsis thaliana* (AtMTA), a small flowering plant widely used as a model research candidate in plant biology (Gehring, 2010). A range of microtubule associated proteins (MTAs) have been reported in higher plants, whereby they are categorized according to their main functions and into two groups: one is involved in the regulation of microtubules (MTs) assembly and disassembly while the other is involved in organization of MT structures and functions (Hamada, 2007).

MTs are most important structural components of the plant cytoskeleton that are composed of α - and β -tubulin heterodimer sub-units, which are assembled into linear protofilaments. The protofilaments associate laterally to form the microtubule, a 25 nm-wide hollow cylindrical polymeric structure (Figure 1.1). These microtubules are essential for a wide variety of cellular functions and are complexly involved in intracellular transport, morphology, cell division and intracellular organization (Desai and Mitchison, 1997). The ability of the MT cytoskeleton to accomplish its versatile cellular functions depends on its inherently dynamic polymer. The

MTAs , are sometimes called 'structural' MTAs because they bind, stabilize and promote the assembly of microtubules, and also because they can be co-purified with tubulin through several cycles of microtubule assembly and disassembly (Sakakibara *et al.*, 2013).

MTAs from animals have been biochemically isolated from brain cells, which contain large amounts of MTs contrary to MTAs from plants, which seem to be difficult to purify, because plants have no MT-rich organs. During the early 1990s, advancement in molecular genetics studies on plant MTAs improved dramatically, leading to the isolation of a large number of mutants with abnormal MT structures and the responsible genes identified (Hamada, 2007).

Previous recombinant MTA expression studies done on various hosts such as yeast, *Escherichia coli* and plant cells, and isolated by column chromatography using the specific binding activity of tags, made it possible for several native MTAs from plant materials to be purified because of a successful preparation of their cytoplasmic extracts (Chang-Jie and Sonobe, 1993). Biochemical analysis using isolated recombinant and native MTAs showed comprehensive characteristics of individual MTAs. Based on the characteristics and localization of these individual MTAs, it was possible to deduce exactly how large numbers of MTAs are involved in the construction of the MT structures in plants (Jiang and Oblinger, 1992).

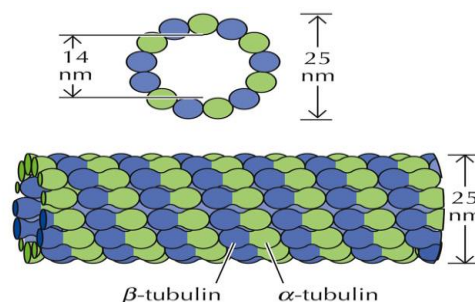


Figure 1.1: Illustration of the composition of a microtubule polymetric structure (adapted from Cellular and Molecular Life Sciences).

In this present study, the recombinant expression and functional characterization of this essential plant protein as a probable adenylate cyclase (AC) molecules was conducted with the aim of possibly gaining and applying the understanding of its functionality within the preamble of the Southern African agricultural context for the possible improvement of crop yields, food productivity and the overall sustenance of food security. The MTA has recently been bioinformatically annotated as a putative higher plant AC (Gehring, 2010) and also predicted as a probable kinase (Durek *et al.*, 2009), and was therefore, a suitable target protein for this study. In this study therefore, we hereby report the molecular cloning and recombinant expression of an AC-containing fragment domain of this protein molecule (AtMTA-AC) followed by its functional characterization. This is because ACs are enzymes capable of generating the second messenger cyclic, 3',5'-adenosine monophosphate (cAMP), that is commonly involved in several plant cellular signal transduction processes, including responses and resistance to stressful environmental conditions.

The world population is on a rapid increase and therefore, the need for food is nowadays a major problem globally, because as the world population increases, the daily demand for food is also estimated to double in the next generation (Fischer *et al.*, 2005). Food security is actually a topic of contention in the sub-Saharan African countries, where the rural population is approximated to be at 70% and farming being the main economic and social activity (Abah *et al.*, 2010). Developing countries are mostly poverty stricken and people have limited access to food (Serageldin, 1999). Therefore, mechanisms to transform agricultural technological systems will be beneficial for allowing sufficient food production in the future and adding new crops to the world food markets (Abah *et al.*, 2010). To this effect therefore, research has been carried out on novel proteins such as ACs and kinases that would bring about a better understanding of the

possible role of second messenger cAMP in plant development, responses to environmental stimuli and resistance to stressful environmental conditions (Gehring, 2010). Food security is therefore, somewhat directly dependent on global warming, as it is predicted that global warming will have serious negative effects on plant growth due to the damaging effects of high temperatures on plant development.

During the years 2015 to 2016, southern African countries experienced weather phenomenon called the El Nino and La Nina weather patterns. These patterns are associated with periods of warming in the central and eastern tropical Pacific that are characterized by deadly and costly climate extremes. The Food and Agriculture Organization (FAO) reported that since the earliest stages of 2015 to 2016, the rainfall season has so far been the driest one in the last 35 years - this having led to a most devastating impact on harvests and thus food security in Southern Africa, contrarily in the first few months of 2019 cyclone Idai and Kenneth swept through some parts of South Africa, Mozambique, Zimbabwe and Malawi, causing devastating floods and ruining crops and once more threatening food security in Southern Africa.

Naturally, plants are sessile and therefore, have to always adjust their cell metabolism in response to environmental fluctuations such as changes in temperature, pH, levels of oxygen and nutrient availability through signal transduction, wherein an external signal activates a specific receptor located on the cell surface or inside the cell and this receptor then generates a biochemical response inside the cell (Hasegawa *et al.*, 2000). A major determinant of plant growth is how plants respond to stressful conditions brought about by biotic and abiotic factors such as pathogenic infections, pest attack, drought, floods and salinity.

The increasing threat of climatological extremes, including very high temperatures and floods might lead to drastic loss of crop productivity and result in wide spread famine. In the arid and semi-arid areas of Southern Africa, these stress factors continue to contribute towards the problem of crop loss and/or yield failure (Denby and Gehring, 2005). According to GrainSA (2015) (www.grainsa.co.za), the wide spread drought in South Africa was expected to result in a 29% decrease of crop production from 2014 to 2015. Crops such as maize and soya, which are directly linked to the production of the staple foods like mealie-meal and oil respectively, were expected to decline in their outputs from about 14.3-million tonnes in 2014 to about 9.8-million tonnes in 2015 (which is an estimated drop of 31%). The most recent gross domestic product (GDP) figures revealed that the agricultural sector has declined by more than 17% quarterly, and mainly because of the wide spread drought throughout the country. This is of course expected to put pressure on food prices, with food hikes likely to be on everything from maize grain to meat, poultry and dairy products.

This prevailing situation, in the face of rapidly mutating pathogens and climate change, will inevitably lead to an increased demand for food and therefore, a consequent rise in the cost of limited resources. Under these circumstances, food security is therefore, heavily dependent onto the development of crop plants with increased resistance to both biotic and abiotic environmental stress factors (Atkinson and Urwin, 2012). Recent advances in the areas of plant biochemistry, plant physiology and plant biotechnology have simplified the process of over-expression and lateral transfer of plant genes capable of increasing the tolerance of plants to stress factors. The approach of plant biotechnology in this regard has been to focus on plant molecules such as the MTA encoded by the gene At3g02930 from *A. thaliana* (AtMTA), which are systemically

known to be involved in the maintenance of homeostasis and adaptation to stress responses (Hussain *et al.*, 2010).

1.1.2 Problem Statement

MTAs have been implicated in plant responses to biotic (disease, pest attack, herbivores or other parasitic plants) and abiotic (drought, floods, heat, light, salt or heavy metals) stress factors as well as in signal transduction pathways (Caplan *et al.*, 2008; Estavillo *et al.*, 2011; Heckathorn *et al.*, 2004; Kagawa *et al.*, 2001; Kasahara *et al.*, 2002; Myouga *et al.*, 2006; Nomura *et al.*, 2012; Sato *et al.*, 1999; Taylor *et al.*, 2009; Yokotani *et al.*, 2010). However, no study to date has yet experimentally demonstrated the ability of these protein molecules to generate cAMP from ATP and/or to transfer a phosphate group to other target proteins. With regard to the AC activity, apart from the *Zea mays* pollen protein (PSiP) (Moutinho *et al.*, 2001), the *A. thaliana* pentatricopeptide repeat protein (AtPPR) (Ruzvidzo *et al.*, 2013), the *Nicotiana benthamiana* adenylate cyclase protein (NbAC) (Ito *et al.*, 2014), the *Hippeastrum hybridum* adenylate cyclase protein (HpAC1) (Świeżawska *et al.*, 2014), the *A. thaliana* K⁺-uptake permease 7 (AtKUP7) (Al-Younis *et al.*, 2015), the *Marchantia polymorpha* adenylate cyclase with phosphodiesterase (MpCAPE) (Kasahara *et al.*, 2016), the *A. thaliana* clathrin assembly protein (AtCAP) (Chatukuta *et al.*, 2018), the *A. thaliana* K⁺-uptake permease 5 (AtKUP5) (Al-Younis *et al.*, 2018) and the recently discovered *A. thaliana* leucine-rich repeat protein (AtLRR) (Bianchet *et al.*, 2019) are the only proteins so far to have been characterized as ACs in plants. As such, a need has therefore been realized to experimentally explore the putative AC activities of the

recently annotated MTA from *A. thaliana* (Gehring, 2010), and possibly determine its functional roles in stress response and adaptation mechanisms.

1.1.3 Research Aim

This study sought to establish whether the MTA from *A. thaliana* is capable of generating cAMP from ATP, and if so, to then assess its potential involvement in essential cellular processes such as plant stress response and adaptation mechanisms.

1.1.4 Research Objectives

The following specific objectives were set to address the research question:

1. To isolate the AC-containing gene fragment domain of the annotated Arabidopsis microtubule associated gene (MTA-AC) into a stable and viable heterologous prokaryotic expression system.
2. To optimize expression strategies of the cloned AC-containing gene fragment domain of the annotated Arabidopsis microtubule associated gene into a recombinant MTA-AC protein.
3. To determine the endogenous AC activity of the recombinant MTA-AC protein.
4. To determine the *in vivo* AC activity of the recombinant MTA-AC protein.
5. To optimize purification regimes of the expressed recombinant MTA-AC protein and determine its *in vitro* AC activity.

6. To further functionally characterize the AC activity of the purified recombinant MTA-AC protein
7. To bioinformatically determine and establish the correlation expressional and functional profiles of the MTA-AC protein in *A. thaliana*.
8. To link up the biochemical and bioinformatic outcomes of the MTA protein and infer its probable role in stress response and adaptation mechanisms.

1.1.5 Significance of the Research Study

Upon a successful completion of this research study, the following significances were set to be realized:

1. The outcomes of the research project would strongly contribute towards the discovery of yet another functional AC in higher plants besides the currently known ACs.
2. The project would produce novel outcomes, which could contribute towards the synthesis and establishment of new literature and further scholarship in the modern field of Plant Sciences.
3. The project would provide a potential contribution towards the moderate integrated management of both biotic and abiotic stressful conditions of agronomically important crops in South Africa.
4. A better understanding of the functionality aspects of this group of enzymes would be of very vital significance to both scientists and agriculturists as this can be strategically

applied into the improvement of crop yields and the subsequent eradication of hunger and poverty.

1.2 LITERATURE REVIEW

1.2.1 *Arabidopsis thaliana* - a Choice Model Plant for this Study

Arabidopsis thaliana has become an essential model plant for plant scientists since the mid-1980s due to the availability of its whole genome sequence, molecular genetic markers and large collections of sequence-indexed DNA-insertion mutants (Meinke *et al.*, 1998). *A. thaliana* is a weed like plant that belongs to the Cruciferae family, commonly known as the mustard family; its different ecotypes have been collected from natural populations and are available for experimental analysis. The Columbia and Landsberg ecotypes are the accepted standards for genetic and molecular studies (David *et al.*, 1998). This plant was a good candidate for use in this study because of its small genome, which has led to a large base of mutants, genetic mapping and gene sequencing (Koncz *et al.*, 1992). The plant is small in size, it self-pollinates, and has a fast-growing entity with a generation time of only 6 weeks (from germination to the first seeds under continuous light). A single plant can produce thousands of seeds within two months that can easily be screened in a single standard Petri dish. Large numbers of the plants can also be grown in a very small space (as much as 100 plants in a pot or a small chamber) and such plants being able to be easily or conveniently mutagenized by chemical mutagens or radiation (Gepstein and Horwitz, 1995).

1.2.2 Cyclic AMP Cellular Signaling in Higher Plants

For many decades, the role of ACs as a key component of the cellular signaling system in synthesizing cAMP from ATP has been studied (Robinson *et al.*, 1968). The molecule, cAMP plays an important role as a signaling molecule in both prokaryotic and eukaryotic organisms. It is a key second messenger in all living organisms ranging from the *Dictyostelium* to *Homo sapiens* (Assmann, 1995). Initially, cAMP was known to be a regulator of glycogen breakdown in the liver and is now known to be a second messenger for a wide variety of cellular responses in animals (Assmann, 1995). Naturally, the enzymes ACs and phosphodiesterases (PDEs) are located in the plasma membrane and cytoplasm respectively, where they are believed to be responsible for the biosynthesis and biodegradation of cAMP respectively (Katayama and Ohmori, 1997; Yashiro *et al.*, 1996).

From the onset, the presence and physiological roles of ACs in higher plant signal transduction were initially very unclear; firstly, due to the lack of information in plants as compared to animals and lower eukaryotes and secondly, as a result of the assays conducted in plants that were not conducive to reach firm conclusions (Amrhein, 1977). Nevertheless, the availability of more and advanced analytical tools has dramatically improved the assaying systems in plants and the affirmation of solid conclusions (Al-Younis *et al.*, 2015). Regardless of all these challenges, the concept that plants have a functional cAMP-dependant signaling system still remained a driving force in higher plant research, largely due to the fact that both cell-permeant 8-Br-cAMP and the stimulation of *albeit* unknown ACs with forskolin could elicit concentration-dependent and time-dependent plant biological responses such as increases in Ca^{2+} influx across the plasma membrane (Kurosaki and Nishi, 1993; Kurosaki *et al.*, 1994). Furthermore, conclusive biochemical evidence demonstrated that crude alfalfa (*Medicago sativa* L.) root extracts could

also show calmodulin-dependent AC activity (Carricarte *et al.*, 1988). The most convincing data for a specific signaling role for cAMP came from whole-cell patch-clamp recordings from *Vicia faba* mesophyll protoplasts, which revealed that an outward K^+ current, could increase in a dose-dependent fashion and as a result of an intracellular application of cAMP but not AMP, cGMP or GMP. This was somewhat indirect evidence indicating that this current modulation was occurring via a cAMP-regulated protein kinase system (Li *et al.*, 1994).

Recently and in higher plants, nine experimentally tested and functionally confirmed ACs were discovered. These are the *Zea mays* pollen protein (PSiP) with a role in polarized pollen tube growth (Moutinho *et al.*, 2001), the *A. thaliana* pentatricopeptide repeat protein (AtPPR) responsible for pathogenic responses and gene expressions (Ruzvidzo *et al.*, 2013), the *Nicotiana benthamiana* adenylate cyclase protein (NbAC) responsible for the tabtoxinine- β -lactam-induced cell deaths during wildfire diseases (Ito *et al.*, 2014), the *Hippeastrum hybridum* adenylate cyclase protein (HpAC1) involved in stress signalling (Świeżawska *et al.*, 2014), the *A. thaliana* K^+ -uptake permease 7 (AtKUP7) responsible for the cAMP-dependent K^+ flux (Al-Younis *et al.*, 2015), the *Marchantia polymorpha* adenylate cyclase with phosphodiesterase (MpCAPE) with a role in male organ and cell development in basal plants (Kasahara *et al.*, 2016), the *A. thaliana* clathrin assembly protein (AtCAP) with a predicted role in plant defence (Chatukuta *et al.*, 2018), the *A. thaliana* K^+ uptake permease 5 (AtKUP5) involved in the increase of cAMP levels in response to K^+ transport activity (Al-Younis *et al.*, 2018), and the *A. thaliana* leucine-rich repeat (AtLRR) protein involved in the defence against biotrophic and hemibiotrophic plant pathogens (Bianchet *et al.*, 2019).

1.2.3 The Presence of Microtubule Associated Proteins in Higher Plants

MTAs play an important role in controlling MT dynamics and organization and therefore, are involved in the regulation of cell expansion (Chan *et al.*, 1996; Hussey *et al.*, 2002; Plett *et al.*, 2009; Wasteneys and Galway, 2003). MTs in plant cells are dynamic structures that play a fundamental role in a range of cellular functions (Zhang *et al.*, 1990). During the stages of the cell cycle, four typical discrete arrays of microtubules within cells appear and disappear sequentially and play distinct roles in the growth and morphogenesis of plant cells (Seagull, 1990; Staiger and Lloyd, 1991; Zhang *et al.*, 1990). During the interphase stage, microtubules lie parallel to and close to the plasma membrane and in this process, the tubes are known as *cortical microtubules*. It has been postulated that one central role of the cortical microtubule array is to regulate the material anisotropy of the cell wall by interacting with cellulose synthase (CESA) complexes to guide the orientation of cellulose microfibrils.

The cellulose microfibrils are a crucial load-bearing constituent of the cell wall. CESA complexes are molecular machines thought to be composed of 36 catalytic subunits that are arranged in symmetrical rosettes with diameters of 25–30 nm, as is observed in freeze-fractured plasma membranes and perform the role of controlling the orientation of cellulose microfibrils within the cell wall (Gunning, 1982; Staehelin *et al.*, 1991), (Figure 1.2). In pre-mitotic cells, microtubules are responsible for the formation of the *pre-prophase band*, which is predicted to be involved in the determination of the site and plane of formation of the cell plate (Gunning and Hardham, 1982; Wick, 1991). During the process of cell division, microtubules appear as *spindle apparatus* and *phragmoplasts* that play key roles in both karyokinesis and cytokinesis (Baskin and Cande, 1990; Gunning and Wick, 1985; Tiwari *et al.*, 1984).

The processes associated with the changes in the arrangement of microtubules and their possible functions have been well documented. On the other hand, the molecular mechanisms by which microtubules can assume such diverse orientations and participate in so many different functions in living plant cells remain unknown, thus making this study important as it would bring in some clarity through the functional characterization of its targeted AtMTA-AC denoted by the gene At3g02930.

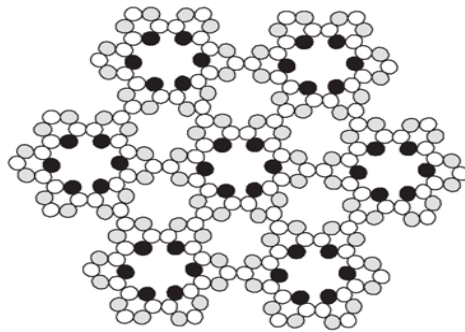


Figure 1.2: Schematic model structure of the cellulose synthase complex in higher plants (Ding and Himmel, 2006).

1.2.4 Cross-talking Systems Between Signalling Pathways

In nature, plants encounter an extensive range of environmental stresses during their typical life cycles and have evolved mechanisms to enhance their tolerance to these factors through both their physical adaptations such as closure of stomata during droughts and interactive molecular and cellular changes such as expression of genes that begin the onset of stress tolerance. The first step in switching on such molecular responses is to perceive the stress factor as it occurs and to relay information about it through a signal transduction pathway (Durrant and Dong, 2004).

The interaction of these signalling pathways via common components can cause the same response to different stimuli or alternatively different signalling pathways could interact and affect each other's outcome negatively or positively. This phenomenon is defined as cross-talking (Knight and Knight, 2001) and in simple terms, biochemical cross-talking can be defined as an interaction of two or more signalling pathways within a given organism (Klimecka and Muszynska, 2007).

Due to the increasing knowledge about plant signalling pathways from stimulus to end response, it has become more apparent that the linear pathways previously studied are actually part of a more complex signalling network with much overlaps between its branches (Taniguchi *et al.*, 2013) for instance, it has become clear that many genes are inducible by more than one stimulus. Whereas previously, stress signalling pathways were studied in isolation from other stresses, in reality, plants encounter stress combinations concurrently and must present an integrated response to them (Knight and Knight, 2001). Cross-talking is thus becoming increasingly significant following the advances in genome sequencing which allow bioinformatic identification and analysis of various plant genes and their protein products. It has also become clear that some plant proteins exist as multi-domain, multi-functional gene products, and this opens up numerous possibilities for some intra- and/or inter-molecular cross-talking. A concerted characterization of such protein candidates can thus aid in the better elucidation of plant signalling pathways, especially as they relate to stress response. An accumulation of knowledge on stress signal transduction would be of great essence for the continuous development of rational breeding and transgenic strategies aimed at improving stress tolerance in agricultural crop plants (Xiong and Zhu, 2002).

Ideally, responses of plants to biotic and abiotic stresses involve nearly every aspect of the plant physiology and metabolism. As such, there exists a complex signalling network underlying plant adaptation to adverse environmental conditions (Zhu, 2001). Some of these particular and poorly understood signal transduction pathways involve ACs, which act as second messengers to coordinate appropriate responses. Recently, a group of protein candidates from *A. thaliana* were bioinformatically identified as probable AC molecules (Gehring, 2010), and these include the protein molecule that was studied here in the study.

1.2.5 The Bioinformatic Identification of the AC Catalytic Centre of At3g02930

In 2010, Gehring made use of a 14-amino acid long GC catalytic centre search motif modified for specificity for ATP binding and with a C-terminal metal-binding residue ([RK][YFW][DE][VIL][FV]X(8)[KR]X(1,3)[DE]) to conduct a BLAST search of the Arabidopsis genome. This motif search retrieved over fourteen putative adenylate cyclase genes (Table 1.1), including a gene at the locus At3g02930 that codes for MAP (Figure 1.3). This gene was targeted mainly to characterize its probable AC activity and linking such function to critical cellular processes such as stress response and adaptation mechanisms.

Table 1.1: Some of bioinformatically identified *Arabidopsis thaliana* proteins containing the AC search motif: [RK][YFW][DE][VIL][FV]X(8)[KR]X(1,3)[DE] (Adapted from Gehring, 2010).

