Molecular Characterization and Elucidation of the Physiological Roles of a Novel Maternal Effect Embryo Arrest Protein from *Arabidopsis thaliana*

D.T. KAWADZA

16232461

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Supervisor: Prof. O. RUZVIDZO

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Declaration

I, David Tonderayi Kawadza, declare that the thesis entitled "Molecular Characterization and Elucidation of the Physiological Roles of a Novel Maternal Effect Embryo Arrest 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 have been acknowledged as indicated in the text and/or list of references.

Name: David T. Kawadza

Date: August 2014

Signature: .....

Date: 10/04/2015

Supervisor.....

Date 10/04/2015

Prof O Ruzvidzo

Dedication to Nthabiseng Mamotho, Taffy, Bangiwe, Amani, Toko, Namara, Charles, Susan, Carol, Khulu Eben and late Mom Winnie.

You ALL spurred me on.

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LIST OF ABBREVIATIONS

2YT: Double strength yeast tryptone medium

AC: Adenylate cyclase

AGI: Arabidopsis Genome Initiative

ANOVA: A one-way analysis of variance

AtCNGC: *Arabidopsis thaliana* cyclic nucleotide-gated channel

ATHENA: *Arabidopsis thaliana* expression network analysis

AtMEE22: *Arabidopsis thaliana* maternal effect embryo arrest (full protein)

AtMEE-AC: *Arabidopsis thaliana* maternal effect embryo arrest (truncated protein)

ATP: Adenosine 5′-triphosphate

BAC: Bacterial Artificial Chromosome

BLAST: Basic Local Alignment Searching Tool

BP: Biological Process

cAMP: Cyclic 3′,5′-adenosine monophosphate

CaM: Calmodulin

CAP: Catabolite gene activating protein

CAPS: Cleaved amplified polymorphic sequences

CC: Cellular component

cGMP: Cyclic 3′,5′-guanosine monophosphate

CHX: Cycloheximide

CLV-WUS: Clavata-Wuschel

CNGC: Cyclic nucleotide-gated channels

CREB: Cyclic AMP response element binding protein

CREM: Cyclic AMP response modulator

CRP: Cyclic AMP receptor protein

CZ: Central zone

DAG: 1, 2-Diacylglycerol

DEG: Differentially expressed genes

DEX: Dexamethasone

ECGG: Expression co-related gene group

EDTA: Ethylenediaminetetraacetic acid

EIA: Enzyme immunoassay

EPAC: Exchange proteins activated by cyclic AMP

ExPaSy: Expert protein analysis system

FACS: Fluorescent-activated cell sorting

GC: Guanylate cyclase

GOI: Gene of Interest

GO: Gene Ontology

GPCR: G-protein coupled receptor

GR: Glucocorticoid receptor

GRN: Gene regulatory networks

GTE: Glucose/tris/EDTA

GTP: Guanosine 5′-triphosphate

hat: Hours after treatment

HD: Homeodomain

HIPVs: Herbivore-induced plant volatiles

HR: Hypersensitive response

HSP: Heat shock proteins

IBMX: 3-isobutyl-1-methyl xanthine

IP3: Inositol 1, 4, 5-triphosphate

IPTG: Isopropyl-β-D-thiogalactopyranoside

KO: Knock out gene

LB: Luria-Bertani

LRR-RLK: Leucine rich repeat-receptor like kinase

β-ME: β-Mercaptoethanol

MEE: Maternal effects embryo arrest

MF: Molecular Function

MOPS: 3-(N-morpholino) propanesulfonic acid

MSMO: Murashige and Skoog basal salt with minimum organics

NAA: Naphthalene acetic acid

Ni-NTA: Nickel-nitrilotriacetic acid

NO: Nitric oxide

OD: Optical density

OC: Organizing centre

PI: Isoelectric point

PIP2: Phosphatidylinositol 4,5-bisphosphate

PKA: Protein kinase A

PMSF: Phenylmethanesulfonyl fluoride

PP: Protein phosphatase

PPR: Pentatricopeptide repeat

PsiP: Pollen signalling protein

PZ: Peripheral zone

RFP: Red fluorescent protein

RLKs: Receptor-like kinases

RNases: Nuclease that catalyzes the degradation of RNA

RTKs: Receptor tyrosine kinases

RT-PCR: Reverse transcriptase-polymerase chain reaction

RZ: Rib zone

SAM: Shoot apical meristem

SDS-PAGE: Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SNK: Student Newman Kuehls

SOC: Super optimal broth with catabolite repression medium

STAND: Signal transduction ATPases with numerous domains

TAIR: The Arabidopsis Information Resource

TF: Transcription factor

TFB 1: Transformation buffer 1

TFB 2: Transformation buffer 2

TILLING: Targeting Induced Local Lesions in Genomes

TM: Transmembrane

UTR: Untranslated regions

XR: Xenobiotic response

YT: Yeast-tryptone

KEY TERMS

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

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

Emb: Embryo defective gene.

Genevestigator: A bioinformatic software used to determine the co-expression systems of organism genes.