ATG No.	Sequence	Annotation
At1g25240	-KWEIFEDDFCFTCKDIKE-	Epsin N-terminal homology
At1g62590	-KFDVVISLGEKMQR--LE-	Pentatricopeptide (PPR) protein
At1g68110	-KWEIFEDDYRCFDR--KD-	Epsin N-terminal homology
At2g34780	-KFEIVRARNEELKK-EME-	Maternal Effect Embryo Arrest 22
At3g02930	-KFEVVEAGIEAVQR--KE-	Microtubule associated 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

The probable AC domain sequences as retrieved by the motif are shown for each of the listed putative AC proteins. The (ATG) accession number and probable AC domain sequence of the MTA is bolded and highlighted yellow.

```

001 MASKIKNGLSDTTLRKSSSTSLRVPRLTRIVTKPDSNSPSPPTQQQSRLSF
051 ERPSSNSKPSTDKRSPKAPTPEKTQIRAVRVSESQPSVQIKEDLKKAN
101 ELIASLENEKAKALDQLKEARKEAEEASEKLDEALEAQKKSLENFEIEKE
151 EVVEAGIEAVQRKEEELKKELENVKNQHASESATLLLVTQELENVNQELA
201 NAKDAKSKALCRADDASKMAAIHAEKVEILSSELTRLKALLDSTREKEII
251 SKNEIALKLGAEIVDLKRDLENARSLEAKVKELEMIIEQLNVDLEAAKMA
301 ESYAHGFADEWQNKAKELEKRLLEEANKLEKCAVSLVSVTKQLEVSNSRL
351 HDMESEITDLKEKIELLEMTVASQKVDLEKSEQKLGIAEEESSKSEKEAE
401 KLKNELETVNEEKTQALKKEQDATSSVQRLLEKKKILSELESSKEEEEK
451 SKKAMESLASALHEVSSSESRELKEKLLSRGDQNYETQIEDLKLVIKATNN
501 KYENMLDEARHEIDVLVNAVEQTKKQFESAMVDWEMREAGLVNHVKEFDE
551 EVSSMGKEMNRLGNLVKRTKEEADASWEKESQMRDCLKEVEDEVIYLQET
601 LREAKAETLKLKGKMLDKETEFQSIVHENDELRVKQDDSLKKIKELSELL
651 EEALAKKHIEENGELSESEKDYDLLPKVVEFSENGYRSAEEKSSKVETL
701 DGMNMKLEEDTEKKEKKERSPEDETVEVEFKMWESCQIEKKEVFHKESAK
751 EEEEDLNVVDQSQKTSPVNGLTGEDELLKEKEKKKKKTLFGKVGNNLLKKK
801 GPVNQK

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Figure 1.3: Amino acid sequence of the microtubule associated protein, encoded by the At3g02930 gene from *Arabidopsis thaliana*. The annotated AC catalytic center is highlighted in red and the priming sites flanking the targeted catalytic center are bolded and highlighted in green.

The genomic sequence and the presented encoded amino acid sequence of the At3g02930 gene were retrieved from The Arabidopsis Information Resource (TAIR) and Protein Expasy websites respectively (<http://www.arabidopsis.org>). This gene is located on chromosome 3 of the *A. thaliana* plant, where it spans about 3859 bps from 654,779 to 658,637 kb. The gene has three transcripts containing a total of 8 exons on the forward strand.

CHAPTER TWO

PRELIMINARY BIOINFORMATIC ANALYSIS OF THE AT3G02930 GENE

ABSTRACT

In order to elucidate the exact physiological and/or biological roles of the microtubule associated (MTA) protein encoded by the At3g02930 gene in *Arabidopsis thaliana*, a combination of various bioinformatic web-based servers and several computer programs such as TAIR, Genevestigator, PHYRE2, GeneMANIA, PSIPRED, NCBI, SUBA3, ARAMEMNON, KEGG, PSORT II, ATTED-II, ThaleMine, and STRING were used. The approach was used to assist us unearth the co-expressional, functional enrichment, stimulus specific and promoter analysis data of the At3g02930 gene that would be used to establish its exact functional roles in plants. According to the TAIR database, the At3g02930 gene encodes a MTA protein described as a weak chloroplast movement under blue light protein and has been implicated in the photosynthetic processes of the plant and reciprocal meiotic recombinations.

2.1 INTRODUCTION

The *Arabidopsis thaliana* genome was the first to be completely sequenced in the year 2000 (Initiative, 2000), thus making it the ‘model system’ in plant biology. Understanding its genome is imperative to the basis for understanding the biology of all plants, including the agronomically important crops such as maize and soya, which we highly depend on for our sustenance. Since the completion of the *A. thaliana* genome in the year 2000 and at the time the sequence was published, only 69% of the genes were assigned to functional categories by sequence similarity. These functional categories were produced automatically using sequence similarity to other proteins of ‘known’ function, with only 9% being characterized experimentally. Therefore, approximately 30% of the genome is still unclassified and has no-closely related sequences of known function.

In recent years, there has been more annotations that have been studied manually since the original annotation and proportion of genes still with no annotation at all has not changed (Lister *et al.*, 2008). Thus, the focus of this chapter was to discuss bioinformatic data available on several biological databases about the MTA protein encoded by the At3g02930 gene from *A. thaliana*. The amount of data available in plant biology has increased immensely, hence, the use of modern technologies is progressively ruling out the traditional way of researching, and in other words, the scientific focus is stirring away from the single-data-domain and problem-oriented methods towards work crossing the borders of data domains. Bioinformatic tools assist to analyse data at a large scale (Roos, 2001). In pursuit of mining data on the biological processes of the At3g02930 gene, a usual literature review of journals and/or text books will not

be sufficient in delivering the required comprehensive information that may provide an extensive representation of the gene and its proteins, thus necessitating the choice and employment of bioinformatic tools in our study.

The term bioinformatics is variously defined as, ‘the application of computational techniques to analyze the information associated with biomolecules on a large-scale’ (Luscombe *et al.*, 2001), and in short, bioinformatics ‘is a management information system for molecular biology and has many practical applications’ as defined by the Oxford dictionary (Dictionary, 2008). Thus, bioinformatics is a discipline that has firmly established itself in molecular biology, and therefore, it covers a wide range of subject areas, from structural biology, genomics, to gene expression studies. It is actually a valuable tool as it enhances the significance of each gene and its protein sequence by providing a wealth of information related to the role of the protein such as its structure, function, subcellular location, interactions with other proteins and domain composition, and in addition, it also provides a wide range of sequence features such as active sites and post-translational modifications (Leinonen *et al.*, 2010). In this regard, software architecture using bioinformatic tools criteria, allowed us to work easily with our gene of interest and multiple lists of other genes at the same time (Barrett *et al.*, 2004). In this study therefore, the At3g02930 gene or its amino acid sequence derived from the TAIR database search query was used in data mining to extract the physiological and/or biological roles of the MTA protein (Lamesch *et al.*, 2011).

2.2 Database Annotations of the At3g02930 Gene

Amino acid sequences of the MTA protein encoded by the At3g02930 gene in *A. thaliana* (AtMTA) were derived from the Arabidopsis Information Resource (TAIR) (Meinke *et al.*, 1998). Apparently, the Arabidopsis annotation was carried out by the Arabidopsis Genome Initiative (AGI) members after which it was continued by The Institute for Genomic Research (TIGR) in partnership with the Lens Fiber Major Intrinsic Protein (MIP). TAIR assumed the responsibility of updating the Arabidopsis annotation, wherein the annotation was done by manual curation from the research literature and direct submissions from the research community. A detailed description of the annotation process is described by Swarbreck *et al.* (2007).

According to the TAIR database, the locus name At3g02930 refers to a gene from the eudicot *A. thaliana* (L) Heynh, and the common name for *A. thaliana* is mouse-ear cress or the thale cress (Berardini *et al.*, 2004). Various annotations of the At3g02930 gene were bioinformatically listed on different databases: The At3g02930 gene is annotated as ‘Cellular Component-Chloroplast’ by the Uniprot database and its accession number: Q9M8T5. Further, the chloroplast is defined on Uniprot as the most common form of a plastid found in all photosynthetic eukaryotes. The wweak chloroplast movement under blue light (WEB) family protein database describes the At3g02930 gene as a ‘chloroplastic protein’, its UniProtKB/Swiss-Prot accession number being Q9M8T5.

Table 2.1: Various annotations of the At3g02930 gene as is bioinformatically listed on different databases.

Database	Locus	Annotation
Ensembl Genomes	At3g02930	Chloroplastic
Expression Atlas	At3g02930	Chloroplastic
NASC Gene ID	At3g02930	Plant protein of unknown function (DUF827)
UniGene	At.53163	Hypothetical protein

2.3 Locus Detail of the At3g02930 Gene

This gene is located on chromosome 3, where it spans over 3859 bps from 654,779 kb to 658,637 kb and it has three transcripts, containing a total of 8 exons on the forward strand as is shown in Figure 2.1 below. Chromosome 3 is described as sub-metacentric and represents about 20% of the Arabidopsis genome. This chromosome 3 has been estimated, using the yeast artificial chromosome (YAC)-based physical MTAs, to be 21-23 MB in size (excluding the centromeric and telomeric regions) (Nacry *et al.*, 1998). The same chromosome 3 also harbours features such as the roughly 5-kb chloroplast DNA insertion, the complete rDNA repeat unit and the telomeric repeat - all in the genetically defined centromere. Even though the biological analysis of the chromosome 3 genes has to be placed in the context of the whole genome, chromosome 3 contains a number of homologues to human disease genes, and the analysis of homologues of human genes can perhaps provide some clues about the function of these genes in plants.

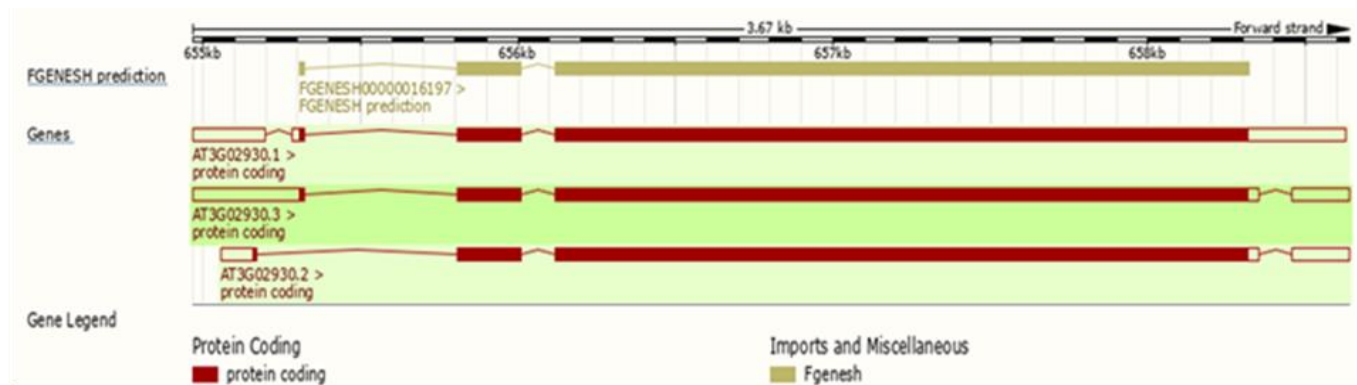


Figure 2.1: Gene model of the At3g02930 gene as is presented by the Ensembl Arabidopsis Plant version 93.11-(TAIR10). The At3g02930 gene is located on chromosome 3, where it spans over 3859 bps from 654,779 kb to 658,637 kb, with three protein coding transcripts; At3g02930.1, At3g02930.2, At3g02930.3 and containing 8 exons on the forward script.

2.4 Gene Expression Profile of Microtubule Associated Proteins

Several studies have been conducted in different known MTA families regarding gene expression. In a study conducted by Smertenko (2008), an MTA65 gene in *A. thaliana* (AtMTA65-1) is ubiquitously expressed both during the cell cycle and in all plant organs and tissues with the exception of anthers and petals. Furthermore, analysis done by Pesquet *et al.* (2011) on a number of MTA gene expression profiling, identified a solitary AtMTA70-5 that was both up-regulated and being specific to the treachery element (TE) differentiation. Another member of the AtMTA70 family, AtMTA70-1, is constitutively expressed in un-induced cells, co-localizing with AtMTA70-5 in cells and binding to it *in vitro*. With regard to the AtMTA gene, baseline experiments conducted by Liu *et al.* (2012) showed that the At3g02930 gene has the highest expression levels in flowers at 28 TPMs and in fruits at 17 TPMs, while the lowest expression levels are found in the leaves at 4 TPMs and the roots at 8 TPMs (Liu *et al.*, 2012).

According to TAIR, the At3g02930 gene is expressed in the growth and developmental stages of the plant, whereby it is expressed during the LP.10 ten 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 the vascular leaf senescent stage. In the plant structure, the At3g02930 gene is expressed in 20 plant structures: the carpel, cauline leaf, collective leaf structure, flower, flower pedicel, guard cell, hypocotyl, inflorescence meristem, leaf apex, petal, petiole, plant egg cell, plant embryo, pollen, root, seed, sepal, shoot apex, shoot system, stamen, and the vascular leaf (Figure 2.2). Data from Genevestigator also shows expression of the At3g02930 gene in 10 different developmental stages of the Arabidopsis plant. Expression is highest during 32-leaf rosette stage and lowest during the time when the pod sheds off its fully developed seeds (Hruz *et al.*, 2008).

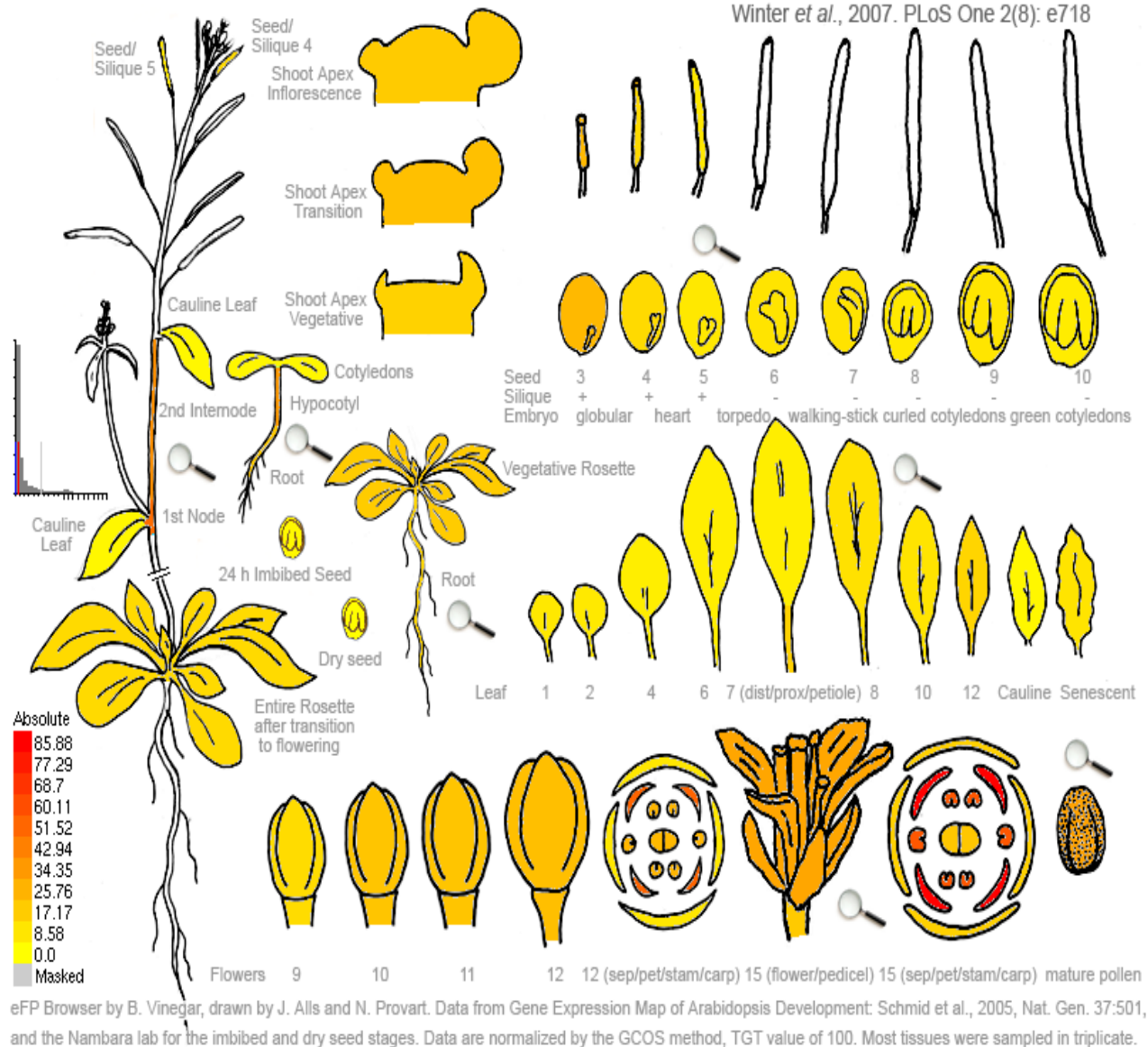


Figure 2.2: The developmental expression profile of the *At3g02930* gene in *A. thaliana* adapted from Winter et al., 2007. The above data shows the expression profile of the *At3g02930* gene at various stages of development beginning from the radicle emerging from the seed coat up to the release of the mature seeds from the pod.

2.5 Subcellular Localization and Function of the At3g02930 Gene

According to UniProt database, the At3g02930 gene belongs to the wweak chloroplast movement under blue light (WEB) family, where its expression product is described as a “chloroplastic protein”. The subcellular location of At3g02930 gene is in the chloroplast, which may suggest that the gene may be involved in the photosynthetic processes of the plant (Ahlerl *et al.*, 2006), (Figure 2.3). Chloroplasts together with mitochondria are important organelles that are a part of the eukaryotic cell. Chloroplasts belong to the organelle group called plastids and are found in land plants, algae and some protists. These chloroplasts are specialized plastids that are responsible for photosynthesis (Cavalier-Smith, 2000).

Chloroplasts move towards weak light, which is known as the accumulation response and the opposite movement away from strong light, which is known as the avoidance response. The quick and accurate movement of chloroplasts in reaction to ambient light conditions is important for efficient photosynthesis and photodamage prevention in the organelle. Chloroplasts are extremely polyploid organelles which means that they contain more than two homologous sets of chromosomes, they comprise circular DNA molecules of 85 to 200 kb organized into discrete membrane-associated nucleoids (Abah *et al.*, 2010; Newman *et al.*, 1991).

In this section we focused on three land plant chloroplast genomes that have been completely sequenced: The dicotyledon tobacco genome from the *Nicotiana tabacum* plant, which is estimated to be 156 kb (Shinozaki *et al.*, 1986), the monocotyledon rice genome from the *Oryza sativa*, which is 135 kb (Hiratsuka *et al.*, 1989) and a liverwort genome from *Marchantia polymorpha*, which is 121 kb (Ohshima *et al.*, 1988), and each of these genomes containing 110 to 120 genes (Sugiura, 1992). These three land plant sequences, in addition to MTAs and partial

sequences from many other species, show that the basic chloroplast genome structure and overall gene order in land plants are highly conserved.

As a result, in this section we looked at the WEB family protein wherein the At3g02930 and compared it with other proteins within the WEB family which include the weak chloroplast movement under blue light 1 (WEB1) (At2g26570) and the plastid movement impaired (PMI15) (At5g38150) proteins from *A. thaliana*. Both the WEB1 and PMI15 are essential for the chloroplast's avoidance response under high intensity blue light. This avoidance response involves the relocation of chloroplasts on the anticlinal side of the exposed cells. WEB1 acts in association with PMI2 to maintain the velocity of the chloroplast photorelocation movement via the cp-actin filaments regulation (Kodama *et al.*, 2010), thus, this affirms that the At3g02930 protein could be implicated in the photosynthetic processes of the plant.

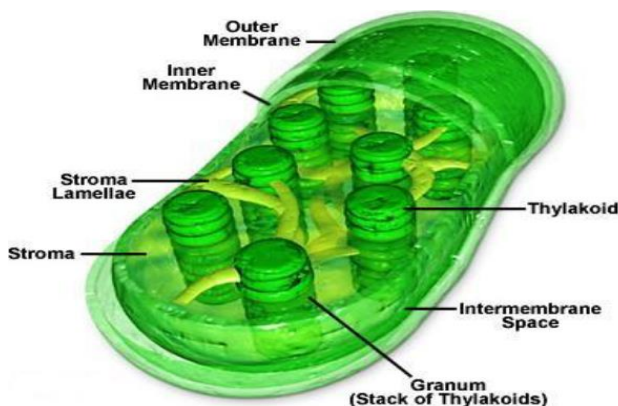


Figure 2.3: Structure of plant cell chloroplast, illustration taken from <http://micro.magnet.fsu.edu>.

The At3g02930 gene is sub-cellularly located in the chloroplast and this perhaps affirming that its expressed protein could be implicated in the photosynthetic processes of the plant that occur within the thylakoids membrane.

2.6 Protein Structure Model Information of the At3g02930 Gene

The protein structure model depicted in Figure 2.4 below indicates the targeted range of the At3g02930 protein from 276 to 473 which is based on template: 3na7A. The sequence identity is at 34%, which points to the expected accuracy of a model between target proteins and template structures, as is confirmed by various studies (Baker and Sali, 2001; Chothia and Lesk, 1986; Koh *et al.*, 2008; Rost, 1999). The resolution of the model is at 2.20 (X-RAY) and the model is a 2D image generated by the ModBase: Database of Comparative Protein Structure Models. Understanding of the predicted accuracy of a protein structure model is of vital importance. The importance of quality estimation in modeling has been emphasized strongly in the literature (Hooft *et al.*, 1996; Schwede *et al.*, 2009). The estimation of the accuracy of homology models is based on two main sources: firstly, the availability of structural information, which is predominantly determined by the evolutionary distance between the At3g02930 protein and template proteins of known structure. This is based on the observation that there is a direct correlation between sequence identity of a pair of proteins and the structural similarity of their common core (Chothia and Lesk, 1986; Rost, 1999). Secondly, estimation of model accuracy is determined from the analysis of the geometry of the model, this is important especially when the sequence identity is low. Individual models may vary considerably from the expected average quality due to various sources of errors in modeling and inaccuracies introduced by the modeling programs.

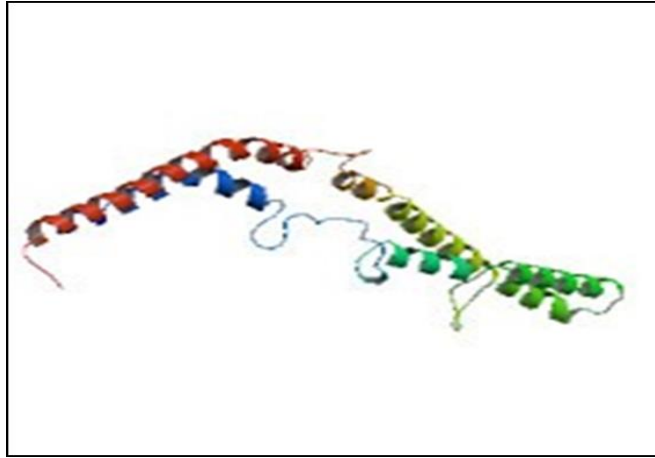


Figure 2.4: 3D model image of the At3g02930 protein, generated by the ModBase: Database of Comparative Protein Structure Models. The targeted range of the At3g02930 protein is from 276 to 473 as is depicted by the template: 3na7A. The resolution of the model is set at 2.20 (X-RAY).

2.7 Protein Orthologues, Homology and Gene Clustering of the At3g02930 Gene

Gene orthologues are genes related by vertical descent from a common ancestor and encode proteins with similar functions from different species. Orthologous genes are homologous genes that diverged after evolution to give rise to different species, an event known as speciation. The genes mostly maintain a similar function to that of the ancestral gene that they evolved from. In this type of homologous gene, the ancestral gene and its function was maintained through a speciation event, though variations may arise within the gene after the point in which the species diverged.

Orthologues are derived from gene trees and are determined using all species in a particular database. In this section, we used the Ensembl plant database. According to the Ensembl plant database, the At3g02930 gene has 137 orthologues, this was generated by databases where the maximum likelihood phylogenetic gene trees play a significant role (Aken *et al.*, 2016). The

evolutionary history of At3g02930 gene families is shown in Figure 2.5, where the genes that are diverged from a common ancestor are presented. These gene trees, reconciled with their species tree, have their internal nodes annotated to distinguish the duplication or speciation events (Vilella *et al.*, 2009).

In the Figure 2.5 below, red squares represent duplication nodes, blue squares represent speciation nodes, giving rise to paralogues and orthologues. Another class of nodes; ambiguous, is shown as a lighter blue square and the gene of interest is highlighted in red. The multiple alignment of the peptides, which are represented by the (green bars) was made using the Ensembl plant database, whereby the green bars show areas of amino acid alignment, while the white areas are gaps in the alignment and the dark green bars indicate consensus alignments.

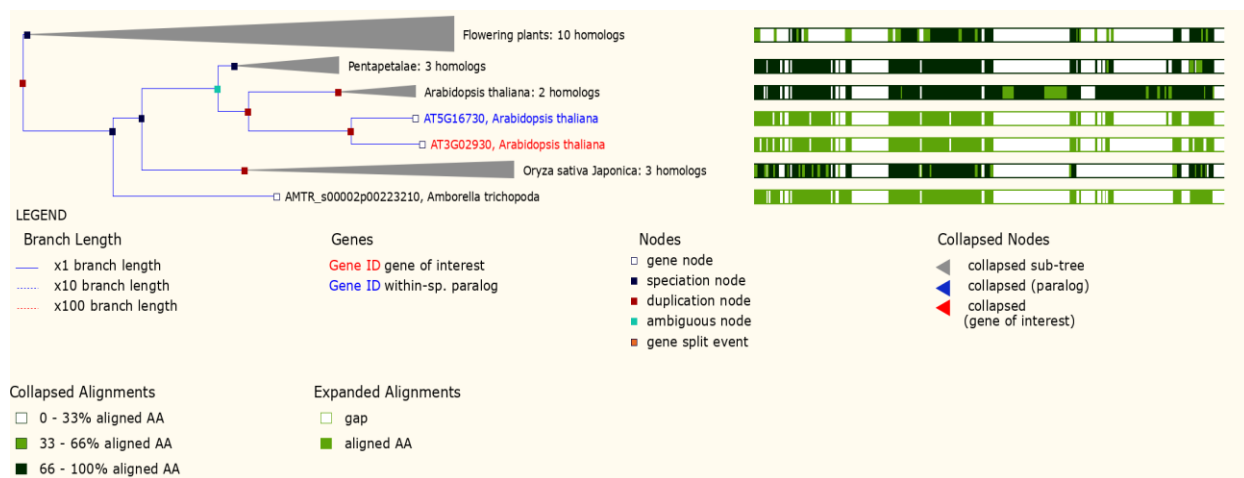


Figure 2.5: Evolutionary history of the At3g02930 gene families as adapted from the Ensembl plant database. The above gene tree shows the At3g02930 gene highlighted in red and the At5g16730 highlighted in blue as well as other genes that have diverged from a common ancestor. Red squares represent duplications nodes and blue squares represent speciation nodes - giving rise to paralogues and orthologues respectively.

Table 2.2: A Summary of orthologues of the At3g02930 gene displaying details for one or more groups of the species.

Species	Target %id	Query %id	GOC Score	WGA Coverage	High Confidence
<i>Amborella trichopoda</i>	37.42 %	38.59 %	n/a	n/a	Yes
<i>Oryza sativa</i> Japonica Group	31.02 %	27.79%	n/a	n/a	Yes
<i>Oryza sativa</i> Japonica Group	31.89 %	30.15 %	n/a	n/a	Yes
<i>Oryza sativa</i> Japonica Group	23.61 %	27.95 %	n/a	n/a	No
<i>Solanum lycopersicum</i>	44.07 %	52.98 %	n/a	n/a	No
<i>Solanum lycopersicum</i>	46.49 %	46.77 %	n/a	n/a	No
<i>Vitis vinifera</i>	50.95 %	53.47 %	n/a	n/a	No

In the Table 2.2 above; Target %id refers to the percentage of the orthologous sequence matching the *A. thaliana* sequence, while the Query %id refers to the percentage of the *A. thaliana* sequence matching the orthologous sequence. GOC score is the Gene Order Conservation (values 0 to 100), the WGA Coverage is the Whole Genome Alignment coverage (values 0 to 100) and lastly, the High Confidence, which refers to homology with high percentage identity and high GOC score or WGA coverage (as available yes or no). The *A. trichopoda* species and the *O. sativa* Japonica Group show a positive (yes) High Confidence, which indicates that both the *A. trichopoda* species and *O. sativa* Japonica Group have homology with high percentage identities. The same then implies that our gene of interest, the At3g02930 gene from *A. thaliana* is closely related and share a common ancestor with both the *A. trichopoda* species and the *O. sativa* Japonica Group.

The At3g02930 gene belongs to the PANTHER Subfamily; Crossover Suppressor on 3 of GOWEN (pthr23160:sf1), which is a myosin heavy chain-related family. Typically, there are four *A. thaliana* genes assigned to this subfamily. Firstly, the At5g16730 gene implicated in the

reciprocal meiotic recombination - this gene is located in the nucleus, microtubule associated complex and chloroplast. Secondly, the At4g27595 gene, which is also implicated in the reciprocal meiotic recombination - this is located in the endoplasmic reticulum and also in the microtubule associated complex and chloroplast. Thirdly, the At1g65010 gene, implicated in flower development and reciprocal meiotic recombination - this is located in the nucleus and the microtubule associated complex. Lastly, the At3g02930, which is our own gene of interest that is also implicated in reciprocal meiotic recombination and is located in essential cellular organelles such as the chloroplast, microtubule associated complex and endoplasmic reticulum (Mi *et al.*, 2016).

Table 2.3: PANTHER Plant Homologs showing genes that have common ancestry with the At3g02930 gene.

Organism	UniProt Accession	Gene
<i>Arabidopsis thaliana</i> (Thale cress)	F4I8B9	At1G65010.1
<i>Arabidopsis thaliana</i> (Thale cress)	F4JJP1	At4G27595.1
<i>Arabidopsis thaliana</i> (Thale cress)	Q9LFE4	At5G16730.1
<i>Glycine max</i> (Soybean)	I1JAN2	GLYMA01G44390
<i>Glycine max</i> (Soybean)	I1LFX6	GLYMA11G01130
<i>Glycine max</i> (Soybean)	I1LJ28	GLYMA11G11580
<i>Glycine max</i> (Soybean)	I1LP66	GLYMA12G01950
<i>Oryza sativa</i> (Rice)	Q6Z7G3	OJ1570_H12.23
<i>Populus trichocarpa</i> (Black cottonwood)	B9I744	POPTR_0013S07650G
<i>Populus trichocarpa</i> (Black cottonwood)	B9IPH4	POPTR_0019S07200G
<i>Solanum lycopersicum</i> (Tomato)	K4BJD6	SOLYC03G097190.2
<i>Solanum lycopersicum</i> (Tomato)	K4C8Z3	SOLYC06G072730.2
<i>Sorghum bicolor</i> (Sorghum)	C5Y0R4	SB04G031190
<i>Vitis vinifera</i> (Grape)	F6HEE0	VIT_16S0039G01650

The GeneMANIA program shows that the At3g02930 protein resides within a cluster of *A. thaliana* proteins with shared domains, Figure 2.6 below. According to this program, the At3g02930 gene cluster shows 20 related genes, with 21 total genes, 0 attributes and 56 total links (Warde-Farley *et al.*, 2010). These *A. thaliana* cluster networking has 66.87% physical interactions, predicted at 14.02%; co-expression at 12.73%; shared protein domains at 3.00%; genetic interactions at 2.87%; and lastly, co-localization at 0.52%

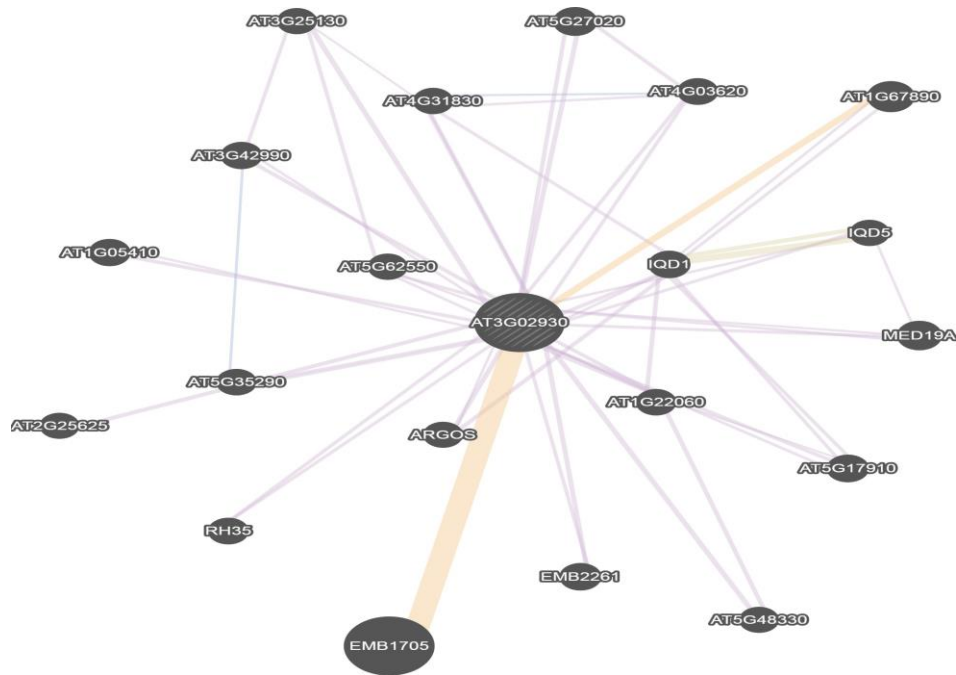


Figure 2.6: A cluster of proteins with domains shared with the At3g02930 (Warde-Farley *et al.*, 2010). The At3g02930 gene cluster showing 20 related genes residing within a cluster of *A. thaliana* proteins with shared domains.

2.8 CONCLUSION

In this chapter, we employed a number of bioinformatics web-based servers and a variety of computer programs to elucidate the exact physiological and/or biological roles of the AtMTA protein encoded by the At3g02930 gene. This protein is a multi-domain, multi-functional molecule. Thus far, there is no experimental evidence to show that this protein product is possibly translated *in vivo*. Furthermore, there is also no experimental evidence for any physical and/or genetic interactions in which the At3g02930 gene is involved. Nevertheless, the gene has been implicated in the photosynthetic processes of the plant and reciprocal meiotic recombination.

CHAPTER THREE

RECOMBINANT EXPRESSION OF THE MICROTUBULE ASSOCIATED PROTEIN AND DETERMINATION OF ITS INHERENT ENDOGENOUS ADENYLATE CYCLASE ACTIVITY

ABSTRACT

Microtubules (MTs) are component of the cytoskeleton, which plays a major role in plant life cycles, including organelle movement, cell division, cell expansion, and organ morphology. Plant MTs may be structurally similar to their eukaryotic counterparts but differ in their organization and dynamics. Microtubule associated proteins (MTAs) interact with MTs to regulate dynamics, stability, and organization of the cell. Expression and characterization of the full set of MTAs in plants would significantly increase our understanding of how various MT arrays are formed and function. In line with this specific notion, our study herein focused on a recently bioinformatically annotated MTA protein encoded by the At3g02930 gene from *Arabidopsis thaliana* (AtMTA), as a putative higher plant adenylate cyclase (AC). However, even though the At3g02930 gene has been bioinformatically annotated as a probable AC molecule, no study to date has ever confirmed its ability to functionally generate cAMP from ATP. In this study therefore, we cloned and partially expressed the AC-containing fragment domain of this annotated gene molecule (AtMTA-AC) in chemically competent *Escherichia*

E. coli EXPRESS BL21 (DE3) pLysS cells and demonstrated its ability to induce the generation of cAMP endogenously.

3.1 INTRODUCTION

Arabidopsis thaliana has become a plant of choice for plant scientists since the mid-1980s due to its many advantages, including the availability of its whole nuclear genome sequence, molecular genetic markers and large collections of sequence-indexed DNA-insertion mutants (Koncz *et al.*, 1992). Consequently, the scientific community has taken keen interest in the investigation of the biological processes of this plant and the molecular characterization of its genes has been of great importance (Meyerowitz and Somerville, 1994). An in-depth analysis of molecular gene function plays a crucial role in understanding physiological processes in living systems (Demain and Vaishnav, 2009), hence the primary goal to recombinant protein production is always to acquire the gene of interest. In this study therefore, we detail the isolation and molecular cloning of the At3g02930 gene fragment harbouring the AC catalytic motif followed by demonstration of the ability of this truncated molecule to induce the generation of endogenous cAMP in a recombinant prokaryotic system.

Cloning was done using a pTrcHis-TOPO expression vector to generate a construct, which was used to transform *E. coli* EXPRESS BL21 (DE3) pLysS cell system. After that, protein expression was then induced using 1 mM IPTG followed by protein characterization using the cAMP-linked enzyme immunoassaying technique (Figure 3.1). At the theoretical level, the steps required for obtaining a recombinant protein are pretty clear-cut however; yet a number of things can go wrong (Makrides, 1996). In this study, the T/A cloning strategy was used, that is the

cloning of PCR products generated by *Taq* DNA polymerase into vectors already having at their ends 3' overhangs comprised of single T's. *Taq* DNA polymerase can catalyze the non-templated addition of a nucleotide (principally adenosine) to the 3' end of PCR-amplified products. This cloning method has been widely used but has proven problematic with regards to the ligation process, where some products fail to be ligated into the vector, as a result, in part, from incomplete T-tailing.

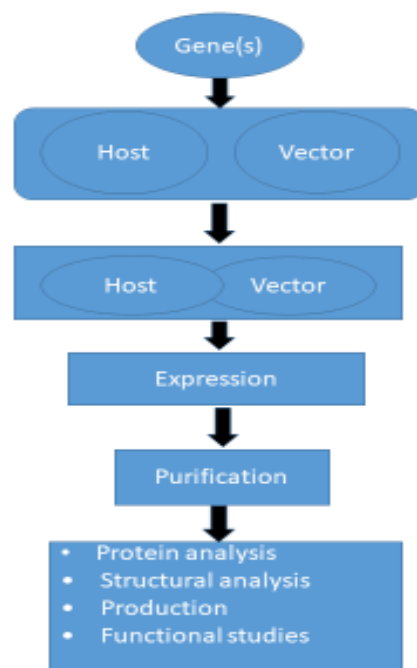


Figure 3.1: Flow chart for the general heterologous expression of recombinant proteins (adopted from Yesilirmak and Sayers (2009))

The challenge of poor cloning efficiency can be resolved through modifications in PCR primer design because PCR products that resist the non-templated addition of nucleotides (adenosine), ligate poorly into the T-tailed vectors (Yin *et al.*, 2007). Brownstein and co-workers (1996) showed that modifying reverse and/or forward primers should result in improved efficient cloning. Likewise, a vector that is completely T-tailed might be produced by linearizing a

plasmid to yield blunt ends with 5' sequences such as GTTTCT. However, there is no suitable restriction enzyme that can be used to make such a product (Brownstein *et al.*, 1996). Therefore, it is recommended to synthesize an oligonucleotide containing the palindromic sequence AAGACAC/GT**G**TCTT and inserting it into the plasmid of one's choice. Substituting the G in bold font for a T causes little or no deterioration in the performance of the resulting tail. Furthermore, Brownstein *et al.* (1996), reported that the non-templated nucleotide addition adjacent to a 3' terminal C is favoured and that addition adjacent to a 3' terminal A is not, and thus the placing of the sequence GTTTCTT on the 5' end of reverse primers resulted in nearly 100% adenylation of the 3' end of the forward strand. This modification or related ones (called "PIGtailing") should facilitate accurate genotyping and efficient cloning in the T/A systems.

A few years ago, our ability to clone and express foreign genes in heterologous host systems, has introduced a remarkable capability to make nearly any protein in abundant quantities to be used in research projects and/or diagnostic agents. However, although heterologous systems are involved in the identification of genes and transfer of corresponding DNA fragments to hosts other than their own original sources for synthesis of the encoded protein, the choice of the expression system and vector is of significant importance as a number of factors have to be taken into account. For instance, selection of an expression system depends on whether the purpose of the study is to produce large quantities of a protein or investigating functional features of the cloned protein, of which the physicochemical properties of the investigated protein may also play a role in such choices (Yin and Liebscher, 2007).

The most primitive description and laboratory isolation of the bacterium now identified as *Escherichia coli*, were made in 1885 by Theodor Escherich. *E. coli* is the most popular and widely used prokaryotic expression system for heterologous protein production (Frommer and

Ninnemann, 1995). Firstly, due to its simplicity, fast growth rate and reasonably low cost, it remains by and large, the first choice expression host. Secondly, nearly all commercially obtainable inducible cloning vectors are compatible with *E. coli* and its widespread biochemical and genetic information is readily available (Sezonov *et al.*, 2007). However, there are disadvantages of using *E. coli* as an expression host, arising from its inability to perform post-translational modifications, which are often required for the correct folding and functional activity of the recombinant protein (Lueking *et al.*, 2000).

The BL21 and BL21 (DE3) strain of *E. coli* have been used throughout many decades and their history has been well documented (Studier and Moffatt, 1986). A couple of genetic characteristics of BL21 are worthy of mentioning; BL21 cells are deficient in the Lon protease, which degrades various foreign proteins (Gottesman, 1996). In addition, the protease OmpT gene coding for the outer membrane is missing from the genome of the ancestors of BL21, its function is to degrade extra cellular proteins. Furthermore, the *hsdSB* mutations that are present in the parental strain (B834) which give rise to the BL21 strain, prevent the loss of plasmids. As a result, DNA methylation and degradation is disrupted. When the gene of interest is placed under a T7 promoter, then T7 RNAP should be provided. In the popular BL21 (DE3) strain, the λ DE3 pro-phage was inserted in the chromosome of BL21 and contains the T7RNAP gene under the *lacUV5* promoter.

3.2 RESEARCH METHODOLOGY

3.2.1 Isolation and Molecular Cloning of the AtMTA-AC Gene Fragment into a Stable Heterologous Prokaryotic Expression System

3.2.1.1 Sterilization and Vernalization of Plant Seeds

About 1 mg (100 seeds) of *A. thaliana* ecotype Columbia seeds were collected in a sterile 1.5 ml Eppendorf tube and washed three times with 500 µl of 70% (v/v) ethanol, then vortexed for 30 seconds using a VX-200 Vortex Mixer (Labnet International Inc., New Jersey, USA). The supernatant was discarded before the seeds were similarly washed further with 500 µl of the seed sterilization buffer (0.1% (w/v) sodium dodecyl sulphate (SDS), 5% (v/v) commercial bleach). This was followed by successive rinsing of the seeds (five times) with 500 µl of the filter-sterilized deionized water before being submerged in 500 µl of the sterile water and then vernalized at 4°C for three days. The vernalization process ensured uniform germination of the seeds according to the principle of Evans and Lack (2001).

3.2.1.2 Plant Re-Generation and Growth Conditions

A. thaliana seeds were planted in Murashige and Skoog media (0.43% MS media, 0.30% sucrose and 0.8% agar, pH at 5.7) using sterile petri dishes. The planted seeds were then allowed to germinate and grow for two weeks in a GC-300TL growth chamber (Lab Companion, Jeio Tech, Seoul, Korea), under greenhouse conditions adjusted to 23/16 day/night for periods of 8/16 hours night/day at 10 000 light lux. After two weeks, the seedlings were transplanted into potting soil containing peat based soil, humus and vermiculite at a ratio of 1:1:1 respectively, watered with sterile distilled water, covered with plastic cling wrap to retain moisture, and then kept in the

growth chamber to grow for a further 2-4 weeks. The annotated At3g02930 gene was then later isolated from these 4-6 week old Arabidopsis plants.

3.2.1.3 Designing and Acquisition of Sequence-specific Primers

The genomic sequence of the At3g02930 gene molecule encoding the putative AtMTA-AC fragment was retrieved from The Arabidopsis Information Resource (TAIR) (<http://www.arabidopsis.org>). Sequence-specific primers (forward and reverse) were then created manually as guided by information in Figure 1.3, and then sent for chemical synthesis and subsequent supply by Inqaba Biotechnological Sciences, Pretoria, South Africa.

3.2.1.4 Extraction of Total RNA from Arabidopsis Plants

Total RNA was obtained by firstly harvesting about 1-2 g of plant leaf material from the 4-6 week old Arabidopsis plants followed by chemically extraction using the Gene Jet Plant RNA Purification Mini Kit, and according to the manufacturer's protocol (Catalogue # K0801; Thermo Scientific Inc., Massachusetts, USA).

Briefly, about 100 mg of freshly harvested leaf material were flash-frozen in liquid nitrogen (Afrox Industrial Gases, Ventersdorp, South Africa) (to protect the RNA in the leaf tissues from degrading), followed by grounding into a fine powder using sterile chilled pestle and mortar. The ground tissue was transferred/decanted into 500 µl of Plant Lysis solution in a 1.5 ml Eppendorf tube and vortexed at high speed for 20 seconds. The mixture was then incubated at 56°C for about 3 minutes and centrifuged at 20,000g (Corning LSE High Speed Microcentrifuge,

Corning Inc., Amsterdam, Netherlands). The lysate/supernatant was transferred into a clean 1.5 ml Eppendorf tube and mixed with 250 µl of 96% (v/v) ethanol by pipetting. The lysate was transferred to a spin column placed in a 2 ml collection tube and centrifuged at 12,000*g* for 1 minute. The flow through was discarded while the spin column was washed three times, firstly by adding 700 µl of Wash buffer 1 and centrifuging at 12,000*g* for 1 minute and the flow through discarded. Secondly, 500 µl of Wash buffer 2 was added to the spin column followed by centrifugation for 1 minute at 12,000*g*, after which the flow-through was discarded and the wash step repeated one more time. Finally, to ensure that no ethanol is carried over during the elution process, the spin column was centrifuging at 12,000*g* for 5 minutes. After this, the spin column was then placed in a new RNase-free 1,5 ml collection tube and 50 µl of RNase-free water added directly onto the spin column membrane to elute the total RNA through centrifugation at 12,000*g* for 1 minute.

The eluted RNA was quantified using a Nanodrop spectrophotometer (Nanodrop 2000, Thermo Scientific, USA) and resolves on a 0.8% w/v agarose gel electrophoretic system (MultiSUB Mini BCMSMINI7, Biocom Ltd, Bridge of Weir, United Kingdom) for 50 minutes at 80 V to confirm its quality and integrity. The resolved RNA was visualized under ultraviolet (UV) illumination using a 2000 UV Trans- illuminator (Bio-Rad Laboratories, California USA) and images captured by the Chemi-Doc Imaging System (Bio-Rad Laboratories Inc., California, USA).

3.2.1.5 Isolation of the AtMTA-AC Gene Fragment

The total RNA extracted from the plant leaves was used as a template to generate copy DNA (cDNA). Together with the generated cDNA, the acquired sequence-specific primers were used to amplify the targeted AtMTA-AC gene fragment in a reverse transcription polymerase chain reaction (RT-PCR) system using the Verso 1-Step RT-PCR Reedy Mix RT-PCR kit and as instructed by the manufacturer (Catalogue # AB-1454/LD/A; Thermo Scientific Inc., California, USA). Amplification of the targeted AtMTA-AC gene fragment was carried out on a C1000 Thermocycling system (Bio-Rad laboratories Inc., California, USA). The used reaction mixtures and their associated cycling conditions are shown in Tables 3.1 and 3.2 below.

Table 3.1: Components of the RT-PCR reaction mixture in a final volume of 50 µl used for amplification of the targeted AtMTA-AC gene fragment.

Composition	Volume (µl)	Final Concentration
Verso Enzyme Mix	1.0	
1-Step PCR Master Mix (10X)	25.0	1X
Forward Primer (10 µM)	1.0	200 nM
Reverse Primer (10 µM)	1.0	200 nM
RT Enhancer	2.5	
Water (PCR Grade)	19.5	
Template (RNA)	1.0	1 ng
Total Volume	50.0	

Table 3.2: The 1-step RT-PCR thermal cycling program used for amplification of the targeted AtMTA-AC gene fragment.

Step	Temperature (°C)	Time (mins)	Cycles
cDNA Synthesis	50	15.00	1
Thermo-start Activation	95	2.00	1
Denaturing	95	0.33	45 
Annealing	60	0.50	
Extension	72	1.00	
Final Extension	72	5.00	1

3.2.1.6 Agarose Gel Electrophoresis of the Amplified AtMTA-AC Gene Fragment

The amplified RT-PCR product was resolved on a 1% agarose gel supplemented with 0.5 µg/ml ethidium bromide. All samples were resolved against a 100 bp Gene-Ruler DNA ladder in 1X TBE buffer at 80 volts and 250 mA current for 50 minutes. The gel was then visualized under UV light using the 2000 UV Trans-illuminator system (Bio-Rad Laboratories Inc., California, USA). Images were finally captured using a Chemi-Doc Imaging System (Bio-Rad Laboratories Inc., California, USA).

3.2.2 Cloning of the Amplified AtMTA-AC Gene Fragment

3.2.2.1 Addition of 3'-adenines Overhangs

A total volume of 1 µl of the 5U *Taq* polymerase solution was added to 40 µl to the RT-PCR product and the mixture incubated at 72°C for 10 minutes on a C1000 Thermocycler system (Bio-Rad Laboratories Inc., California, USA). The resultant reaction mixture was then kept on ice for further use.

3.2.2.2 Ligation of the Adenylated AtMTA-AC Gene Fragment Insert into the pTrcHis-TOPO Expression Vector

An aliquot (4 µl) of the adenylated PCR product was collected and transferred into a fresh PCR tube before 1 µl of the pTrcHis-TOPO expression vector (Invitrogen, Carlsbad, USA) was added. The ligation mixture was incubated at room temperature for 5 minutes before its subsequent use for the transformation process of competent propagation *E. coli* cells.

3.2.2.3 Transformation of the Competent One Shot *E. coli* Cells with the pTrcHis-TOPO: AtMTA-AC Gene Fragment Construct

Immediately after the ligation process, about 2 µl of the ligation mixture (pTrcHis-TOPO:AtMTA-AC) fusion construct was added into an ice-cold Eppendorf tube containing 40 µl of the One Shot chemically competent *E. coli* cells (Invitrogen Corp., New York, USA). The reaction mixture was gently mixed and incubated on ice for 30 minutes. The reaction mixture was then heat-shocked on a dry-bath heating block at 42°C for 30 seconds (Labnet International Inc., New Jersey, USA) before being immediately incubated on ice for 5 minutes. Subsequently, the reaction mixture was supplemented with 250 µl of SOC medium (2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM Mg₂SO₄ and 20 mM glucose) and incubated in a shaking incubator at 200 rpm at 37°C for 30 minutes (SI-600 Bench-top Shaking Incubator, Lab companion GMI Inc., Minnesota, USA). This incubation step is always carried out so as to allow cells to produce the enzyme β-lactamase, which later and during the screening process, detoxify ampicillin. The mixture was then plated (80 µl and 20 µl)