Guanylate cyclase (GCs): Enzymes capable of converting guanine-5′-triphosphate (GTP) to cyclic 3′,5′-guanosine monophosphate (cGMP).

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

β-Mercaptoethanol: This is a reducing agent that will irreversibly denature RNases by reducing its di-sulfide bonds and destroying the native conformation required for enzyme functionality.

Motif: A short conserved region in a DNA or protein sequence.

Ortholog: A homolog with identical function in a different organism.

Primers: Short synthetic nucleic acid sequences capable of forming base pairs with 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 specific signalling molecule.

Reverse transcription polymerase chain reaction (RT-PCR): A molecular method used to amplify 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.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE): A molecular biology technique used to separate protein molecules according to their sizes and migration capacities in a polyacrylamide gel system subjected to a strong electrical field.

Two-dimensional gel electrophoresis (2-D gels): An electrophoretic molecular biology method used to separate protein molecules according to their migrational sizes and iso-electrical points (PI-values).

Xenobiotic response: Plant response to synthetic chemicals eliciting expression of detoxifying enzymes such as glutathione transferases (GSTs).

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CHAPTER 1

Literature Review and General Overview

1.1 Introduction

One of the natural characteristics of living organisms is the ability to sense and discern stimuli, then appropriately responding to them (Avila, 1995). This aspect forms the basis of homeostasis, which is defined as the dynamic constancy of the internal environment of an organism (Widmaier *et al.*, 2008). For multicellular organisms, the internal environment is the intracellular and the extracellular milieu within which the cell finds itself. Generally, the arrival of a signal/ligand onto the cell surface and its perception, are mainly dependent on the chemical nature of both the signal and the cell surface. The responses of the cell to such arriving signals/ligands are influenced mainly, by the presence of receptors which specifically bind to these signalling molecules, and such responses are highly specific (Widmaier *et al.*, 2008). For example, the catecholamine hormones only affect certain cell types and not all cell types affected by these hormones respond in the same way. This specificity in response may typically be explained by three particular variables: 1) the hormone receptor system; 2) the second messenger system; and 3) the protein kinase system (Steer, 1975). Only those cells possessing the proper receptors are capable of binding to a given signal and then directing the subsequent and specific downstream signalling systems (Steer, 1975).

Ligands of a lipid-soluble nature, such as steroid hormones, have intracellular receptors while water-soluble ligands have cell-membrane receptors (Cooper, 2000). Binding of environmental or developmental signal molecules causes a conformational change in the receptor, which then triggers the subsequent signalling cascade that recruits specific transcription factors. These transcriptional factors activate downstream executor genes, which in turn carry out the required response(s) (Vogler and Kuhlemeier, 2003). The

transcription factors in essence, control or influence the final cellular responses by either activating or deactivating RNA polymerases (Phillips and Hoopes, 2008).

According to this concept, the arriving stimuli usually activate the receptor molecules thereby initiating a complex series of downstream signalling networks which exhibit cross-talking, and in order to respond to the various environmental and developmental cues in an appropriate and integrated manner (Osakabe *et al.*, 2013). Apparently, the effect of a signal may lead to the activation of the formation of 3′,5′-cyclic adenylate monophosphate (cAMP); 3′,5′-cyclic guanylate monophosphate (cGMP); 1,2 diacylglycerol (DAG); inositol 1,4,5-triphosphate (IP_3); a variety of phophoinositides and the ionized calcium (Ca^{2+}) (Steer, 1975).

In this chapter, we present the general overview of the cell signalling systems in living organisms, and particularly on the various biological molecules that typically have central roles in this system. The chapter begins by providing some brief and generalized background on nucleotide cyclases and their cyclic nucleotide products. This is followed by a direct and comprehensive focus on adenylate cyclases - their signalling systems, their receptor systems, their effector systems as well as their different forms, and with specific anchorage to their role in cell transduction and signalling systems. This is then followed by an overview of what is currently available in plants, on both ACs and their enzymatic product cAMP, and the pointing out of possible knowledge gaps in this specific area of study. Finally, the chapter then wraps up by presenting and exploring the prospects of studying a novel protein candidate termed the maternal embryo effect arrest protein from *Arabidopsis thaliana* (AtMEE22) as a potential AC candidate in plants, and particularly, in relation to important plant processes like growth, development and responses to environmental stress factors.

1.2 Nucleotide Cyclases and Cyclic Nucleotides

Cyclic nucleotide monophosphates (cNMP) and specifically, the 3′,5′-cyclic adenylate monophosphate (cAMP) and the 3′,5′-cyclic guanylate monophosphate (cGMP), and to a

lesser extent, the 3′,5′-cyclic cytidine monophosphate (cCMP) are known to regulate a number of metabolic and developmental processes in all living organisms (Martinez-Atienza *et al.*, 2007, Lemtiri-Chlieh *et al.*, 2011). Naturally, there are two main types of nucleotide cyclases – enzymes that generate cNMP and these are the adenylate cyclases (ACs) that produce cAMP plus a pyrophosphate and guanylate cyclases (GCs) that synthesize the corresponding analogue cGMP plus a pyrophosphate (Shenoy *et al.*, 2002). Both enzymes utilize their respective nucleotide 5′-triphosphates, adenosine 5′-triphosphate (ATP) for ACs and guanosine 5′-triphosphate (GTP) for GCs) as substrates, and require a metal co-factor, usually Mg^{2+} or Mn^{2+} metal, for catalysis (Tang and Hurley, 1998).