onto two Luria Bertani (LB) agar plates (1% (w/v) agar, 1% (w/v) tryptone powder, 0.5% (w/v) yeast extract, 0.5% w/v NaCl) supplemented with 50 µg/ml ampicillin and 0.5% glucose. The plates were incubated at 37°C overnight.

3.2.2.4 Extraction of the pTrcHis-TOPO:AtMTA-AC Fusion Construct from the Transformed One Shot Chemically Competent *E. coli* Cells

Single colonies from the plated LB plates were used to prepare overnight cultures, whereby a colony of cells was picked and used to inoculate 10 ml of fresh double strength yeast-tryptone (2YT) media (0.8% (w/v) tryptone powder, 0.5% (w/v) yeast extract and 0.25% (w/v) NaCl) supplemented with 50 µg/ml ampicillin and 0.5% glucose in 50 ml falcon tubes. The tubes were incubated overnight at 37°C while shaking at 200 rpm. The next morning, cells were harvested by centrifuging at 800g using a TD3 800B Low Speed Table Top Centrifuge (Xilangyi Laboratory Instrument Development Ltd., Hunan, China) for 5 minutes at room temperature and the supernatant discarded. Plasmid extraction was then performed at room temperature using the Gene Jet Plasmid Miniprep Kit (Thermo Fisher Scientific Inc., California, USA).

Briefly, the pelleted cells were resuspended into 250 µl of the resuspension solution (supplemented with RNase) by pipetting up and down or until no cell clumps remained, and the mixture then transferred into a sterile Eppendorf tube. This was followed by the addition of 250 µl of lysis solution and mixing thoroughly by inverting the tube 4-6 times or until the solution was viscous and slightly clear. Thereafter, 350 µl of the neutralization solution was added and mixed immediately and thoroughly by inverting the tube 4-6 times. The cells were then pelleted by centrifuging for 5 minutes at 16 300g and the supernatant transferred into the supplied

GeneJET spin column. The spin column, fitted into a collection tube, was centrifuged for 1 minute and its flow-through discarded while the column was re-placed into the same collection tube. About 500 µl of wash solution (diluted with ethanol prior to first use) was added to the GeneJET spin column and the column further centrifuged for 30-60 seconds. The flow-through was discarded and the column was placed back into the same collection tube. The washing procedure was repeated twice before the GeneJET spin column was centrifuged for an extra 1 minute to remove any residual wash solution. The washed and dried GeneJET spin column was then transferred into a sterile Eppendorf tube, where the plasmid DNA was eluted by adding 50 µl of elution buffer directly to the center of the GeneJET spin column membrane, followed by an incubation of 2 minutes at room temperature and centrifugation at 16 300g for 2 minutes. The used column was discarded while the purified plasmid DNA was stored at -20°C for further use.

3.2.2.5. Assessing for Positive Clones

Confirmation of positive clones was carried out by PCR and in accordance with the standard MyTaq Mix protocol (Bioline, London, UK) to confirm if the AtMTA-AC gene inserts were successfully cloned into the pTrcHis-TOPO expression vector (i.e., successful ligation and correct orientation). The used reaction mixtures for these two particular processes are shown in Tables 3.3 and 3.4 respectively while the associated thermal cycling conditions for both processes are shown in Table 3.5 below.

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

Component	Volume (µl)	Final Concentration
Template (Construct DNA)	1	2 ng
Insert Forward Primer	1	200 nM
Insert Reverse Primer	1	200 nM
MyTaq™ Red Mix (10X)	25	1X
Water (sdH ₂ O)	Up to 50	

Table 3.4: Components of a PCR reaction mixture to confirm the correct orientation of the AtMTA-AC gene insert in the pTrcHis-TOPO expression vector.

Component	Volume (µl)	Final Concentration
Template (Construct DNA)	1	2 ng
Insert Forward Primer	1	200 nM
Vector Reverse Primer	1	200 nM
MyTaq™ Red Mix (10X)	25	1X
Water (sdH ₂ O)	Up to 50	

Table 3.5: Thermal cycling program for the step by step assessment profile of the successful cloning of the AtMTA-AC gene insert into the pTrcHis-TOPO expression vector.

Step	Temperature (°C)	Time (mins)	Cycles
Initial Denaturation	95	1.00	1
Denaturing	95	0.25	35
Annealing	60	0.25	
Extension	72	0.17	

3.2.2.6 Agarose Gel Electrophoresis of the Positively Confirmed AtMTA-AC Gene Fragment

The PCR products from both Tables 2.3 and 2.4 were resolved on an ethidium bromide-stained 1% agarose gel as disused already in section 3.2.1.6 above. A double amplification of the targeted AtMTA-AC gene fragment (insert) in both reaction samples (Table 3.3 and 3.4 respectively) was going to correspondingly confirm its successful and positive cloning into the pTrcHis-TOPO expression vector.

3.2.3 Transformation of Chemically Competent *E. coli* EXPRESS BL21 (DE3) pLysS Cells with the pTrcHis-TOPO:AtMTA-AC Fusion Expression Construct

After the successful cloning process of the AtMTA-AC gene fragment into the pTrcHis-TOPO expression vector was positively confirmed, the resultant fusion expression construct (pTrcHis-TOPO:AtMTA-AC) was then used to transform some chemically competent *E. coli* EXPRESS BL21 (DE3) pLysS expression cells. This transformation was carried out in a sterile Eppendorf tube and in accordance with the manufacturer's protocol and instructions (Lucigen, Wisconsin, USA). In brief, 1 µl of the pTrcHis-TOPO:AtMTA-AC fusion construct was aseptically added to 40 µl of the ice-cold *E. coli* BL21 (DE3) pLysS cells. The mixture was incubated on ice for 30 minutes before being heat-shocked for 45 seconds at 42°C and immediately placed on ice for 2 minutes. The reaction mixture was supplemented with 960 µl of the expression recovery medium and incubated in a shaking incubator at 37°C and 200 rpm for 1 hour (this incubation step is carried out to allow cells to produce the β-lactamase enzyme, which will later and during

the selection process, detoxify ampicillin). The transformation reaction mixture was then plated onto LB agar plates supplemented with 100 µg/ml ampicillin and 34 µg/ml chloramphenicol. The plates were incubated overnight at 37°C.

3.2.4 Partial Expression of the AtMTA-AC Recombinant Protein

A transformed *E. coli* BL 21 (DE3) pLysS cell colony from the above plates, harbouring the successfully designed pTrcHis-TOPO:AtMTA-AC fusion expression construct was used to inoculate 10 ml of fresh 2YT media supplemented with 0.5% glucose, 34 µg/ml chloramphenicol and 100 µg/ml ampicillin in a 50 ml falcon tube. The falcon tube was incubated overnight at 37°C and shaking at 200 rpm. On the subsequent day, 200 µl of overnight transformation culture was used to inoculate 20 ml of fresh 2YT media containing 34 µg/ml chloramphenicol, 100 µg/ml ampicillin and 0.5% glucose. The culture was incubated at 37°C, shaking at 200 rpm and until an OD₆₀₀ of 0.5 was reached and as measured by the Hekios spectrophotometer (Merck, Gauteng, RSA). Immediately, the culture was split into two 50 ml falcon tubes each containing 10 ml of the split culture. One culture was induced to express the intended AtMTA-AC recombinant through the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma-Aldrich Corp, Missouri, Country) while the other culture was left un-induced (control). The split cultures were then shaken in an incubator (200 rpm) at 37°C for 3 hours. After the 3 hours, 500 µl samples of each culture were transferred into 1.5 ml Eppendorf tubes and these, together with the rest of the bulk cell cultures, were centrifuged at 8 000g for 5 minutes to pellet the cells. The pelleted small samples were analyzed by sodium dodecyl sulphate-polyacrylamide gel

electrophoresis (SDS-PAGE) while the pelleted bulk cultures were stored at -20°C for further use.

3.2.5 Determination of the Endogenous Activity of the Recombinant AtMTA-AC Protein

The pelleted bulk cultures in the above section 3.2.4, were each lysed in 1 ml lysis buffer 1 (Amersham Healthcare, California, USA) supplemented with 2 mM 3-isobutyl-1-methylxanthine (IBMX) (Sigma-Aldrich Corp., Missouri, USA) to inhibit phosphodiesterases (PDEs). The samples were shaken at 100 rpm at 37°C for 30 minutes in an orbital shaker to intensify the cell lysis process. The samples were then centrifuged at 9.200xg for 5 minutes and their lysates transferred into fresh Eppendorf tubes where 200 µl of the lysis buffer 2 (Amersham Healthcare, California, USA) was added and mixed. After that, 220 µl portions of the sample mixtures were then transferred into fresh Eppendorf tubes, where 11 µl of the acetylating reagent (Sigma-Aldrich Corp., Missouri) was added before the mixtures were gently mixed by pulsing. The endogenous cAMP contents from the lysates were then measured by a cAMP-linked enzyme immunoassay kit (Catalogue # CA201; Sigma-Aldrich Corp., Missouri, USA) following the acetylation version of its protocol and as described by the manufacturer's manual. The measurements were taken using a Microplate Reader (Labtech International Limited, East Sussex, UK) at 405 nm and the obtained results then subjected to the statistical analysis of variance (ANOVA) in triplicate forms.

3.3 RESULTS

3.3.1 Generation of the *Arabidopsis thaliana* Plants

Wild-type *Arabidopsis thaliana* ecotype Columbia seeds were surface sterilized and germinated for 2 weeks on solidified MS media (Figure 3.2 A) that mimic soil conditions in a GC-300TL growth chamber (Lab Companion, Jeio Tech, Seoul, Korea). The germinated seedlings were then transplanted to sterile potting soil and kept in the growth chamber to grow for a further 2-4 weeks (Figure 3.2 B). The leaf material from these 6 week-old plants was later harvested for isolation of total RNA.

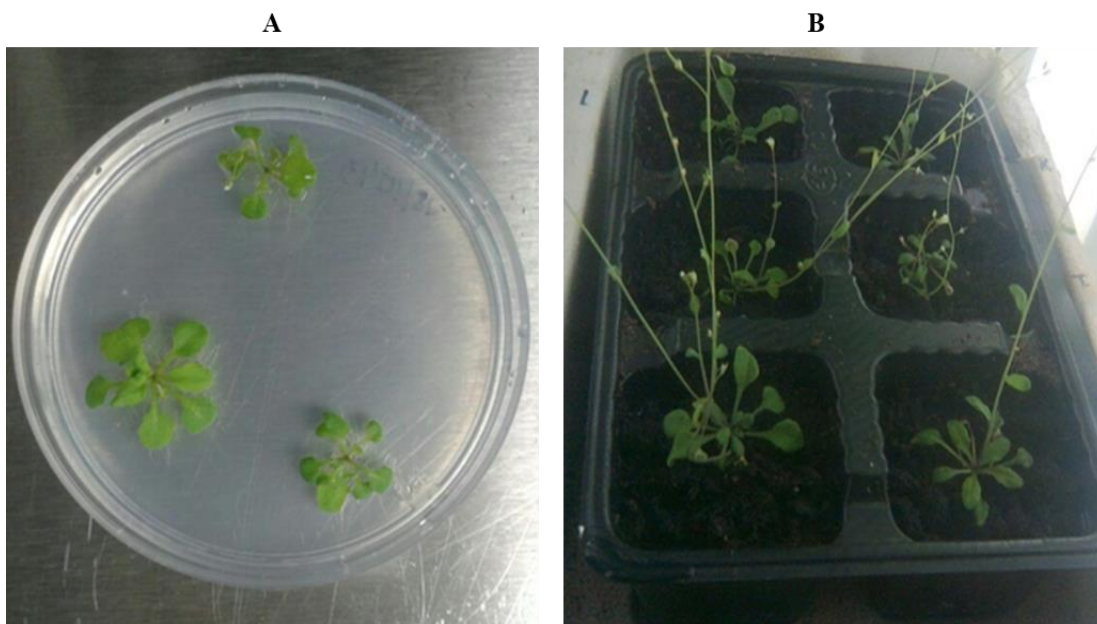


Figure 3.2: Generation of the *Arabidopsis thaliana* plants. (A) Newly germinated *Arabidopsis* seedlings on MS media under the growth chamber conditions. (B) Some six weeks old *A. thaliana* plants in potting soil from which total RNA was later extracted.

3.3.2 Molecular Isolation of the AtMTA-AC Gene Fragment

The cDNA of the At3g02930 gene that was generated from the *A. thaliana* total RNA, together with the manually designed sequence-specific primers, were used to isolate the targeted AtMTA-AC gene fragment (Figure 3.3) using a specialized 1-step RT-PCR technique (Thermo Scientific Inc., Burlington, Canada).

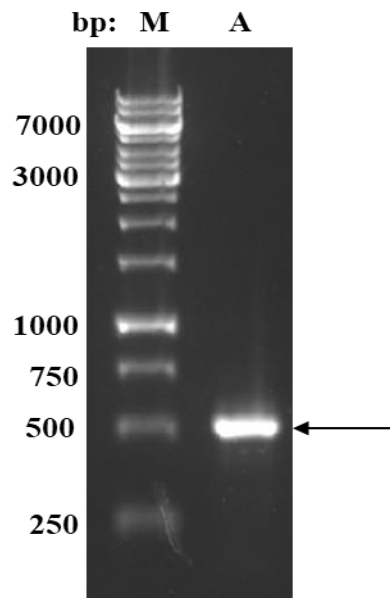


Figure 3.3: Molecular isolation of the AtMTA-AC gene fragment. The targeted and desired AtMTA-AC gene fragment was isolated by amplifying it from the total *A. thaliana* mRNA via a specialized RT-PCR system followed by its resolution on a 1% agarose gel. Lane 1 (M) represents the 100 bp GeneRuler ladder (Thermo Scientific Inc., Burlington, Canada), lane 2 (A) represents the amplified AtMTA-AC gene fragment while the arrow is marking the resolved AtMTA-AC fragment gene.

3.3.3 Confirmation of the Cloning Success of the AtMTA-AC Gene Fragment

After the successful isolation of the AtMTA-AC gene fragment in Figure 3.3 above, the gene fragment was ligated into a pTrcHis-TOPO expression vector to yield the pTrcHis-TOPO:AtMTA-AC fusion construct. After that, the cloning success of this gene fragment into the expression vector was then checked and ascertained by standard PCR using a specialized MyTaq reaction system. The successfully cloned and molecularly confirmed AtMTA-AC gene fragment is shown below (Figure 3.4).

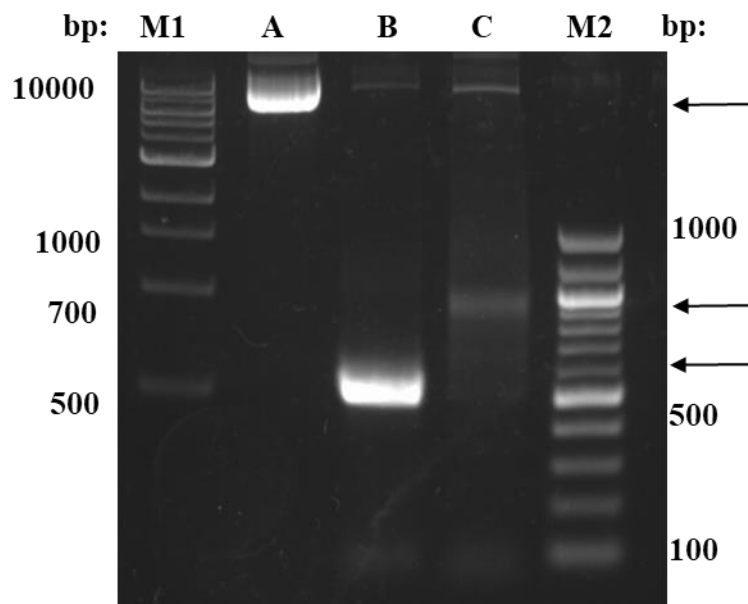


Figure 3.4: Confirmation of the cloning success of the AtMTA-AC gene fragment. The AtMTA-AC gene fragment was ligated into the pTrcHis-TOPO expression vector and re-amplified with its own sequence-specific primers as well as by one of its own primers and one of the vector primers followed by its resolution on a 1% (w/v) agarose gel system. Lane 1 (M1) represents the 1 kb Gene-Ruler ladder (Thermo Scientific Inc., Burlington, Canada), lane 2 (A) represents the pTrcHis-TOPO:AtMTA-AC gene expression construct, lane 3 (B) represents the AtMTA-AC gene fragment amplified with its own sequence-specific primers, lane 4 (C) represents the AtMTA-AC gene fragment amplified with its own forward primer and the vector reverse primer, while lane 5 (M2) represents the 100 bp Gene-Ruler ladder (Thermo Scientific Inc., Burlington, Canada). The three arrows mark sizes of the various amplified and resolved AtMTA-AC gene fragments.

3.3.4 Partial Expression of the AtMTA-AC Recombinant Protein

The successfully confirmed pTrcHis-TOPO:AtMTA-AC fusion construct (section 3.3.3 above) was used to transform some chemically competent *E. coli* EXPRESS BL21 (DE3) pLysS cells followed by the induction of protein expression with 1 mM IPTG. As is shown in Figure 3.5 below, the desired AtMTA-AC recombinant protein was successfully expressed.

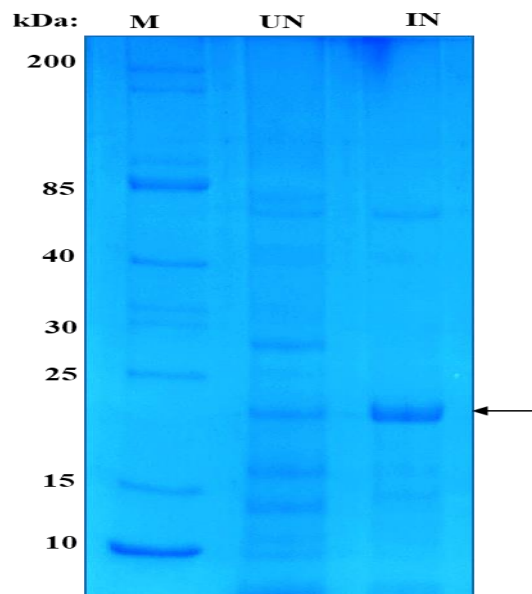


Figure 3.5: Partial expression of the recombinant AtMTA-AC protein. An SDS-PAGE of protein fractions expressed in chemically competent *E. coli* EXPRESS BL21 (DE3) pLysS cells transformed with the pTrcHis-TOPO:AtMTA-AC fusion construct, where lane 1 (M) is the unstained protein standard, broad range low molecular marker (BioLabs Intc, New England), while lane 2 (UN) represents the un-induced control cell culture and lane 3 (IN) representing the bacterial cell culture treated with 1 mM IPTG to induce the partial expression of the targeted and desired recombinant AtMTA-AC protein. The arrow marks the 19.8kDa partially expressed recombinant AtMTA-AC protein.

3.3.5 Determination of the Endogenous Activity of the AtMTA-AC Recombinant Protein

After successfully expressing the targeted AtMTA-AC recombinant protein in the transformed chemically competent *E. coli* EXPRESS BL21 (DE3) pLysS cells, its ability to generate cAMP from ATP within these prokaryotic expression systems (endogenously) was assessed and determined. The resultant endogenous AC activity of this partially expressed recombinant AtMTA-AC protein is presented below in Figure 3.6.

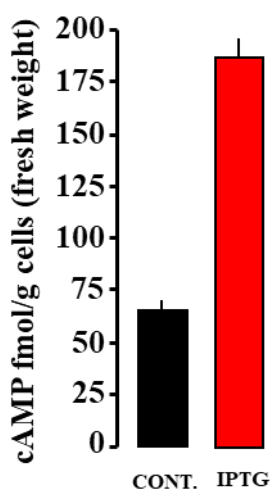


Figure 3.6: Determination of the endogenous adenylate cyclase activity of the recombinant AtMTA-AC protein. Some chemically competent *E. coli* EXPRESS BL21 (DE3) pLysS cells transformed with the successfully designed pTrcHis-TOPO:AtMTA-AC fusion construct were grown under various culturing conditions followed by an assessment of the expressed AC recombinant protein's ability to generate cAMP from ATP under such different culturing conditions within this prokaryotic expression system. CONT represents cAMP levels generated in cells cultures the were not induced to express the recombinant AtMTA-AC protein while IPTG represents cAMP levels generated in cell cultures induced with 1 mM IPTG to express the recombinant AtMTA-AC protein. For all cultures, the generated cAMP levels were determined using a cAMP-linked enzyme immunoassaying system (Sigma-Aldrich Inc., Missouri, USA) and based on its acetylation protocol. Analyses were undertaken in triplicate sets ($n = 3$) using ANOVA, where error bars represent the standard errors of the replicate means.

3.4 DISCUSSION

As previously mentioned in chapter 1, microtubule associated (MTA) protein encoded by the At3g02930 gene from *Arabidopsis thaliana* is one of the putative candidates that contain an adenylate cyclase (AC) catalytic motif. In order to target the AC region of the AtMTA gene, we firstly, retrieved the At3g02930 gene sequence from TAIR and determined its expressible cDNA sequence. Secondly, the targeted gene fragment (AC-containing segment of the At3g02930 or AtMTA-AC) was then amplified using total mRNA (as a template) extracted from the *A. thaliana* ecotype Columbia, grown under greenhouse conditions (Figure 3.2). In essence, the actual amplification of the AtMTA-AC was achieved by combining the generate cDNA and the forward and reverse sequence-specific primers for its AC region via a specialized RT-PCR system. The sequence-specific primers were designed in such a way to favour effective cloning by adding a non-template nucleotide adjacent to a 3' terminal C, which is favoured as compared to the addition adjacent to a 3' terminal A, which is not favoured. This was done by placing the sequence GTTTCTT on the 5' end of reverse primers, it was noted that it always resulted in adenylation of the 3' end of the forward strand, which then facilitated accurate genotyping and efficient T/A cloning (Brownstein *et al.*, 1996).

In our case, the AC gene region of the AtMTA gene (AtMTA-AC) was amplified at its specific annealing temperature of 60 °C, and when resolved on a 1% agarose gel, the expected size of the AtMTA-AC gene fragment of approximately 540 bp as shown in Figure 3.3 was obtained. Subsequent to that, the amplified PCR product was then ligated into the pTrcHis-TOPO expression vector to yield a pTrcHis-TOPO:AtMTA-AC fragment fusion construct. Ideally, the

pTrcHis-TOPO vector utilizes the TA cloning system, which is the simplest and most efficient method for the cloning of PCR products (Zhou and Gomez-Sanchez, 2000). The procedure utilizes the terminal transferase activity of certain thermophilic DNA polymerases, including *Thermus aquaticus* (*Taq*) polymerase, which was used in this study, whereby single 3'-adenine overhangs are normally added first to the ends of each of the PCR product by the enzyme *Taq* polymerase (Powell *et al.*, 1987). The *Taq* has a non-template dependent activity that preferentially adds a single adenosine to the 3'-ends of a double stranded DNA molecule, so that it becomes complementary to the 5'-thymine overhangs on both ends of the expression vector, thus allowing PCR inserts to ligate efficiently into the vector (Saiki *et al.*, 1985). This is the most common method of cloning and sub-cloning whereby DNA ligase is required to covalently link the compatible ends of the DNA fragment and its corresponding linearized plasmid, forming a single cyclic molecule that is capable of autonomously replicating in host cells (Sambrook *et al.*, 1989).

After cloning, identification of positive colonies or cloning success usually can be carried out either by restriction mapping of the mini-prepared plasmids or screening by PCR, of which in this study, the cloning success was carried out via PCR screening. Generally, it is very important to verify positive clones due to the possibility of the non-directional cloning aspect of the T/A system since both ends of the vector are complementary to either ends of the insert (Chen and Janes, 2002). In our case, PCR screening was carried out with primers that flank the vector cloning sites, so that the final length of the amplified PCR product of a positive clone will then be the size of the actual insert plus the bases flanking the two primers on the plasmid (Zhou and Gomez-Sanchez, 2000). Thus, the cloning success of the targeted fragment was confirmed by firstly, amplifying the fragment with its own forward primer and reverse (self-primers) and then

with one of its own primer plus a vector primer via a standard PCR system, using a specialized MyTaq reaction system. The first amplification confirmed ligation of the fragment insert into the vector while the second amplification confirmed correct orientation of the fragment insert in the vector (Figure 3.4).

After confirming the correctly designed expression construct of the AtMTA-AC gene fragment, the construct was then used to transform some competent BL21 *E. coli* cells for protein expression. The transformed BL21 cells were the most suited hosts for protein expression because they are a non-T7 expression strain and therefore, very appropriate for both plasmid transformation and protein expression. This strain does not express the T7 RNA polymerase, which is only expressable upon addition of IPTG to a final concentration of 1 mM that induces a high-level of protein expression (Lucigen Corporation, 2015). The expected AtMTA-AC protein was successfully expressed as a His-tagged fusion product of approximately 19.8 kDa (Figure 3.5).

After successfully expressing the desired and targeted recombinant AtMTA-AC protein in the chemically competent *E. coli* EXPRESS BL21 (DE3) pLysS cells, its capacity to produce cAMP from ATP within these prokaryotic expression systems was then assessed and determined using a cAMP-linked enzyme immunoassaying system. As is shown in Figure 3.6, cells that were induced with 1 mM IPTG had a cAMP generation increase of up to 5.4-fold compared to the un-induced cells, signifying the inherent endogenous AC activity for the recombinantly expressed AtMTA-AC protein. These obtained findings are very comparable to those of Moutinho *et al.* (2010), where the PSiP coding region of a pollen-specific putative AC from *Zea mays* was cloned and expressed into some competent bacterial cells and in turn, increasing the inherent cellular cAMP levels by a factor of up to 3.0 folds. The results are also in line with

those of Ruzvidzo *et al.* (2013), whereby a pentatricopeptide (AtPPR-AC) protein from *A. thaliana* was cloned into some bacterial cellular host cells followed by cell induction with 1 mM IPTG. The induced cells then increased their cAMP content by a factor of up to 2.7-fold as compared to the un-induced cells (Ruzvidzo *et al.*, 2013).

3.5 CONCLUSION

In conclusion, the results obtained in this study suggest that the *A. thaliana* MTA protein is possibly a *bona fide* AC capable of directly converting ATP to cAMP or else, it is just another non-AC functional plant molecule capable of stimulating other resident AC (like the bacterial ones in this case) to produce cAMP.

CHAPTER FOUR

DETERMINATION OF THE *IN-VIVO* ADENYLATE CYCLASE

ACTIVITY OF THE RECOMBINANT AtMTA-AC PROTEIN

ABSTRACT

Adenylate cyclases (ACs) are enzymes which play central roles in numerous and diverse physiological responses and processes in prokaryotes and eukaryotes. ACs catalyze the formation of cyclic 3',5'-adenosine monophosphate (cAMP) from adenosine 5'-triphosphate (ATP), which is one of the most comprehensively studied second messengers in animals, lower eukaryotes and bacteria. Nowadays, there is adequate physiological and biochemical evidence for the existence of this group of enzymes in plants however, only nine of these enzymes have so far been proven to exist but given the wide spectrum of processes and activities regulated by cAMP in plants, it is highly unlikely that there is only a few of such ACs in plant species. Hence much interest was developed in the investigation of the recently annotated microtubule associated protein (MTA) from *Arabidopsis thaliana*. In this chapter we describe the recombinant cloning of the AC-containing fragment domain of the protein (AtMTA-AC) into a non-lactose fermenting mutant strain of *Escherichia coli* (*cyaA* SP850 cells) and confirm its practical ability to generate cAMP in this strain, which in turn then complemented the mutant to behave as if it was a wild type strain, capable of fermentation of lactose.

4.1 INTRODUCTION

Specialized enzymes termed adenylate cyclases (ACs) are capable of converting adenosine 5'-triphosphate (ATP) to the second messenger cyclic 3',5'-adenosine monophosphate (cAMP) (Sutherland *et al.*, 1968). Since the late 1960s, the question of whether or not cAMP exists and functions in plants was strongly debated despite the fact that in animals and other lower eukaryotes, ACs and their cAMP had been proven to play key roles as signaling molecules in important cellular processes such as stress response and signal transduction (Gerisch and Wick, 1975). Early published experimental data and countless claims from several laboratories proposing comparable roles for cAMP in higher plants were very much criticized firstly, due to the insufficiency of specificity in the effects elicited by cyclic nucleotides, secondly, due to the doubts about cyclic nucleotide-generating enzymes, and thirdly, due to the indefinite identification of endogenous putative cyclic nucleotides largely as a result of inadequate chromatographic confirmations (Amrhein, 1977, Newton and Smith, 2004). However, it is worth stating that in the early 1970s, similar techniques for tissue extraction, purification and detection as employed in animals were applied to plant tissues without taking into consideration the specific characteristics of the biology and/or chemistry of plants. This then led to unnecessary discrepancies that were only resolved in the late 1970s when experimental techniques to analyze cAMP were modified to suit the plant cellular and extracellular environments (Lemtiri-Chlieh *et al.*, 2011). Consequently, a number of plant scientists have reported more reproducible data, for instance, the localization of AC activity in meristems of young pea (*Pisum sativum*) hypocotyl (Hilton and Nesius, 1978) and the activity of

phosphodiesterase (PDE) to hydrolyze 3',5'-cAMP through their enzyme inhibition reactions by methylxanthines (Farber *et al.*, 1979).

Most recently, in higher plants, only a few studies have concretely proven the existence of ACs, whereby to date, only a couple of them have been functionally and experimentally confirmed. These are the *Zea mays* pollen (PSiP) protein responsible for the polarized growth of pollen tubes (Moutinho *et al.*, 2001), the *A. thaliana* pentatricopeptide repeat (AtPPR) protein responsible for pathogen responses and gene expressions (Ruzvidzo *et al.*, 2013), the *Nicotiana benthamiana* adenylyl cyclase (NbAC) protein responsible for the tabtoxinine- β -lactam-induced cell deaths during wildfire diseases (Ito *et al.*, 2014), the *Hippeastrum hybridum* adenylyl cyclase (HpAC1) protein involved in stress signalling (Swiezawska *et al.*, 2014), the *A. thaliana* K⁺ uptake permease 7 (AtKUP7), which plays a role as a cAMP-dependent K⁺ flux sensor (Al-Younis *et al.*, 2015), the *Marchantia polymorpha* (MpCAPE), which plays an important role in male organ and cell development in basal plants (Kasahara *et al.*, 2016), the *A. thaliana* clathrin assembly protein (AtClAP) with a predicted role in plant defence and endocytosis (Chatukuta *et al.*, 2018), the *A. thaliana* K⁺ uptake permease 5 (AtKUP5) responsible for K⁺ transport (Al-Younis *et al.*, 2018) and the *A. thaliana* leucine-rich repeat (AtLRR) protein involved in defence against biotrophic and hemibiotrophic plant pathogens (Bianchet *et al.*, 2019).

In *E. coli*, cAMP is synthesized from ATP by the AC, *cyaA* (Yang and Epstein, 1983), and the regulation of this *cyaA* activity is coordinated by the phosphoenolpyruvate (PEP)-carbohydrate phosphotransferase system (PTS) (Reddy *et al.*, 1985). The PTS phosphorylation cascade transfers a phosphoryl group from PEP via a chain of proteins (EI, HPr, EIIA and EIIB) until it is transferred to the sugar that is transported through the EIIC (Deutscher *et al.*, 2006; Escalante *et al.*, 2012). When PTS sugars are absent or low in concentration, the glucose-specific EIAGlc

protein is phosphorylated and work together with *cyaA* to activate cAMP synthesis (Reddy and Kamireddi, 1998). When PTS sugars are high in concentration, the EIIAGlc is dephosphorylated and *cyaA* is not activated, leading to decreased cAMP levels and a reduced expression of genes encoding enzymes that process secondary carbon sources such as sucrose and lactose (Reddy and Kamireddi, 1998). This regulatory system is known as catabolite repression (Deutscher *et al.*, 2006; Escalante *et al.*, 2012).

In bacterial systems, cAMP is primarily involved in the positive regulation of the *lac* operon, whereby it accumulates and binds to the allosteric site of the cAMP receptor protein (CRP), a transcription activator protein in an environment of a low glucose concentrations. Once the CRP is in its active configuration, it binds to a *cis*-element upstream of the *lac* promoter and activates transcription. At high glucose concentrations, cAMP concentration decreases and the CRP disengages from the *lac* operon promoter (Meiklejohn and Gralla, 1985).

E. coli SP850 is a *cyaA* deletion mutant and thus lacks both the AC and cAMP systems. The SP850 strain cannot utilize lactose (Lac⁻), produces colourless colonies on Mac-Conkey lactose agar, and is also kanamycin-resistant. As a result of this, the SP850 strain provides a very suitable platform for testing the possible AC activities of foreign recombinant proteins via complementation, wherein the system is used in order to determine if two recessive mutations that produce the same phenotype are located on the same gene. In practise, when the *cyaA* mutant strain is transformed with a foreign gene capable of encoding for a functional AC activity, it produces deep red/purple coloured colonies on the McConkey agar, which is indicative of a complementation of its AC deletion (Cotta and Troya, 1998), leading to the production of cAMP, which in turn restores the normal utilization of lactose as a carbon source

(Perlman and Pastan, 1969). Otherwise mutant cells that cannot ferment lactose produce colourless to yellowish cells on MacConkey agar (Perlman and Pastan, 1969).

Some previous studies that were successfully undertaken in higher plants utilizing the complementation system include the one by Moutinho *et al.* (2001) on the PSiP protein from *Zea mays*, the one by Ruzvidzo *et al.* (2013) on the AtPPR-AC protein from *A. thaliana*, the one by Swiezawska *et al.* (2014) on the HpAC1 protein from *Hippeastrum hybridum*, the one by Al-Younis *et al.* (2015) on the AtKUP7 protein from *A. thaliana*, the one by Kasahara *et al.* (2016) on the MpCAPE from *Marchantia polymorpha*, the one by Chatukuta *et al.* (2018) on the AtCIAP from *A. thaliana*, the one by Al-Younis *et al.* (2018) on the AtKUP5 from *A. thaliana* and the one by Bianchet *et al.* (2019) on the AtLRR protein from *A. thaliana*.

As a result of the success in the above mentioned studies our research project therefore, focused on the expressed recombinant AtMTA-AC protein that was successfully demonstrated in the previous chapter to have the ability to generate cAMP from ATP within the transformed *E. coli* EXPRESS BL21 (DE3) pLysS cells, whereby its expression construct (the pTrcHis2-TOPO:AtMTA-AC gene fragment) was used to transform chemically competent SP850 *cyaA* *E. coli* mutant cells that systematically could not ferment lactose as a result of their inability to generate the most required cAMP. Consequently, the transformed *cyaA* cells were then assessed for their ability to now ferment lactose and as a result of their rescuing by the then inherent and expressed recombinant AtMTA-AC protein.

4.2 RESEARCH METHODOLOGY

In chapter 3, the partially expressed recombinant AtMTA-AC protein managed to demonstrate its ability to generate cAMP from ATP within the transformed *E. coli* EXPRESS BL21 (DE3) pLysS cells though a firm conclusion could not be reached on whether it is a *bona fide* AC or not. Therefore, in order to further assess it, its expression construct (the pTrcHis2-TOPO:AtMTA-AC) was used to transform chemically competent SP850 *cyaA* *E. coli* mutant cells that systematically cannot ferment lactose as a result of their incapability to produce cAMP, followed by analysis of their probable rescue into wild type counterparts.

4.2.1 Preparation of Competent *cyaA* Cells and Phenotypic Scoring

4.2.1.1 Preparation of Competent Cells

Some *Escherichia coli cyaA* cells obtained from the Coli Genetic Stock Center (Yale University, Connecticut, USA), were prepared to become chemically competent. Briefly, about 10 ml of fresh Luria Bertani (LB) broth [1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1.0% (w/v) NaCl, 0.5% (w/v) glucose, 15 µg/ml kanamycin, pH: 7.0] were inoculated with the mutant *E. coli cyaA* SP850 cells and incubated at 37°C overnight at 200 rpm in an SI-600 Bench-top Shaking Incubator (Lab companion GMI Inc., Minnesota, USA). On the subsequent day, 10 ml of the fresh pre-warmed LB media were inoculated with 1 ml of the overnight culture and the mixture shaken at 200 rpm at 37°C and up until an OD₆₀₀ of 0.6 was reached as measured by the Hekios Spectrophotometer (Merck, Modderfontein, RSA). At this OD, the culture was cooled on ice for 5 minutes and then centrifuged at 4000xg, for 5 minutes at 4°C. Thereafter, the supernatant was

discarded and the cells were re-suspended in 30 ml of the ice-cold Transformation Buffer 1 (TFB1) [30 mM KAc, 50 mM MnCl₂, 100 mM RbCl, 10 mM CaCl₂, 15% glycerol, pH: 5.8] and kept on ice for 90 minutes. Following that, the cells were harvested by centrifugation at 4000xg for 5 minutes at 4°C and kept on ice. The supernatant was discarded while the harvested cells were resuspended in 4 ml of the ice-cold Transformation Buffer 2 (TFB2) [10 mM MOPS, 75 mM CaCl₂, 10 mM RbCl, 15% glycerol, pH: 6.8]. Finally, aliquots of 100 µl were then prepared in Eppendorf tubes and kept on ice for further use.

4.2.1.2 Phenotypic Scoring

A MacConkey agar plate [2% (w/v) peptone, 1% (w/v) lactose, 0.5% (w/v) bile salts, 0.0075% (w/v) neutral red, 1.5% (w/v)] supplemented with 15 µg/ml kanamycin and 0.1 mM IPTG was prepared and then sub-divided into 3 segments using a permanent marker (Figure 4.1). The first segment was kept un-streaked (no cells or control) while the second segment was streaked with the *cyaA* mutant cells transformed with the pTrcHis-TOPO:AtMTA-AC expression construct and the third segment streaked with the non-transformed *cyaA* mutant cells (Figure 4.1 below). The streaked plate was then inverted and incubated at 37°C for 40 hours. After incubation period, all the segments were then visually inspected for various phenotypic characteristics.

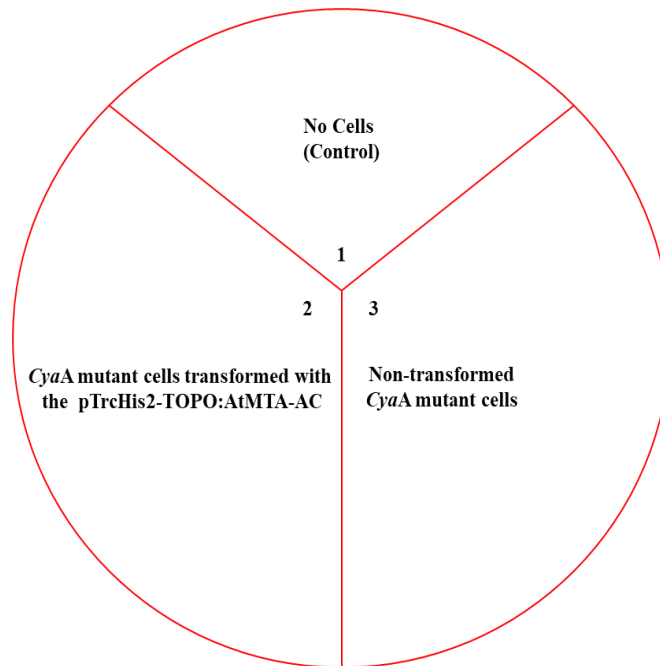


Figure 4.1: The designed culture distribution of the *cyaA* SP850 mutant cells on a MacConkey agar plate to undertake a complementation test. The 1st segment was going to contain no cells/control, the 2nd segment was going to contain the *cyaA* mutant cells transformed with the pTrcHis2-TOPO:AtMTA-AC expression construct while the 3rd segment was going to contain the non-transformed *cyaA* mutant cells.

4.3 RESULTS

The Figure below shows a complementation test conducted using the SP850 *cyaA* *E. coli* mutant host strain (Coli Genetic Stock Center, Yale University, Connecticut, USA), whereby an assessment of the recombinant AtMTA-AC's ability to convert the non-lactose fermenting SP850 *cyaA* *E. coli* cells into lactose-fermenting wild types was undertaken. The settings were then visually assessed on MacConkey agar (Figure 4.2) where the white or yellowish colonies of the mutant cells then changed into deep red or purple coloured colonies reminiscent of wild type cells after transformation with the pTrcHis2-TOPO:AtMTA-AC expression construct.

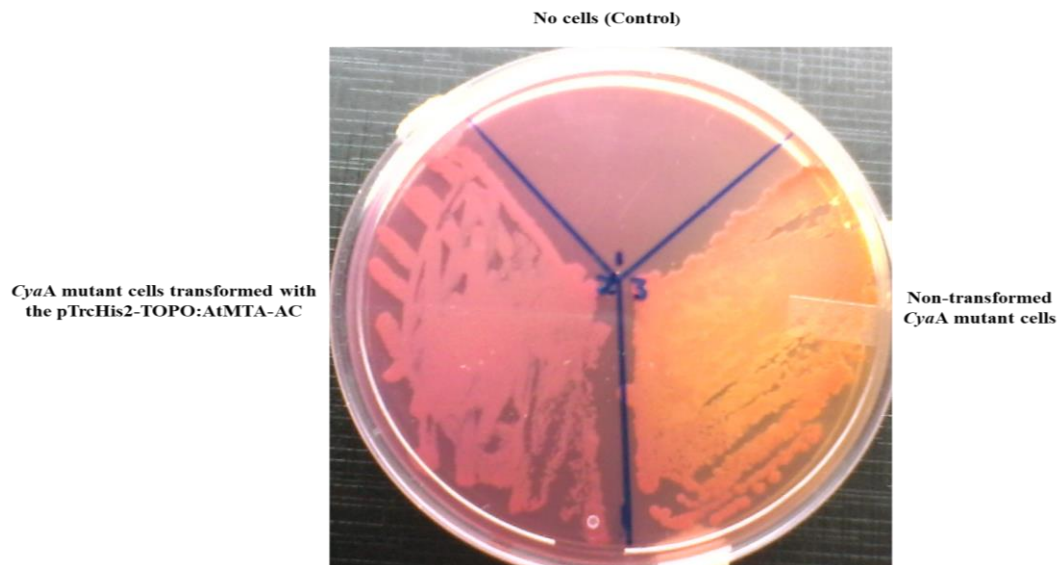


Figure 4.2: Determination of the Endogenous Adenylate Cyclase Activity of the Recombinant AtMTA-AC Protein. Differently prepared chemically competent *E. coli* *cyaA* mutant cells were plated onto three different segments of a MacConkey agar supplemented with 15 µg/ml kanamycin and 0.1 mM IPTG, and the plate incubated at 37°C for 40 hours. The 1st segment of the plate contains no cells/control, the 2nd segment contains the *cyaA* mutant cells transformed with the pTrcHis2-TOPO:AtMTA-AC expression construct while the 3rd segment contains the non-transformed *cyaA* mutant cells. Cells in the 2nd segment of the plate then picked a deep red or purple phenotype - a characteristic signifying their acquired ability to now ferment lactose and as a result of the then presence and functional activity of the now included AtMTA-AC protein, which now essentially generates the most required cAMP for this process. Cells in the 3rd segment are non-lactose fermenters and therefore remained white or yellowish in colour.

4.4 DISCUSSION

For this study, complementation testing was conducted on the AC fragment domain of a microtubule associated protein from *Arabidopsis thaliana* (AtMTA-AC) that previously had been bioinformatically annotated as a putative AC candidate (Gehring, 2010). After the

successful expression of the recombinant AtMTA-AC protein and its demonstrated ability to generate cAMP from ATP within the transformed *E. coli* EXPRESS BL21 (DE3) pLysS cells, its expression construct (the pTrcHis-TOPO:AtMTA-AC gene fragment) was then used to transform chemically competent SP850 *cyaA* *E. coli* mutant cells that systematically cannot ferment lactose as a result of their inability to produce the most essential cAMP. As a result, the transformed SP850 *cyaA* cells were then assessed for their ability to now ferment lactose and as a result of their rescuing by the then inherent and expressed recombinant AtMTA-AC protein. A visual analysis of the cell phenotypes on a selective and differential MacConkey agar medium was performed, where it was found that the cells harbouring the induced pTrcHis-TOPO:AtMTA-AC fusion construct picked up a deep red or purple phenotype as opposed to those that were non-transformed, which appeared pale yellowish. This result therefore convincingly affirmed that the recombinant AtMTA-AC protein is indeed a *bona fide* higher plant AC capable of generating cAMP from ATP (Figure 4.2).

These results are significantly important because they are consistent with findings from other previous studies, where other related higher plant ACs were also tested and confirmed using a similar approach. Such ACs include the *Z. mays* PSiP with a role in polarized pollen tube growth (Moutinho *et al.*, 2001), the *A. thaliana* AtPPR-AC responsible for pathogenic responses and gene expressions (Ruzvidzo *et al.*, 2013), the *H. hybridum* HpAC1 involved in stress signalling (Świeżawska *et al.*, 2014), the *A. thaliana* AtKUP7 with a role as a cAMP-dependent K⁺ flux sensor (Al-Younis *et al.*, 2015), the *M. polymorpha* MpCAPE with a role in male organ and cell development in basal plants (Kasahara *et al.*, 2016), the *A. thaliana* AtCIAP with a predicted role in plant defence (Chatukuta *et al.*, 2018), the *A. thaliana* AtKUP5 involved in the increase of cAMP levels in response to K⁺ transport activity (Al-Younis *et al.*, 2018) and the *A.*

thaliana AtLRRAC1 involved in defence against the biotrophic and hemibiotrophic plant pathogens (Bianchet *et al.*, 2019). In this regard therefore, the AtMTA protein thus becomes the sixth ever AC molecule to be identified in *A. thaliana*, the ninth ever AC molecule to be identified in higher plants and the tenth ever AC molecule to be identified in plants. Essentially also, its ability to generate cAMP may perhaps suggest for its possible involvement in critical cellular processes such as growth, development and the perception of extracellular biotic and/or abiotic stress stimuli for subsequent transduction and coordination of such signals using cAMP.

4.5 CONCLUSION

Findings of this chapter provide strong confirmation that the AtMTA-AC protein is indeed a *bona fide* functional AC molecule capable of generating cAMP from ATP.

CHAPTER FIVE

AFFINITY PURIFICATION OF THE RECOMBINANT AtMTA-AC

PROTEIN AND CHARACTERIZATION OF ITS ACTIVITY

ABSTRACT

In recent years, several plant genome sequencing projects have been completed and the next logical step was to perform experimental assessments of the protein products encoded by these genomes. To date, protein composition of different cell types, tissues, and isolated cellular organelles of a range of plant species have been investigated, making use of several techniques such as peptide mass spectrometry and pattern matching to genomic data. These studies have employed a variety of methods to separate proteins, though isoelectric focussing (IEF) and chromatography are but some of the most common techniques used. In this chapter, we detail the affinity purification of a recombinant version of the adenylyl cyclase (AC) containing fragment of a microtubule associated (MTA) protein encoded by the At3g02930 in *Arabidopsis thaliana* (AtMTA-AC) using the nickel nitrilotriacetic acid (Ni-NTA) affinity chromatographic system followed by characterization of its inherent AC activity *in vitro*.

5.1 INTRODUCTION

In the late 1950's, Earl Sutherland, made a significant discovery that was going to change the course of biochemistry as a subject, for the better. Actually, he was the founder of the important second messenger, cyclic 3',5'-adenosine monophosphate (cAMP) (Sutherland and Rall, 1958). Decades later, and specifically in the mid-70s, cAMP together with adenylyl cyclases (ACs), which are enzymes that catalyse its formation from ATP were established as important molecules that play crucial roles in the regulation of a variety of cellular responses in both animals and lower eukaryotic organisms. These molecules have been implicated in the regulation of a range of cellular events, including cell growth, cell-cycle progression, glycogen metabolism, and heat shock sensitivity. Although cAMP and ACs were entirely established in the animal and lower eukaryotic organisms, records on these two molecules were not readily available in the plant kingdom. Firstly, this was due to the fact that the levels of cAMP detected in plants were significantly lower (<20 pmol/g fresh weight) (Ashton and Polya, 1978) as compared to those found in animals (>250 pmol/g wet weight) (Robinson *et al.*, 1968) and therefore, firm conclusions could not be reached. Secondly, due to the lack of conclusive results in plant assays, most probably, due to the methodological difficulties encountered in earlier years when methods and techniques that were developed for the analysis of cAMP in mammalian tissues were simply implemented in plants. Nevertheless, the idea that plants would also most likely have a functional cAMP-dependent signal system comparable to that of animals and lower eukaryotes still remained alive.

In that regard therefore, several efforts were then made in attempting to either isolate intact plant proteins and/or expressing their recombinants in various expression system followed by some comprehensive functional characterizations. Amongst all other possible expression systems, the

use of plants as expression hosts for their own recombinant proteins has been an increasingly attractive option. Over the years, different plant protein extraction and purification protocols have also been reported. In 2002, Ros *et al.* reported a novel OFFGEL electrophoresis (OGE) system, which uses the isoelectric point (pI) based fractionation concept that allows the separation of proteins and peptides according to their native pIs (Ros *et al.*, 2002). This method was further enhanced and modified to a multi-well device by Michel *et al.* in 2003. This multi-well system uses a set of wells placed over an immobilized pH gradient (IPG) gel strip, which after rehydration, the IPG strip wells are filled up with the protein extract diluted in suitable solution and covered (Michel *et al.*, 2003).

A variable electrical field is then applied and charged proteins migrate through the IPG gel strip, from well to well (pI protein pH gel) until they reach the well where their pI is equal to the pH of the gel. At this stage, proteins come out of solution or precipitate and consequently, can be easily recovered and used or combined with a broad range of other downstream analysis systems. The length and pH gradient range of any selected IPG strip determines the separation resolution. OGE has been used as a reproducible alternative approach for protein purification and identification in different types of samples, including proteins extracted from plant tissues under reducing/denaturing conditions (Vincent and Solomon, 2011). However, if the research objective comprises, e.g., enzymatic activity characterisation, then the purified proteins therefore need to be in their native conditions and thus, the use of OGE in non-denaturing conditions seemed important and a step forward in the fractionation of plant protein extracts.

In this reported research project, we focused our attraction on plant ACs, in particular the MTA from *A. thaliana*, which is one of the nine bioinformatically identified putative AC candidates in the Arabidopsis genome Gehring (2010). Up to now however, only a handful of ACs have been

experimentally verified and functionally established across a variety of plant species such as the *Zea mays* where, Moutinho *et al.* (2001) reported a PSiP protein responsible for the polarized growth of pollen tubes; secondly an AtPPR-AC protein from *A. thaliana* with a role in pathogenic responses and gene expressions (Ruzvidzo *et al.*, 2013); thirdly, an NbAC protein from *Nicotiana benthamiana* with a role in the tabtoxinine- β -lactam-induced cell death that takes place during the development of the wildfire disease (Ito *et al.*, 2014); fourthly, an HpAC1 protein from *Hippeastrum hybridum* responsible for increased expression rate in response to mechanical damage and the *Phoma narcissi* infection (Swiezawska *et al.*, 2014); fifthly, an AtKUP7 protein from *A. thaliana* with a role in potassium ion transmembrane transport activities (Al-Younis *et al.*, 2015); sixthly, an MpCAPE protein from *Marchantia polymorpha* with a role in male organ and cell development in basal plants (Kasahara *et al.*, 2016); seventhly, an AtCIAP protein from *A. thaliana* with a predicted role in plant defence mechanisms (Chatukuta *et al.*, 2018); eighthly, an AtKUP5 protein from *A. thaliana* with a cytosolic function for K^+ transport (Al-Younis *et al.*, 2018); and lastly an LRRAC1 protein involved in defence against the biotrophic and hemibiotrophic plant pathogens (Bianchet *et al.*, 2019).

Specifically, in this chapter, we detail the steps taken in purifying, refolding and biochemically characterizing the recombinant AtMTA-AC protein that was previously expressed and shown to possess endogenous AC activity in Chapter 3 and a complementation role in Chapter 4. Biochemical characterization involved a further determination and establishment of how this recombinant protein's AC activity is mediated and/or modulated at the molecular level by an array of other molecular and/or ionic components.

5.2 RESEARCH METHODOLOGY

5.2.1 Over-expression and Affinity Purification of the Recombinant AtMTA-AC Protein

5.2.1.1 Large-scale Recombinant Protein Expression

For large scale protein expression, a cell colony of the *E. coli* EXPRESS BL21 (DE3) pLysS expression cells harbouring the pTrcHis-TOPO:AtMTA-AC fusion expression construct was used to inoculate 20 ml of 2YT media, containing 100 µg/ml of ampicillin and 34 µg/ml of chloramphenicol. The culture was grown overnight at 37°C in a Labcon shaking incubator (Labex, Lab design Engineering) at 200 rpm. On the subsequent day, in a 300 ml Erlenmeyer flask, 100 ml of the pre-warmed 2YT containing 100 µg/ml ampicillin with 34 µg/ml chloramphenicol was inoculated with 1 ml the overnight culture and immediately incubated at 37°C in the Labcon shaking incubator at 200 rpm. The culture was left to grow until it reached an OD₆₀₀ of 0.6 as was measured by the Hekios Spectrophotometer (Merck, Modderfontein, RSA). Immediately, the culture was split into two portions, one containing 295 ml while the other had 5 ml. The bulk culture was then induced with 4 mM IPTG (Sigma-Aldrich Corp, Missouri, USA) to over-express the targeted AtMTA-AC recombinant protein while the other small culture was left un-induced (control). The split cultures were then shaken in an incubator for 3 hours at 200 rpm at 37°C. After the 3 hours, small portions (20 µl) of the cell cultures were collected for analysis by SDS-PAGE while the bulk induced culture was centrifugation at 9 200xg for 10 minutes to pellet out the cells. The pelleted cells were then stored at -20°C for further use.

5.2.2 Determination of the Soluble or Insoluble Nature of the Recombinant AtMTA-AC

In order to determine the solubility or insoluble nature of the recombinant AtMTA-AC protein, the pelleted bulk induced cell culture was lysed and the protein component of its lysate analysed by SDS-PAGE, following the methods of the QIAexpressionist (06/2003) protocol 6. In brief, the pelleted cultures were re-suspended in 5 ml phosphate buffer saline (PBS) (140 mM NaCl, 3 mM KCl, 4 mM Na₂HPO₄·2H₂O, 1.5 mM KH₂PO₄) supplemented with 10 mM imidazole and 0.5 mM PMSF, and then sonicated for 6 minutes (10 seconds pulsing and 10 seconds chilling cycles) to solubilize the cell contents. The solubilized cell contents were then centrifuged at 9 200xg for 10 minutes and both its supernatant and pellet were separately kept as the soluble and insoluble protein fractions respectively. An aliquot (20 µl) of each of these two fractions was then analyzed by SDS-PAGE and in order to determine the presence and relative proportion of the expressed recombinant AtMTA-AC proteins in each one of them. A presence of the recombinant protein in the lysate fraction would indicate its soluble nature while its presence in the pellet fraction would on the other hand, indicate its insoluble nature.

5.2.3 Affinity Purification of the Recombinant AtMTA-AC Protein

5.2.3.1 Binding of the Recombinant AtMTA Protein

Protein purification was then performed under non-native denaturation conditions because the expressed recombinant protein was wholly found to be in its insoluble form. The pellet fraction from above was re-suspended in 5 ml of a urea lysis buffer (8 M urea, 100 mM NaH₂PO₄, 10 mM Tris-HCl; pH 8.0, 500 mM NaCl, 20 mM β-mercaptoethanol and 7.5% (v/v) glycerol and 10 mM imidazole), supplemented with 10 mM imidazole through vigorous vortexing for an

hour. The produced crude lysate was then centrifuged at 10 000xg for 30 minutes at room temperature to pellet out the cell debris while at the same time, generating a cleared lysate, which was then collected for further processing on the Ni-NTA beads following the supplier's protocol (QIAexpressionist, 2006). Alongside this, about 2 ml of the Ni-NTA slurry matrix/beads (Thermo Scientific., Rockford, USA) were washed twice with 5 ml sterile distilled water on a rotary mixer for 5 minutes. The Ni-NTA beads were then equilibrated with 1 ml of the urea lysis buffer supplemented with 10 mM imidazole. The equilibrated beads were then pelleted out through a low speed centrifugation for 15 seconds and the supernatant discarded. The cleared lysate was then mixed up (binding) with the Ni-NTA beads on a rotary mixer for 1 hour at room temperature. After an hour, the flow-through of the cleared lysate was removed and its small portion (20 µl) kept aside for analysis by SDS-PAGE.

5.2.3.2 Washing of the Bound AtMTA-AC Protein

After binding the recombinant AtMTA-AC protein onto the equilibrated Ni-NTA beads, the bound AtMTA-AC protein was washed three times with the wash buffer (100 mM $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 10 mM Tris-Cl, 8 M urea, pH 6.3 (adjusted with HCl), 500 mM NaCl, 20 mM β -mercaptoethanol, 7.5% (v/v) glycerol, and 10 mM imidazole) as described by the manufacturer protocol (Catalogue # P6611; Sigma-Aldrich Inc., Missouri, USA). Briefly, 2 column volumes (cv or 4 ml) of the wash buffer were added to the bound affinity beads and the suspension mixed for 10 minutes at 30 rpm on a bench revolver (Labnet International, Inc Whitehead Scientific (Pty) Ltd., USA). The suspension was then allowed to settle by gravity for 20 minutes before the supernatant was removed and discarded. This washing step (wash 1) was repeated two more times (wash 2 and 3) and for each wash, aliquots of 20 µl were kept aside at 4°C for later

analysis by SDS-PAGE. In addition, and after washing, a 20 µl aliquot of the washed bound beads was also collected and kept at 4°C for further analysis by SDS-PAGE.

5.2.4 Refolding of the Purified and Denatured Recombinant AtMAP-AC Protein

5.2.4.1 Preparation of the Refolding Column

The washed Ni-NTA affinity beads carrying the bound and purified recombinant AtMTA-AC protein were firstly resuspended in 10 column volumes (cv) of a 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 was then loaded into an empty XK16 column (Bio-Rad Laboratories Inc., California, USA) with the column tap at the bottom closed. The beads were then allowed to sediment by draining out 8 cv of the buffer through the tap before the loaded column was connected to a BioLogic DuoFlow Chromatography System (Bio-Rad Laboratories Inc., California, USA) for the initiation of a gradient refolding process.

5.2.4.2 The Chromatography Refolding Gradient System

A linearized refolding gradient system was created and the protocol subsequently ran on the BioLogic DuoFlow Chromatography System based on the parameters listed in Table 5.1 below. In this system, the starting concentration of 8 M urea lysis buffer or Buffer A 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 sulfobetaine, and 0.5 mM

PMSF). A refolding concept based on a decreasing urea concentration gradient is vital as it provides a gentle and easily controllable environment for protein re-naturation.

Table 5. 1: Conditions for the Refolding Process of the Recombinant AtMTA-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 time 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 weigh	2.00 cv
Length of gradient	600 min
Clean after gradient with (Washing Buffer)	10.00 ml
Elute protein with (Elution Buffer)	1.00 ml

5.2.4.3 Elution of the Refolded and Purified Recombinant AtMTA-AC Protein

The refolded and fully purified AtMTA-AC protein was eluted from the Ni-NTA affinity matrix by firstly, washing the bound beads in the column with 10 cv of washing buffer (200 mM NaCl, 50 mM Tris-HCl; pH: 8.0, 20% (v/v) glycerol, 0.5 mM PMSF), which removes any residual refolding agents from the refolded protein. The washed and refolded recombinant AtMTA-AC protein was then eluted in 2 ml elution buffer (200 mM NaCl, 50 mM Tris-HCl; pH 8.0, 250 mM imidazole, 0.5 mM PMSF, and 20% (v/v) glycerol), using an elution process that was directly

linked to the DuoFlow system. The resultant supernatant containing the eluted protein was then collected and kept at 4°C for further use.

5.2.4.4 De-salting and Concentration Determination of the Recombinant AtMTA-AC

The eluted recombinant AtMTA-AC protein sample was both concentrated and de-salted by transferring the whole 2 ml eluent to the upper chamber of the Corning® Spin-X UF concentrator device with a molecular weight cut off (MWCO) of 3.0 kDa (Product # 431482, Corning Life Sciences, USA) and spun down at 2540xg, at 4°C using a swing-out bucket rotored Hermle Z300k centrifuge (Hermle Labortechnik, Germany) and monitored until the final volume of the protein sample was at about 0.2 ml. The concentrated and de-salted protein fraction was then transferred into a sterile 1.5 ml Eppendorf tube and protein concentration determined with a 2000 Nanodrop Spectrophotometer (Thermo Scientific Inc., Massachusetts, USA). A small fraction (20 µl) of the eluted protein fraction was then set aside for analysis by SDS-PAGE while the rest was stored at -20°C for further downstream use.

5.2.5 Characterization of the Enzymatic Activity of the Recombinant AtMAP-AC Protein

Functional characterisation of the AC activity of the purified recombinant AtMTA-AC protein was determined *in vitro* by assessing for its ability to convert ATP into cAMP in the presence of various molecular and chemical components known to affect ACs. The assessment was carried out using the cAMP-linked enzyme immunoassaying system based on its acetylation protocol and in accordance with the manufacturer's instructions (Catalogue # CA201; Sigma-Aldrich Inc.,

Missouri, USA). Essentially, this characterization process was determined by setting up a 200 μ l reaction containing 10 μ g of the purified recombinant AtMTA-AC, 50 mM Tris-HCl; pH 8.0, 2 mM IBMX, 5 mM Mg^{2+} and/or 5 mM Mn^{2+} , 1 mM GTP, 1M $NaHCO_3$, 100 μ M Ca^{2+} , 1 mM ATP and/or 1 mM GTP (for detail refer to section 5.2.5.1 and Table 5.2 below). Detection and quantification of the residual cAMP levels was done by setting a control reaction containing all incubation components except for the AtMTA-AC protein. Measurements were then undertaken at 405 nm in triplicate sets ($n = 3$) using the Microplate Reader (Labtech, International Limited East Sussex, UK) and results subjected to statistical analysis – one-way analysis of variance (ANOVA) (Super-Anova, Stats Graphics Version 7, 1993, Stats Graphics Corporation, USA).

5.2.5.1 Samples Preparation and Enzyme Immunoassaying

Several 200 μ l reaction mixes were prepared in sterile 1.5 ml microfuge tubes and as is detailed in Table 5.2 below. Each tube was made to contain 50 mM of Tris-HCl (pH: 8.0) for buffering purposes as well as 2 mM of IBMX to prevent the degradation of cAMP by phosphodiesterases (PDE). Each reaction mix was then incubated at room temperature for 20 minutes and the reaction system stopped by adding 1 mM EDTA to chelate out all the divalent metal ions and hence removing the co-factors necessary for the enzymatic action of the recombinant AtMTA-AC. Additionally, the samples were boiled at 100°C for 5 minutes in order to inactivate the protein. The inactivated samples were then clarified by centrifugation at 16,300 $\times$ g for 5 minutes. Their cAMP contents were then acetylated by adding the acetylating reagent (2:1 trimethylamine to acetic anhydride (v/v)) at a volumetric ratio of 1:20 acetylating reagent to sample. The

acetylated reaction solutions were then vigorously mixed through vortexing at high speed for 2 seconds and their inherent cAMP levels subsequently measured by enzyme immunoassaying.

Table 5.2: Molecular Characterisation of the Recombinant AtMTA-AC Protein.

	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5	Tube 6	Tube 7	Tube 8
Tris	50 mM	50 mM	50 mM	50 mM	50 mM	50 mM	50 mM	50 mM
IBMX	2 mM	2 mM	2 mM	2 mM	2 mM	2 mM	2 mM	2 mM
MnCl₂	5 mM	5 mM	-	5 mM	5 mM	5 mM	5 mM	5 mM
Protein	-	25 µg	25 µg	25 µg	25 µg	25 µg	25 µg	25 µg
ATP	1 mM	1 mM	1 mM	1 mM	1 mM	1 mM	-	1 mM
MgCl₂	-	-	5 mM	-	-	-	-	-
CaCl₂	-	-	-	250 µM	-	-	-	-
GTP	-	-	-	-	-	1 mM	1 mM	-
NaHCO₃	-	-	-	-	50 mM	-	-	-
NaF + AlCl₃	-	-	-	-	-	-	-	10 + 30 µM

5.2.5.2 Statistical Analysis of Enzyme Immunoassaying Data

The results of the enzyme immunoassaying system were all based on the means of three replicates, whereby all outcomes of each assay were subjected to one-way 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 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/Insolubility Nature of the Recombinant AtMTA-AC

Escherichia coli is a bacteria of choice for the expression of recombinant proteins due to its many advantages such as easy growth on cost-effective carbon sources, rapid biomass accumulation, and relative ease for increasing the production scale (Makrides, 1996). However, protein expression in a prokaryotic system such as this usually results in the accumulation of the protein product in inactive insoluble deposits inside the cells, called inclusion bodies. In this study and after expressing the AtMTA-AC recombinant protein in *E. coli* EXPRESS BL21 (DE3) pLysS expression cells, its expression status was first assessed to establish if it was in its soluble or insoluble form before the purification process was undertaken. As is shown below in Figure 5.1 the AtMTA-AC recombinant was wholly expressed as an insoluble product.

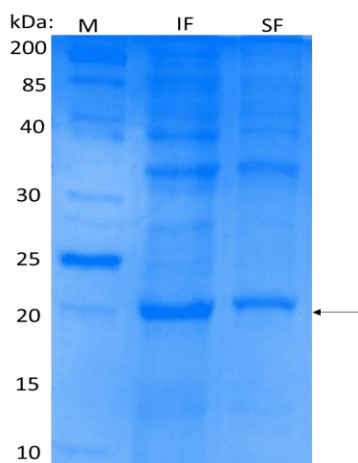


Figure 5.1: Determination of the Solubility or Insolubility Nature of the Recombinant AtMTA-AC Protein. An SDS-PAGE of the recombinant AtMTA-AC protein fractions collected from the pellet and supernatant of the cell content. In this presentation, M represents the unstained protein standard, broad range marker (BioLabs Intc, New England), IF represents the insoluble fraction on the cells content, SF represent the soluble fraction on the cell content. The arrow marks the AtMTA-AC recombinant protein that is wholly expressed as an insoluble product.

5.3.2 Purification of the Recombinant AtMTA-AC Protein

After determining that the recombinant AtMTA-AC protein was entirely expressed in the form of insoluble inclusion bodies as shown in Figure 5.1 this insoluble poly-histidine C-tagged fusion protein was then purified under non-native denaturing conditions using a Ni-NTA affinity matrix system (Hochuli *et al.*, 1987) and based on the manufacturer's protocol (Catalogue # P6611; Sigma-Aldrich Inc., Missouri, USA) and as is shown in Figure 5.2 below.

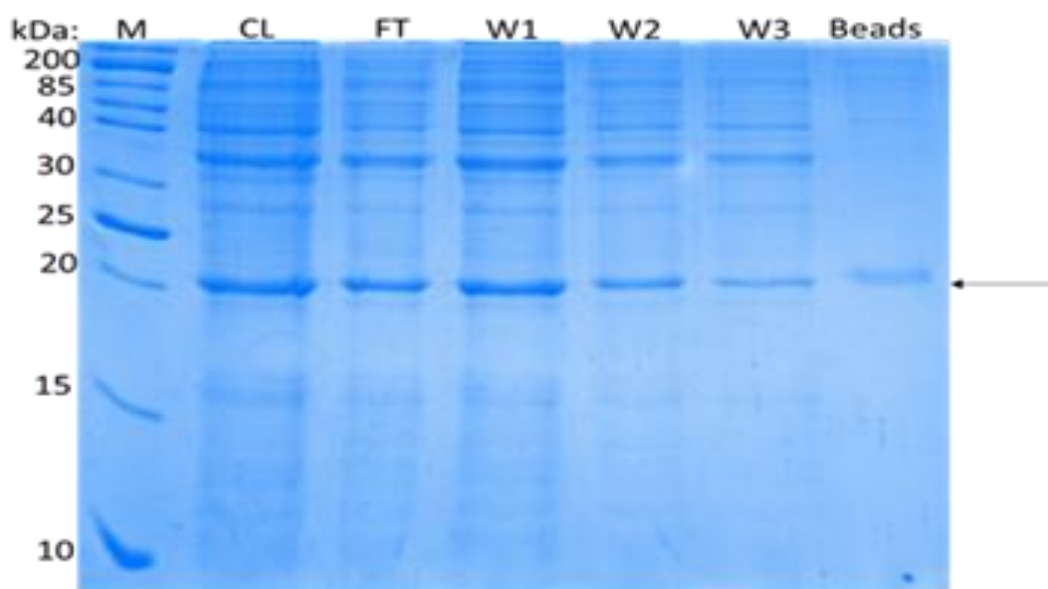


Figure 5.2: Purification of the Recombinant AtMTA-AC Protein. An SDS-PAGE of the recombinant AtMTA-AC protein fractions collected at the different stages of its purification process using the Ni-NTA affinity matrix system (Sigma-Aldrich Inc., Missouri, USA). In this presentation, M represents the unstained protein standard, broad range marker (BioLabs Intc, New England), CL represents the cleared cellular lysate before its passage through the Ni-NTA affinity matrix, FT represents the flow-through of the cleared lysate after it was passed through the Ni-NTA matrix, W1 is the first wash of the bound AtMTA-AC recombinant protein onto the Ni-NTA affinity matrix with the wash buffer, W2 and W3 are the second and third washes respectively, and Beads is the purified and bound AtMTA-AC recombinant protein. The arrow marks the resultant purified AtMTA-AC recombinant protein product.

5.3.3 Refolding and Chemical Elution of the Purified Recombinant AtMTA-AC Protein

Subsequent to the success of purifying the AtMTA-AC protein by the Ni-NTA affinity matrix system under non-native denaturing purification conditions, it was thus imperative that the denatured and non-functional protein be restored into its native and biologically active molecule state. This was accomplished by using the DuoFlow BioLogic system (Bio-Rad Laboratories Inc., California, USA), where beads containing the bound and purified AtMTA-AC protein were refolded by a linearized refolding gradient system. This system utilized, an 8 M urea buffer into which the recombinant AtMTA-AC protein was initially suspended whereby it was slowly diluted over a 600-minute time period with a non-denaturing buffer to a final concentration of 0 M urea and thereby refolding the protein back into its native active state. The refolded AtMTA-AC protein was then eluted off the Ni-NTA affinity matrix in 2 ml Elution Buffer (200 mM NaCl, 50 mM Tris-HCl; pH 8.0, 250 mM imidazole, 0.5 mM PMSF, and 20% (v/v) glycerol), using an elution process that was directly linked to the DuoFlow system. The resulting supernatant containing the eluted protein was then de-salted and concentrated. When analysed by SDS-PAGE, the eluted refolded purified AtMTA-AC was resolved within its expected size range of approximately 22.8 kDa (Figure 5.3 below).

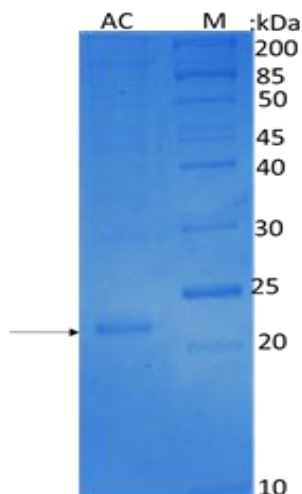


Figure 5.3: Refolding and Chemical Elution of the Recombinant AtMTA-AC. An SDS-PAGE of the refolded purified recombinant AtMTA-AC protein fraction eluted off the Ni-NTA affinity matrix with 250 mM imidazole, where AC is the refolded purified AtMTA-AC recombinant protein and M is the unstained low molecular weight marker (Fermenters Int., Burlington, Canada). The arrow is marking the resultant purified and chemically eluted recombinant AtMTA-AC protein.

5.3.4 Characterization of the Enzymatic Activity of the Recombinant AtMTA-AC Protein

The AC activity of the resultant refolded purified recombinant AtMTA-AC protein was then further functionally characterized *in vitro* and via enzyme immunoassaying, based on its ability to generate cAMP from ATP in the presence of other various chemical elements (GTP, Mg^{2+} , Mn^{2+} , Ca^{2+} , CO_3^{2-} , and F^-) that are known to influence AC activity. The detailed outcome of such an assessment is clearly presented below in Figure 5.4.

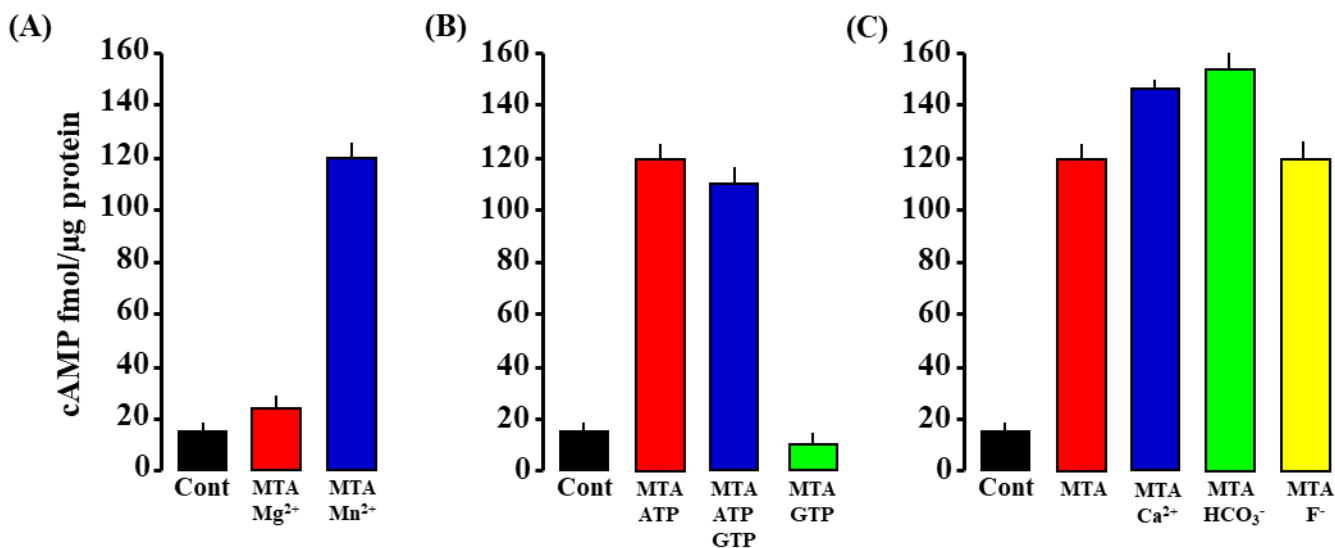


Figure 5.4: Molecular Characterization of the Enzymatic Activity of the Recombinant AtMTA-AC Protein. A reaction mixture containing 10 μg of the refolded purified recombinant AtMTA-AC protein, 50 mM Tris-Cl, pH: 8.0, 2 mM IBMX, 5 mM Mn²⁺, 1 mM ATP and/or other additives was prepared and incubated at room temperature for 20 minutes. The generated cAMP levels were then measured with a cAMP-specific enzyme immunoassaying kit (Sigma Aldrich Inc., Missouri, USA) based on its acetylation protocol. (A) cAMP levels generated with the purified recombinant AtMTA-AC protein in the presence of Mg²⁺ or Mn²⁺ ions, (B) cAMP levels generated with the purified recombinant AtMTA-AC protein in the presence of either ATP and/or GTP when Mn²⁺ ion is the co-factor, and (C) cAMP levels generated with the purified recombinant AtMTA-AC protein in the presence of either calcium, bicarbonate or fluoride ions when Mn²⁺ ion is the co-factor. All assays were undertaken in triplicate sets and the error bars represent the standard errors of the triplicate means (n = 3).

5.4 DISCUSSION

Heterologous gene expression in *Escherichia coli* has been conducted for many years due to the ease with which this bacterium can be transformed into various plasmids and tested for the desired growth phenotypes and/or screened using DNA colony hybridization techniques - thus making it the organism of choice for gene cloning (LaVallie and McCoy, 1995). In our study, an

E. coli EXPRESS BL21 (DE3) pLysS expression cell colony harbouring the pTrcHis-TOPO:AtMTA-AC fusion expression construct was cultured and subsequently induced with 4 mM IPTG to over-express the AtMTA-AC recombinant protein. Following this expression, the solubility and/or insolubility nature of the expressed protein was then undertaken to determine whether the recombinant AtMTA-AC protein was or not expressed in the form of an insoluble product termed inclusion bodies. Formation of inclusion bodies indicates limitation of *E. coli* to completely remove the amino-terminal initiator methionine residues, thus the formation of insoluble aggregates of the recombinant proteins in the bacterial cytoplasm. As is shown in Figure 5.1, the recombinant AtMTA-AC protein was wholly expressed in form of those inclusion bodies that are insoluble and non-functional.

Therefore, the strategy that was then used to address this limitation was to carry out an affinity protein purification process under non-native denaturation conditions using the Ni-NTA affinity matrix system. Urea salt was used as the denaturant and just like GnCl, it can denature tagged proteins but still at the same time, allowing such proteins to carry out their affinity binding onto selected columns such as the Ni-NTA system (Hochuli *et al.*, 1987). The His-tagged recombinant AtMTA-AC protein was then successfully bound and purified using this approach and as is shown in Figure 5.2 The purification of recombinant proteins by the use of affinity tags is a useful and an extensively applied technology (LaVallie and McCoy, 1995), as it allows for the protein contaminants to be effortlessly washed off the bound protein on the affinity matrix, even under the strongly and harsh denaturing conditions required to solubilise the inclusion bodies (Crowe *et al.*, 1994).

After purification, a specialized refolding system (Singh and Panda, 2005) (Table 5.1), was then used to restore the native and functional status of the denatured and non-functional AtMTA-AC

protein. Actually, the DuoFlow BioLogic system (Bio-Rad Laboratories Inc., California, USA) is the one that was used to refold the denatured protein, in a process that involved a controlled linearized gradient system. In this gradient system, a high concentration of a denaturant (e.g. 8 M urea or 6 M GnCl) is slowly reduced to 0 M concentration and thus facilitating protein refolding and functional regaining. After refolding, the renatured purified AtMTA-AC recombinant was then eluted off the Ni-NTA affinity matrix (Figure 5.3) for subsequent use in the intended functional characterization assays.

After refolding, the ability of the eluted recombinant AtMTA-AC protein to generate cAMP from ATP *in vitro* and in the presence of other various chemical elements such as GTP, Mg^{2+} , Mn^{2+} , Ca^{2+} , CO_3^{2-} , and F^- was assessed. Firstly, the enzymatic activity of the recombinant AtMTA-AC protein was assessed with regard to Mg^{2+} or Mn^{2+} metal ions as co-factors for enzymatic activity and it was found that the protein activity was more dependent on Mn^{2+} (120 fmols) than Mg^{2+} (20 fmols) metal ion as co-factor (Figure 5.4). This outcome is not unusual because in a previous study done by Al-Younis *et al* (2018), using another Arabidopsis recombinant protein, AtKUP7 with AC activity, it was shown that this other protein could generate about 42.5 fmols of cAMP in the presence of Mn^{2+} and 27.0 fmols of cAMP in the presence of Mg^{2+} . Additionally, it was also shown in another study by Chatukuta *et al* (2018), that another related Arabidopsis protein, AtCIAP-AC, could generate about 110.0 fmols of cAMP when Mn^{2+} metal ion was the co-factor of activity and approximately 31.0 fmols of cAMP when Mg^{2+} metal ion was the co-factor of activity. These findings thus closely correlate with our study, where the Mg^{2+} metal ion had no apparent effect onto the enzymatic activity of the recombinant protein AtMAP-AC while the Mn^{2+} metal ion significantly enhanced its enzymatic activity to a factor of approximately 6-fold (Figure 5.4A). Further studies undertaken

by Steer and Levitzki (1975) also further affirmed our findings that despite the fact both the Mg^{2+} and Mn^{2+} metal ions can at times be able to activate other known ACs in various organisms, (Tesmer *et al.*, 2000; Geng *et al.*, 2005), the recombinant AtMTA-AC protein is strictly dependent onto the Mn^{2+} metal ion as its sole co-factor for enzymatic activity. Additionally, Braun and Dods (1975), also showed a sAC from the rat testis that was strictly Mn^{2+} -dependent

After establishing the co-factor preference for the AtMTA-AC recombinant, its substrate specificity with regards to two structural analogs, ATP and GTP, was also tested. The generated cAMP levels from ATP were 6.5 times more than the levels generated from GTP thus indicating the strict specificity of the AtMAP-AC for ATP than GTP. On the other hand, the presence of GTP in an ATP reaction (ATP/GTP) had an apparent effect of decreasing the cAMP production with a factor of about 4-fold (Figure 5.4B), suggesting a competitive effect between the two analogs for binding onto the catalytic center but not for catalysis. These findings of ours closely correlate with those of Chatukuta *et al* 2018, where it was shown that ATP alone could increase the levels of cAMP production with a factor of approximately 7-fold while GTP alone drastically decreased the cAMP production by almost 33%. Our study indicates that, even though the GTP could have had been structurally competing with ATP for the AtMTA-AC binding site, ATP is typically the preferred substrate for the AtMTA-AC protein.

After establishing both the co-factor preference and substrate specificity of the recombinant AtMTA-AC, its activity was then further assessed against various ionic and molecular components naturally known to affect ACs. As is shown in Figure 5.4C, it was found that the recombinant AtMAP-AC protein could significantly cyclize ATP into cAMP in the presence of both Ca^{2+} and CO_3^{2-} ions as its functional modulators than when the fluoride ion was a

modulator. The presence of calcium ions may lead to an increase in activity of the AtMTA-AC recombinant protein perhaps as a result of the responsive activities of the cyclic nucleotide-gated ion channels (CNGCs), which play a very central part in the development of plants through specialized signalling pathways that involve cyclic nucleotides (Talke *et al.*, 2003). According to previous studies, calcium ions have been shown as second messenger molecules that activate physiological changes in plants by defining mechanisms in response to abiotic stimuli such as oxidative stresses and phytohormones that then result in signalling transduction (Ma and Berkowitz, 2007; McAinsh and Pittman, 2009). Previous studies done in support of this idea, reported that the modification of cAMP levels was always accompanied by an increase in Ca^{2+} , which might suggest that the two signaling pathways are interconnected (Malhó *et al.*, 2000).

The recombinant AtMTA-AC also displayed an increase in cAMP production in the presence of carbonate ions therefore, suggesting the positive stimulatory effects of these ions on the enzymatic activity of protein. Notably, this finding was not surprising at all since hydrogen carbonate is known to increase cAMP generation by several AC molecules in a pH-independent manner, which is noted to directly modulate the enzymes (Chen *et al.*, 2000). Finally, the AtMTA-AC protein fragment was characterised with respect to its modulation by the fluoride ion. The results revealed that this ion has no significant effect onto the catalytic activity of the AtMTA-AC protein (Figure 5.4C). Finally, the AtMTA-AC protein fragment was characterised with respect to its modulation by the fluoride ion. The results revealed that this ion has no significant effect onto the catalytic activity of the AtMTA-AC protein (Figure 5.4C).

Nonetheless, all these observations presented in Figure 5.4 are expected because in all living organisms, the AC system is characterized by its two forms, the soluble AC (sAC) and the transmembrane AC (tmAC) (Kamenetsky *et al.*, 2006), both of which are also well represented in the

plant system (Lomovatskaya *et al.*, 2008). It is known that all sACs are functionally activated by the calcium and bicarbonate metal ions (Garty and Salomon, 1987; Carricarte *et al.*, 1988; Chen *et al.*, 2000; Visconti *et al.*, 1990) but not the fluoride ion and forskolin, which are the main activators of all tmACs (Rall and Sutherland, 1958; Robison *et al.*, 1971; Wuttke *et al.*, 2001; Newton and Smith, 2004). In addition, the activity of all sACs is strictly dependent onto the Mn^{2+} metal ion as a co-factor (Braun and Dods, 1975) as compared to tmACs that flexibly depend on both the Mn^{2+} and Mg^{2+} metal ions (Zippin *et al.*, 2004). Furthermore, even though all tmACs are mediated by the second messenger, cAMP via control mechanisms that are typically regulated by the GTP-binding protein, all sACs are mediated by the second messenger, cAMP via control mechanisms that are specifically regulated by the calcium-binding protein, calmodulin (Kamenetsky *et al.*, 2006).

5.5 CONCLUSION

Our findings in this chapter show that the recombinant AtMTA-AC protein is indeed a functional sAC, whose activity is essentially modulated by the Mn^{2+} , Ca^{2+} and CO_3^{2-} ions while, on the other hand, it is also totally insensitive to the Mg^{2+} , and F^- molecular ions. Thus, the physiological and biochemical activities of the MTA protein in *A. thaliana* or other related plants may be mediated by cAMP possibly, via a calmodulin-dependent signalling pathway.

CHAPTER SIX

CONCERTED BIOINFORMATIC EXPRESSIONAL ANALYSIS OF THE AtMTA PROTEIN

ABSTRACT

A combination of various web-based computational tools was used in an attempt to bioinformatically elucidate the probable physiological roles of the AtMTA protein encoded by the At3g02930 gene in *Arabidopsis thaliana* and other related higher plants. Observations made from analysis indicated that this putative gene is co-expressed with three genes, which are the At5g52280 and At1g64330 that function as myosin heavy chain-related proteins and the At3g53350 which functions as a repressor of primer (ROP) interactive partner 4. Ideally, since co-expressed genes are generally expected to be involved in related and/or similar cellular functions, the AtMTA gene may potentially be a myosin heavy chain-related protein and/or a ROP interactive partner 4. Myosin heavy chain-related proteins are essentially involved in reciprocal meiotic recombinations, regulation of starch biosynthesis and starch biosynthetic processes, thus our gene of interest in this study could as well be implicated in these specific cellular processes.

6.1 INTRODUCTION

Gene expression profiling is an important tool in functional bioinformatics. Over the past decade, DNA microarrays has emerged as the foremost transcript profiling technology in the global analysis of biological systems (Cao *et al.*, 2004; Peng *et al.*, 2003). mRNA of any cell, under a given set of conditions, can be used to provide DNA microarray data of the level of expression of all genes in the cell. This data can be used to study a variety of biological processes such as development or evolution, and additionally, it can be used to determine the function of new genes such as the At3g02930 gene that encodes a microtubule associated (MTA) protein studied in this work. The At3g02930 was retrieved in 2010 by Gehring using a 14-amino acid long guanylate cyclase catalytic centre search motif modified for specificity for ATP binding and with a C-terminal metal-binding residue ([RK][YFW][DE][VIL][FV]X(8)[KR]X(1,3)[DE]) to conduct a BLAST search of the Arabidopsis genome. This motif search retrieved over fourteen putative AC candidate genes, including the At3g02930. Furthermore, DNA microarrays data can also be used to gather information on the role that individual genes may play in plants life cycles and to monitor the effect of biotic and abiotic stress factors on gene expression (Lee *et al.*, 2004).

Several improvements have been made in the gene expression profiling of *A. thaliana* due to the availability of its whole genome sequences and thus, DNA arrays constructed with oligonucleotides are a suitable tool for quantitative monitoring of gene expression on a large scale. For *A. thaliana*, the techniques that have been used predominately included cDNA glass microarrays, membrane-spotted microarrays, serial analysis of gene expression (SAGE) and oligonucleotide-based arrays (Donson *et al.*, 2002). In this chapter we profiled genes that were directly connected with the At3g02930 gene on the microarray network.

In addition, we also detail the co-expression of the AtMTA gene, which is fundamentally important as it offers essential information to better understand complex biological systems (Eisen *et al.*, 1998). In co-expression of genes, we study genes that are assumed to have related expression patterns across a set of samples and are suggested to have a functional relationship (Eisen *et al.*, 1998). Several studies have shown that co-expression is interconnected with functional relationships such as physical interaction between the encoded proteins (Kemmeren *et al.*, 2002). Biological functions of genes are characteristically determined by collaborations of gene products or proteins, and therefore, genes in related biological processes are frequently expressed cooperatively. Co-expression databases for plant species such as ATTED-II and GENEVESTIGATOR were used to provide a parallel view of multiple co-expression data sets with the network analysis tools (Jansen *et al.*, 2002).

6.2 RESEARCH METHODOLOGY

6.2.1 Expression Profile of the AtMTA Protein

In an attempt to conduct the bioinformatic analysis of the AtMTA protein and determination of its possible and/or exact physiological roles in *A. thaliana*, a number of bioinformatic web-based servers and programs were applied. GENEVESTIGATOR Version V3 (www.genevestigator.com/gv/) (Zimmermann *et al.*, 2004), was utilised to reveal the expression intensities of the At3g02930 gene in 111 tissues of the *A. thaliana*. Transcriptome information from the Affymetrix Arabidopsis ATH1 Genome Array platform using the 260011_At probe was conducted to provide data that was used in this analysis.

6.2.2 Anatomical Expression Analysis of the AtMTA Protein

The gene expression omnibus (GEO) database was used to illustrate the levels of expression of the gene of interest, which stores some gene expression profiles derived from curated GEO DataSets. Respectively, each profile is presented as a chart that shows the expression level of one gene across all samples within a DataSet. Experimental context is provided in the bars along the bottom of the charts, and making it possible to get a glance of whether or not a gene is differentially expressed across the different experimental conditions (Edgar *et al.*, 2002).

6.2.3 Developmental Expression Analysis of the AtMTA Protein

Genevestigator database was used in order to determine the expressional levels of the At3g02930 gene at different stages of its development, whereby the gene was queried against 10 developmental stages of the plant. Such developmental stages were set from the radicle emerging from the seed coat up to the release of the mature seeds from the pod (Zimmermann *et al.*, 2004).

6.2.4 Co-expressed Protein Network Around the AtMTA Protein (Microarray)

In order to determine the co-expressional levels of the AtMTA protein with the other *A. thaliana* proteins, the ATTED-II (<http://atted.jp>) co-expression database for plant species was used to detail relationships between the genes directly co-expressed with At3g02930 gene on the microarray network. ATTED-II (version 9) is a reliable tool as it provides 16 co-expression platforms for 9 plant species, which includes 7 species that are supported by both microarray-

and RNA sequencing (RNAseq)-based co-expression data. Furthermore, the assessment of the reproducibility of co-expression is enabled by two independent sources of co-expression data. The latest co-expression data for Arabidopsis (Ath-m.c7-1 and Ath-r.c3-0) showed the highest reproducibility (Jaccard coefficient = 0.13) among previous co-expression data in ATTED-II.

6.2.5. Stimulus-specific Microarray Expression Analysis of the AtMTA Protein

The expression profiles of the AtMTA-ECGG50 were initially screened over all of the available ATH1:22K array Affymetrix public microarray data in the Genevestigator V3 version (<https://www.genevestigator.com>) using the stimulus/perturbations tool (Zimmermann *et al.*, 2004). In order to achieve greater resolution of gene expression profiles, the normalized microarray data were subsequently downloaded and analyzed for experiments that were found to induce differential expression of the genes. The data was downloaded from the following repository sites: GEO (NCBI) <http://www.ncbi.nlm.nih.gov/geo/>), (NASCArrays (<http://affymetrix.arabidopsis.info/narrays/experimentbrowse.pl>) and TAIR-ATGenExpress (<http://www.ebi.ac.uk/microarray-as/ac/>). The array data was then analyzed and fold-change (log2) values calculated for each experiment. Expression values were then generated using the Multiple Array Viewer program from the Multi-Experiment Viewer (MeV) software package (version 4.2.01) created by The Institute for Genomic Research (TIG).

6.3 RESULTS

6.3.1. Analysis of the Expression Profile of the AtMTA Protein

Expression analysis of the At3g02930 gene using Genevestigator showed that the AtMTA protein is significantly expressed in 111 plant tissues of the Arabidopsis plant. Notably, the protein is highly expressed in the giant cells and suspensor and lowly expressed in the petal and the nonmicropylar endosperm Figure 6.1.

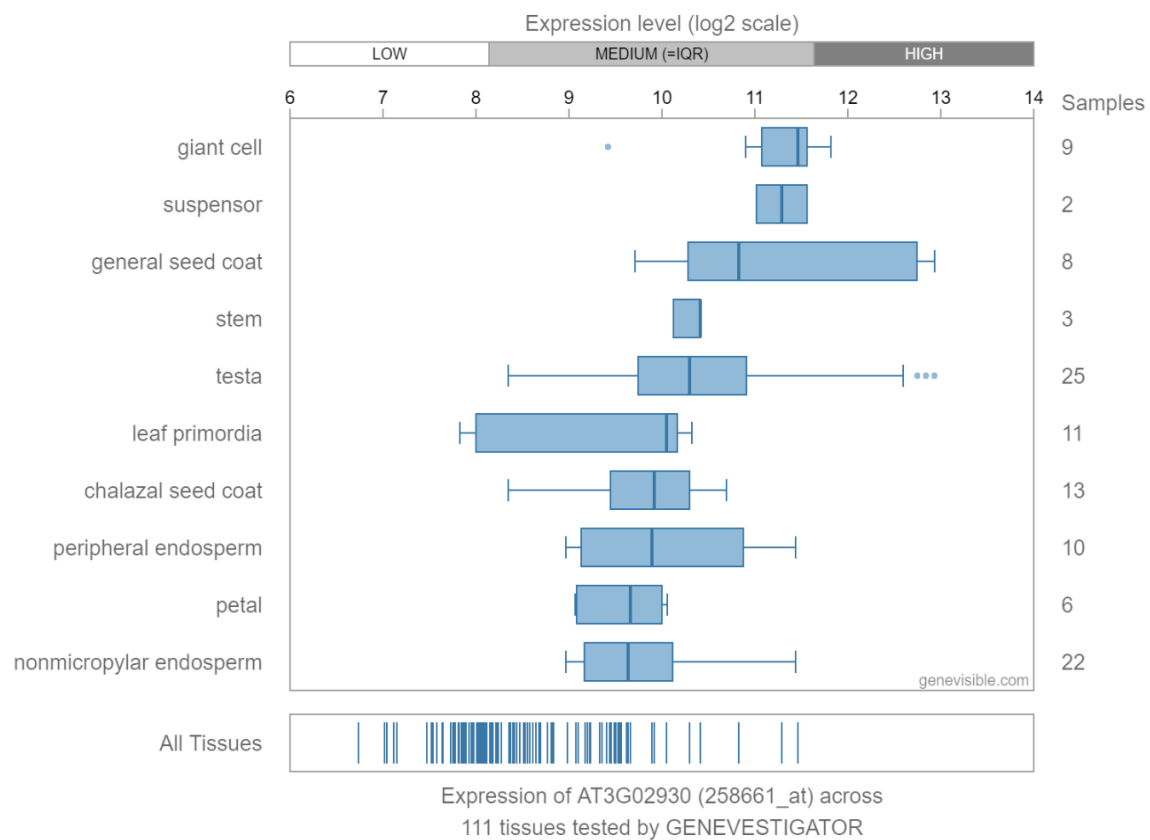


Figure 6.1: Expression intensity levels of the AtMTA protein in various tissues of the Arabidopsis plant. The presentation shows expression intensity levels of the At3g02930 gene in various parts of the Arabidopsis. The presentation shows considerably elevated expression levels in the giant cell and the suspensor, and moderately in the stem and testa, and low levels in the petal and the nonmicropylar endosperm (data obtained from the Genevestigator anatomy tool (Zimmermann *et al.*, 2004)).

6.3.2 Analysis of the Anatomical Expression of the AtMTA Protein

After determining the expression profile of the AtMTA protein, analysis of the At3g02930 gene expression in Arabidopsis leaf, stem and flower tissues was then undertaken (Edgar *et al.*, 2002). As is shown in Figure 6.2 and show Table 6.1 below, the AtMTA protein is highly expressed in the stem tissue, moderately expressed in the flower tissue and least expressed in the leaf tissue.

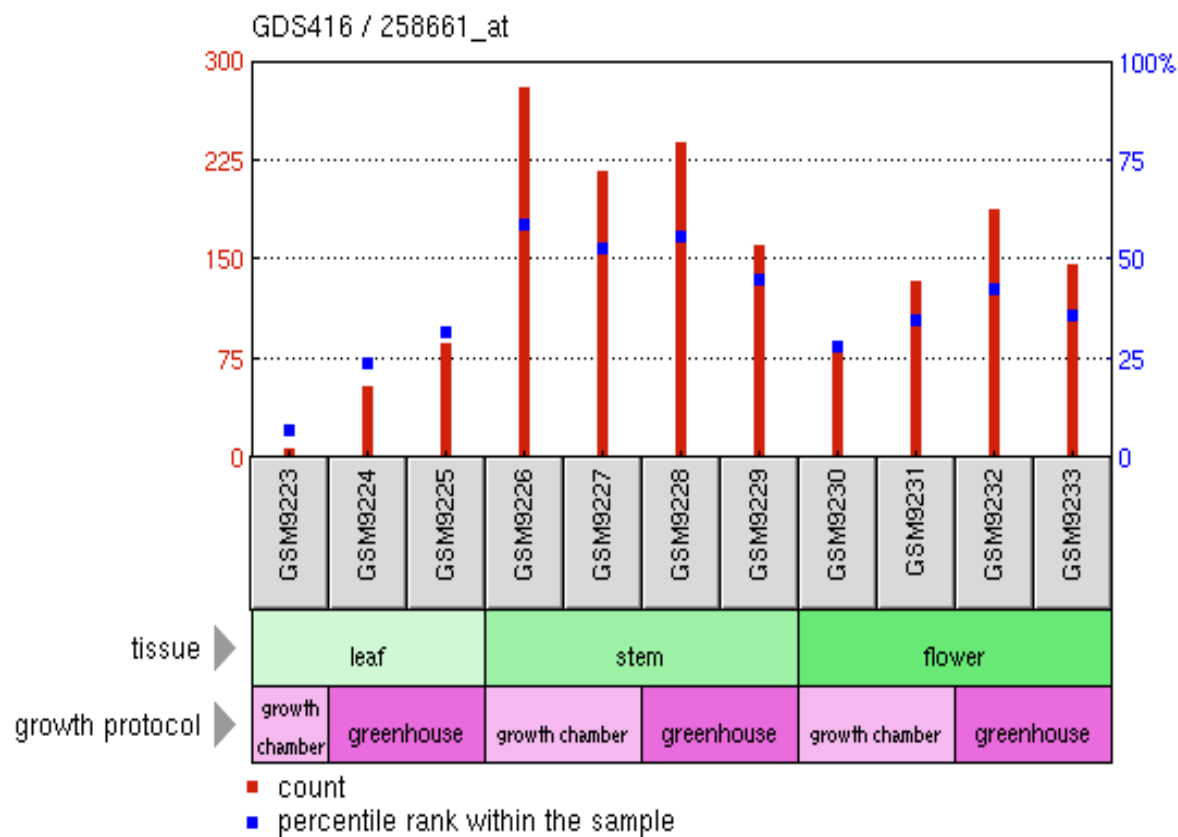


Figure 6.2: Expression levels of the AtMTA protein across the leaf, stem, and flower tissues of *A. thaliana*. Red column: Each column represents the expression measurement extracted from the value column of one original submitter-supplied sample record. The original sample accessions (GSMxxx) are listed in the grey boxes along the bottom of the chart. Blue square: Represents rank order of expression measurements. All values within a sample are rank ordered, and then placed into percentile, the blue rank squares on charts give an indication of where the expression of that gene falls with respect to all other genes on that array.

Table 6.1: Expression levels of the AtMTA protein across the leaf, stem, and flower tissues of *A. thaliana*.

Sample	Title	Value	Rank
GSM9223	Leaf_GC2	8.2	7%
GSM9224	Leaf_GH1	54.4	24%
GSM9225	Leaf_GH2	88.5	32%
GSM9226	STEM_GC7	281.6	59%
GSM9227	STEM_GC8	217.6	53%
GSM9228	STEM_GH7	240.2	56%
GSM9229	STEM_GH8	161.2	45%
GSM9230	FLOWER_GC5	85.4	28%
GSM9231	FLOWER_GC6	134.6	35%
GSM9232	FLOWER_GH5	188.6	43%
GSM9233	FLOWER_GH6	147.1	36%

6.3.3 Determination of the Developmental Expression of the AtMTA Protein

A comprehensive expressional profile of the At3g2930 gene during the different stages of development was obtained by using Genevestigator database, whereby screening using microarray data analysis was performed. The results shown in Figure 6.3 below, illustrate 10 developmental stages of the At3g02930 gene from data selection: AT_AFFY_ATH1-0, indicating that the AtMTA protein is highly expressed during the flowering stage of the plant and least expressed in the seed pots.

Dataset: 10 developmental stages from data selection: AT_AFFY_ATH1-0
Showing 1 measure(s) of 1 gene(s) on selection: AT-0

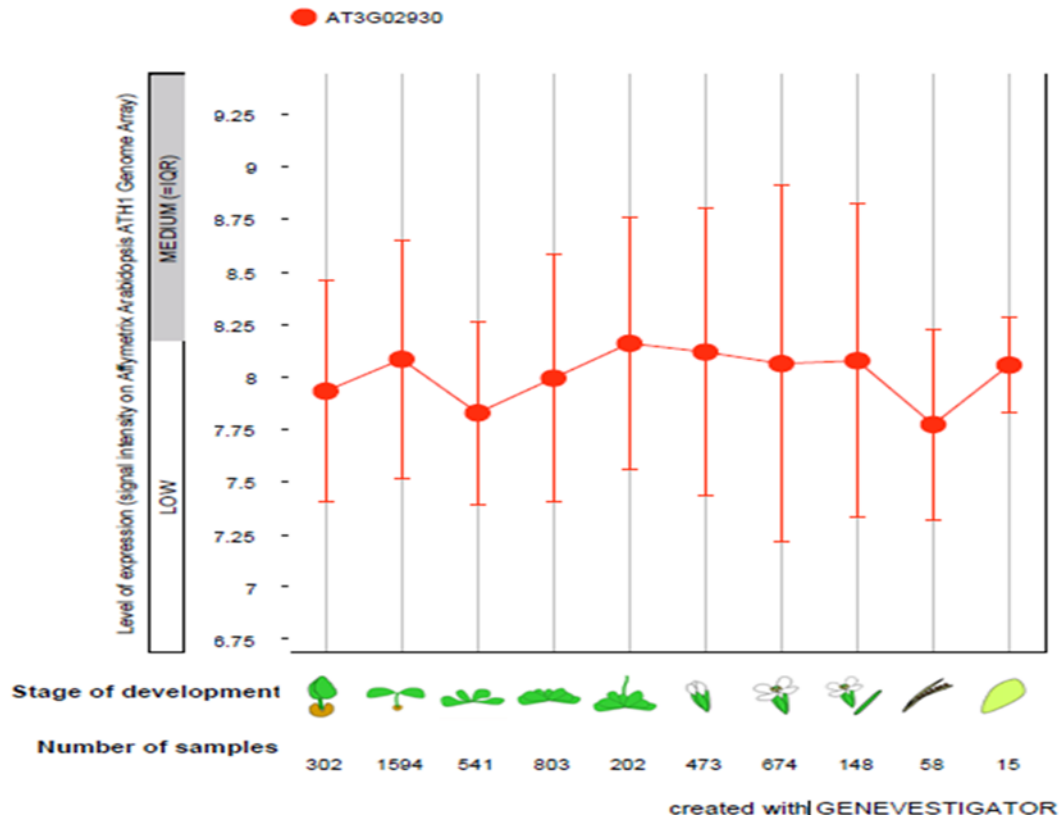


Figure 6.3: Ten developmental stages of the At3g02930 gene and its AtMTA protein from data selection: AT_AFFY_ATH1-0. The above data shows measures of expression levels in relation to the various stages of development beginning from the radicle emerging from the seed coat up to the release of the mature seeds from the pod (data obtained from the Genevestigator anatomy tool (Zimmermann *et al.*, 2004)).

6.3.4 Co-expressional Analysis of the AtMTA Protein

In order to determine other proteins that are co-expressed with the AtMTA protein, co-expression profile was assessed and determined using the ATTED-II (version 9) program. Apparently and in nature, the function of every gene depends on that of other genes and therefore in this case, our gene of interest, the At3g02930, was found to be directly connected to three

other genes on the microarray network as is shown below in Figure 6.4. These genes are the At5g52280 and At1g64330 that function as myosin heavy chain-related proteins and the At3g53350 which functions as a repressor of primer (ROP) interactive partner 4. Co-expressed genes are generally expected to be involved in related cellular functions, thus the AtMTA protein may potentially be a myosin heavy chain-related protein and/or a ROP interactive partner 4.

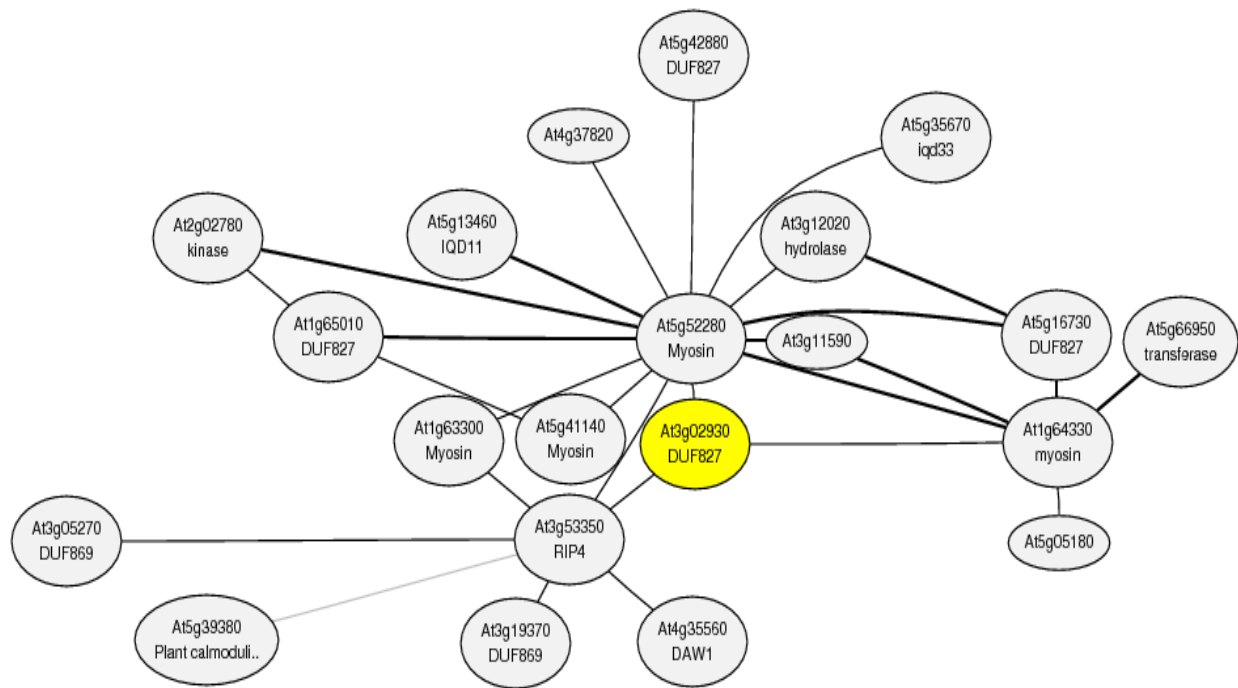


Figure 6.4: Co-expressed gene network around the At3g02930 gene. The query gene is highlighted in yellow while its co-expression partners are indicated in grey ((Microarray) data from ATTED II).

Table 6.2 below displays the mutual ranking of the three genes directly connected to the At3g02930 gene on the microarray network, of which amongst them, the At3g53350 gene, which functions as a repressor of primer (ROP) interactive partner 4, shows the highest mutual rank (MR) at 19.9%, which maybe an indication that the functionality of our gene of interest might be similar to that of the At3g53350 gene. According to TAIR, the At3g53350 gene is closely

related to the AtMTA, which is described by TAIR as a microtubule-binding protein that is anchored to the plasma membrane domains and promoting local microtubule disassembly, forming as specific patterns of secondary walls in the xylem vessel cells. On the contrary, the mutual rankings of the At5g52280 and At1g64330 genes are 12.1% and 14.3% respectively which are slightly lower than that of the At3g53350 gene.

Table 6.2: Genes directly connected with At3g02930 on the microarray network.

Mutual Rank (MR)	Locus	Function
12.1	At5g52280	Myosin heavy chain-related protein
14.3	At1g64330	Myosin heavy chain-related protein
19.9	At3g53350	ROP interactive partner 4

6.3.5 Determination of the Stimulus-specific Expression of the AtMTA Protein

After determining the co-expression status of the At3g02930 gene and its AtMTA protein, this gene and its 50 top-most co-expressed genes were subjected to an *in silico* global expression analysis against specific experimental conditions that are capable of inducing differential expression (Vuleta *et al.*, 2015). The heat map generated from the microarray expression analysis was in accordance with the co-expression and GO analysis of the At3g02930 gene, and revealed that the transcriptional processes of the At3g02930 gene and its associated AtMTA-ECGG50 set are generally and collectively induced in response to a wide range of biotic and abiotic stress factors as is shown in Figure 6.4 below.

Dataset: 35 perturbations from data selection: AT_AFFY_ATH1-0
Showing 1 measure(s) of 1 gene(s) on selection: AT-0

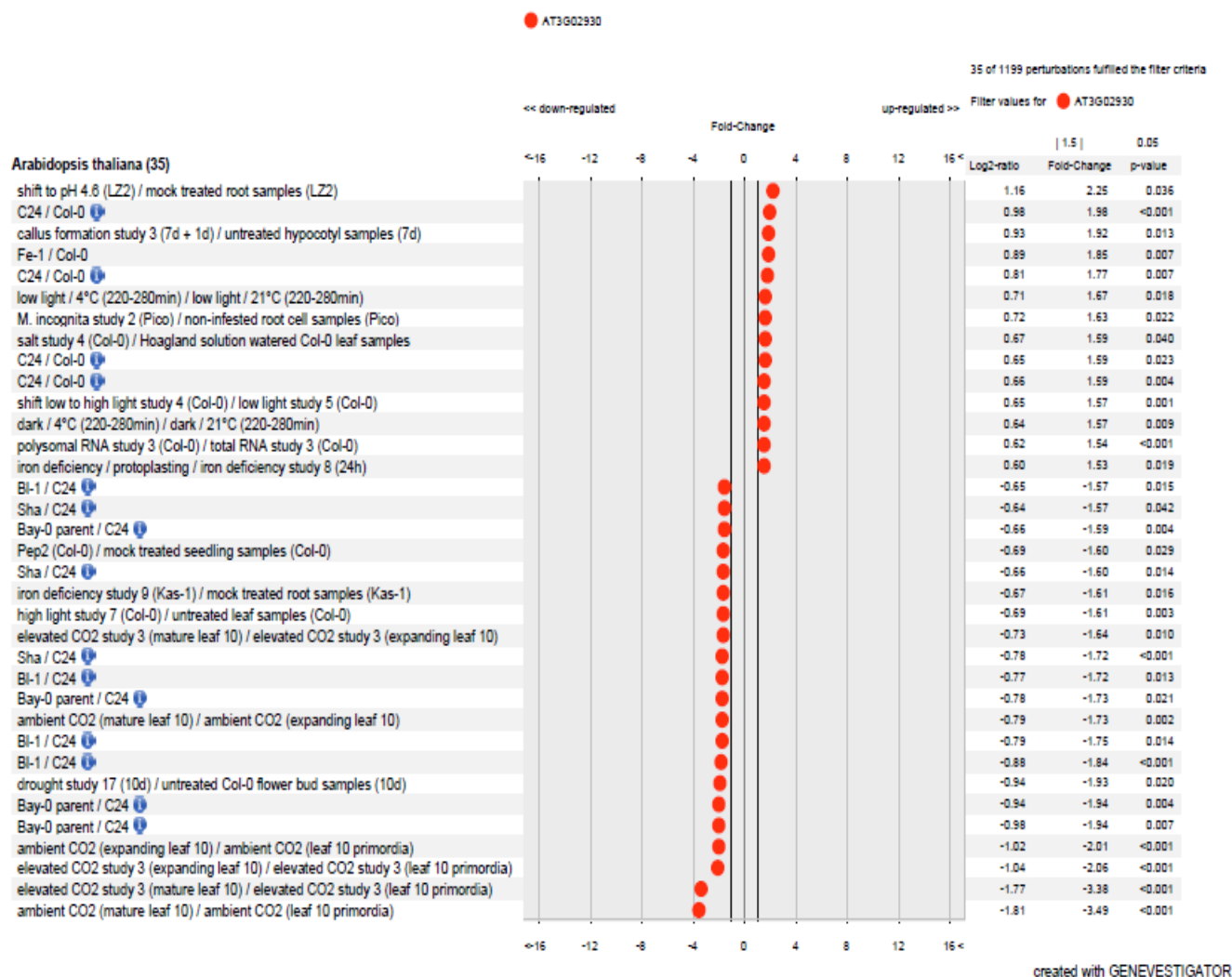


Figure 6.5: Expression profile of the AtMTA protein in *Arabidopsis thaliana* in response to various stimulus-specific conditions. The profile shows differential induced expression of the protein in response to a wide range of biotic and abiotic stress factors (data obtained from the Genevestigator anatomy tool (Zimmermann *et al.*, 2004)).

6.4 DISCUSSION

Results of the analysis of the expression profile of the AtMTA protein indicates that it is significantly expressed in 111 plant tissues of the Arabidopsis plant. Notably, it is highly expressed in the giant cells and suspensor and least expressed in the petal and the nonmicropylar endosperm Figure 6.1. Regarding anatomical expression analysis of the At3g02930 gene, GEO database was used to assess and illustrate the levels of expression of our gene of interest. Figure 6.2, displays the expression levels of the At3g02930 gene across three key plant tissues leaves, stems, and flowers, with expressional analysis indicating the At3g02930 gene is mainly expressed in the stem followed by the flowers and lastly, the leaf tissues. This is in line with some baseline experiments conducted by Liu *et al.* (2012) that showed that the At3g02930 gene has the highest expression levels in flowers and fruits at 28 and 27 TPMs respectively while the lowest expression levels are found in the leaves and roots at 4 and 8TPMs respectively (Liu *et al.*, 2012).

Furthermore, determination of developmental expression of the AtMTA protein was conducted using the Genevestigator database, microarray data analysis of the At3g02930 gene was screened and the results obtained indicates that during the 10 developmental stages of the At3g02930 gene, it is highly expressed during the flowering stage of the plant and least expressed in the seed pods Figure 6.3. With regards to co-expressional analysis of the At3g02930 gene that was undertaken and showed that the gene is directly co-expressed with three other genes on the microarray network, which are the At5g52280 and At1g64330 genes that function as myosin heavy chain-related proteins and At3g53350 gene, which functions as a repressor of primer (ROP) interactive partner 4 (Figure 6.4). Since these three genes are co-expressed and therefore are generally expected to be involved in related cellular functions, thus this therefore, strongly

suggests that the AtMTA gene may potentially be a myosin heavy chain-related protein and/or a ROP interactive partner 4.

These findings were substantial because the function of every gene depends on that of another related gene and our results, according to ATTED II, the At3g53350 gene, which is a repressor of primer (ROP) interactive partner 4 has the highest mutual rank (MR) of 19.9% with the At3g02930 gene - a situation which may indicate that the functionality of our own gene of interest maybe and/or similar to that of the At3g53350 gene. The At3g53350 gene is involved in the following biological process: cortical microtubule organization and plant-type cell wall organization this implies that since it is closely co-expressed with the At3g02930 gene, it may have similar functions Smertenko *et al.* (2008). With respects to stimulus-specific expression of the AtMTA gene naturally plants respond to various stress factors through a direct programming of their gene expressions by their respective transcription factors, which lead to a response in metabolic and phenotypic changes (Singh, 1998; Alves *et al.*, 2014). These transcription factors are very important nuclear targets for cellular signal transduction systems. Notably, according to microarray expression analysis, co-expression and GO analysis of the At3g02930 gene, it was found that the transcriptional processes of the At3g02930 gene and its associated AtMTA-ECGG50 set are generally and collectively induced in response to a wide range of biotic and abiotic stress factors Figure 6.5.

6.5 CONCLUSION

Thus and in light of all these findings, we therefore, proposed that the AtMTA protein could essentially be involved in organizing cytoskeletal arrays and/or cortical microtubule organization

and plant-type cell wall organization that are mediated by the second messenger cAMP. By linking up this to all the other findings from this whole study, it is really apparent that the AtMTA protein performs its functions via cAMP, which it literally generates by itself as a functional sAC molecule.

CHAPTER SEVEN

CONCEPTUAL SYNTHESIS AND OVERALL CLOSING REMARKS

In recent years, sequencing of several plant genomes has paved the way for studies such as our own to compare protein genes across species. In other words, the sequencing of genomes has provided plant scientists with the opportunity for more detailed studies of microtubule associated (MTA) proteins in various species, which should cast light on their function in economically important crops such as maize and soy. Microtubules (MTs) are components of the cytoskeleton in eukaryotic cells, which play a vital role in cell life systems, such as cell morphogenesis and maintenance, cell migration, intracellular transport, signal transduction and cell division (Gardiner, 2013). Their functions are accomplished with a range of microtubule associated (MTA) proteins, and the dynamic interaction between MTs and MTA proteins regulates a number of functions in the plant life cycles such as the aggregation and de-polymerization of MTs, the dynamic activities of MTs through spatial organization of MT arrays and the orientation changes of MT arrays. MTA proteins control cell division and expansion - two essential biological processes important for growth and development of all eukaryotes, including plants (Hamada, 2014).

In this study, however, we focused our attention on the MTA protein from *Arabidopsis thaliana* (AtMTA) that has in recent years been shown to harbour an AC catalytic centre (Gehring, 2010). The AtMTA segment containing the AC catalytic domain was targeted and then cloned for functional characterization. In order to target the catalytic AC centre in the AtMTA sequence,

we first retrieved the genomic sequence of the At3g02930 gene encoding the putative AtMTA-AC from The Arabidopsis Information Resource (TAIR) database and thereafter, designed some sequence-specific primer sets (forward and reverse) manually as guided by information in Figure 1.3 of Chapter 1. Ideally, the primers were designed to specifically amplify at least 50 amino acid residues away from both sides of the AC-containing region of the At3g02930 gene. In detail, the targeted AtMTA-AC gene fragment was isolated from total RNA extracted from the *A. thaliana* ecotype Columbia, leaves whereby the extracted total RNA was first used as a template to generate copy DNA (cDNA) and the generated cDNA then used together with the sequence-specific primers to amplify the targeted AtMTA-AC gene fragment in a specialized reverse transcription polymerase chain reaction (RT-PCR) system. When resolved on a 1% agarose gel, the gene fragment of an expected approximate size of 540.0 bp was obtained (Figure 3.3; Chapter 3).

After the successful isolation of the AtMTA-AC gene fragment (Figure 3.3), the resulting gene fragment was ligated into the pTrcHis-TOPO expression vector to form the pTrcHis-TOPO:AtMTA-AC expression constructs, and then cloning success was checked and verified by standard PCR, using a specialized MyTaq reaction system. The successfully cloned and molecularly confirmed AtMTA-AC gene fragment is shown Figure 3.4 of Chapter 3. The formed pTrcHis-TOPO:AtMTA-AC expression construct was then used to transform some chemically competent *E. coli* EXPRESS BL21 (DE3) pLysS cells and subsequently, partial expression of the targeted and desired AtMTA-AC recombinant protein was then conducted by inducing the transformed cells with 1 mM IPTG at an OD₆₀₀ of 0.6. The expressed recombinant protein was then resolved by SDS-PAGE and as expected, a recombinant fusion His-tagged protein product of approximately 19.80 kDa was obtained (Figure 3.5; Chapter 3).

To assess if the AC catalytic centre in the AtMTA-AC protein had any enzymatic activity, an endogenous assaying of the cAMP levels generated within the induced cells was undertaken and such cells showed that they could generate cAMP levels of up to 5.4-fold as compared to the un-induced cells (Figure 3.6; Chapter 3), thereby exhibiting the property of being either a *bona fide* soluble AC (sAC) capable of generating cAMP from ATP by itself or else as a functional plant molecule capable of stimulating other resident AC (*E. coli* ACs in this case) to produce cAMP.

Therefore, in order to determine if the recombinant AtMTA had any specific functional AC activity, an *in vivo* assessment of the AtMTA-AC activity was then undertaken through a functional complementation test. This experiment was carried out using an SP850 mutant *E. coli* *cyaA* strain that is deficient in endogenous AC activity and therefore, cannot catabolize lactose. When this strain is grown on MacConkey agar, it produces white/yellowish colonies as compared to the red/purple colonies produced by its wild-type counterpart (Moutinho *et al.*, 2001; Ruzvidzo *et al.*, 2013; Swiezawska *et al.*, 2014; Al-Younis *et al.*, 2015; Kasahara *et al.*, 2016; Chatukuta *et al.*, 2018; Al-Younis *et al.*, 2018). In this regard the pTrcHis-TOPO:AtMTA-AC gene fragment was used to transform chemically competent SP850 *cyaA* *E. coli* mutant cells and the transformed cells then assessed for their ability to ferment lactose and as a result of their rescuing by the then inherent and expressed recombinant AtMTA-AC protein. A visual cell phenotype analysis was then conducted on MacConkey agar medium, where it was found out that the cells harbouring the induced pTrcHis-TOPO:AtMTA-AC fusion construct had picked up a deep red/purple colour as opposed to the no transformed cells, which appeared white/pale yellow in colour (Figure 4.2; Chapter 4). This result therefore, affirmed that the recombinant AtMTA-AC protein is indeed a higher plant AC capable of generating cAMP from ATP. In order to further validate this outcome and further characterize the AC activity of this

recombinant, the AtMTA-AC protein was purified via a charged Ni-NTA affinity matrix (Figure 5.2; Chapter 5) followed by its concerted functional characterization *in vitro* (Figure 5.4; Chapter 5). From our findings (Figure 5.4; Chapter 5), it is apparent that AtMTA-AC protein is indeed a sAC, whose activity is solely modulated by the Mn^{2+} , Ca^{2+} and CO_3^{2-} ions while, on the other hand, it is also totally insensitive to the Mg^{2+} , and F^{-} ions. Hence, we could conceivably conclude that the AtMTA-AC protein is actually a sAC, whose physiological and biochemical activities may be mediated by cAMP and possibly, via a calmodulin-dependent signalling pathway.

Finally, a bioinformatic expressional analysis of the at3g02930 gene revealed that the AtMTA protein is highly expressed in the stem tissue, moderately expressed in the flower tissue and least expressed in the leaf tissue and it is directly co-expressed with three other genes on the microarray network, which are the At5g52280 and At1g64330 genes that function as myosin heavy chain-related proteins and At3g53350 gene, which functions as a repressor of primer (ROP) interactive partner 4 (Figure 6.4; Chapter 6). Since co-expressed genes are generally expected to be involved in related and/or similar cellular functions, the AtMTA protein may potentially be a myosin heavy chain-related protein and/or a ROP interactive partner 4 that functions via cAMP-mediated signal transduction systems.

Results from this whole research study firmly confirm the MTA protein, annotated as a probable AC and encoded by the At3g02930 gene from *A. thaliana* (AtMTA protein), as a functional soluble higher plant AC molecule and thus becoming one of the few recently identified higher plant ACs after the only nine currently known ones. These findings therefore, provided the basis for further possible characterisations and explorations of the exact physiological roles played by the AtMTA in higher plants by assessing its possible cross-talking between its intrinsically

generated cAMP and other signaling molecules such as NO, Ca^{2+} and/or HCO_3^- . Therefore, this study has succeeded to provide a novel outcome with substantial significance to both the current and future scientific literature and academic scholarship in the whole broader domain of Modern Sciences.

Findings from this study permit for the following possible and practically recommendations:

- Firstly, since the At3g02930 gene has been firmly confirmed as a functional sAC, it is important that its particular physiological functions in signal transduction and cell communication systems are further investigated so that its exact mode of action, particularly in plant stress (biotic and abiotic) responses and adaptation mechanisms, is properly determined.
- Secondly, further assays involving the activities of the recombinant AtMTA-AC protein must be conducted in living plant tissues (*in planta*) using either protoplasts and/or whole plant tissues).
- Thirdly, further studies on AtMTA knockout or knockdown mutants as a future screen for the gene at a whole plant level.
- Fourthly, further studies on the gene expression using qPCR analysis must be conducted as this will validate the findings with regards to spatial and temporal gene expression of the AtMTA.
- Furthermore, more unknown AC candidates should be enzymatically and functionally characterized in the agronomically important crop plants, to elucidate their particular

physiological and biological roles in biotic and abiotic stress responses and adaptation mechanisms, thereby providing methods and processes that would enable plants to tolerate the on-going hostile environmental extremes brought about by climate change. Idealistically, this could contribute towards the possible eradication and/or sustainable alleviation of hunger and poverty as a result of improved crop production and yields.

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