Cyclic AMP is arguably one of the most extensively studied second messengers in animals, lower eukaryotes and bacteria, where it has critical roles in regulating the metabolic status. In prokaryotes, cAMP is involved in the regulation of the *lac* operon where in an environment of low glucose, it accumulates and binds to the allosteric site of the cAMP receptor protein (CRP), a transcription activator protein. 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 CRP disengages from the *lac* operon promoter stopping the transcription (Meiklejohn and Gralla, 1985). Besides having a central role in *E. coli*, cAMP signalling is also critical for many aspects of development in the slime mold, *Dictyostelium discoideum* that grows unicellularly, but develops as a multicellular organism (Kimmel and Firtel, 2004, Mcmains *et al.*, 2008).

1.2.1 Cyclic AMP as a Second Messenger

In 1958, Sutherland and co-workers discovered cyclic AMP and shortly thereafter adenylate cyclase (Sutherland *et al.*, 1962). These researchers proposed the "second messenger" concept to explain how hormones, binding to the external surface of the target cell plasma membrane, might affect cellular responses (Sutherland and Robison, 1966). According to

this concept, the circulating hormone is termed the "first messenger", and when this first messenger binds to its receptor, the adenylate cyclase in the plasma membrane is then stimulated to convert ATP into cAMP. The newly formed cAMP is then released from the internal surface of the plasma membrane into the cytoplasm. The cAMP is therefore considered the "second messenger" because it moves from the plasma membrane into the cell. Eventually, the cAMP itself then binds to protein kinases in the cytoplasm, which upon binding, become activated to stimulate or inhibit various metabolic reactions. This, in turn, would lead to specific cellular responses which, depending upon the cell and cell type, may involve changes in enzyme secretion, ion transport, muscle contraction or hormone synthesis (Robison *et al.*, 1971). Ultimately, the second messenger cAMP is then further metabolized to 5'-adenosine monophosphate (5'-AMP) by the enzyme cyclic nucleotide phosphodiesterase, and the stimulus of the "second messenger" is thus dissipated (Figure 1.1).

1.2.2 The Adenylate Cyclase Signalling System

Principally, the adenylate cyclase system has the following specific features: firstly, hormone receptors binding specific hormones (the receptors, which may either be stimulatory or inhibitory are coupled to a heterotrimeric $\alpha\beta\gamma$ G-protein); secondly, an adenylate cyclase catalytic unit catalyzing the formation of cAMP; thirdly. regulatory sites on the enzyme altering the response of the catalytic unit to stimulation; fourthly, cAMP dependent protein kinases (PKA) binding cAMP and thereby becoming active from being inactive; fifthly, intracellular enzymes which are activated or inhibited when they are phosphorylated by the activated cAMP-dependent protein kinases; sixthly, phosphoprotein phosphatases which dephosphorylate the intracellular enzyme systems that originally had been phosphorylated by

the cAMP-dependent protein kinases; and lastly, the cyclic nucleotide phosphodiesterases which break down the cAMP by converting it to 5'-AMP (Defer *et al.*, 2000, Berridge, 2008).

The formation of cAMP can be initiated by a myriad of extracellular stimuli, such as neurotransmitters and hormones originating in other cells or tissues (Zippin *et al.*, 2001). All these stimuli are detected by G protein-coupled receptors (GPCRs) that use heterotrimeric G proteins, which are the transducers that are responsible for either activating or inhibiting the enzyme AC. In the case of AC stimulation, the external stimulus binds to the GPCR that functions as a guanine nucleotide exchange factor (GEF) to replace GDP with GTP, which eventually dissociates the heterotrimeric complex into its Gβγ and Gα subunits. The $G_{\alpha s}$-GTP complex activates ACs, whereas the Gαi-GTP inhibits ACs. The Gα subunits have GTPase activity that hydrolyses GTP to GDP, thus terminating their effects on AC (Figure 1.1). The heterotrimeric G proteins are made up from sixteen Gα, five Gβ and eleven Gγ genes. These proteins are extremely versatile signalling elements, and both the Gα subunit and the Gβγ subunit are able to relay information to downstream components (Berridge, 2008).

1.2.3 The Adenylate Cyclase Receptor System

A key feature of signal transduction is the convergence of a very wide range of extracellular stimuli to an extremely limited number of intracellular second messengers, such as cAMP, cGMP, inositol (1,4,5)-triphosphate [Ins(1,4,5)P3], diacylglycerol (DAG) and Ca^{2+} (Zaccolo and Pozzan, 2003). Naturally, there are four main cell-surface AC receptor types, namely the G protein-coupled receptors, the ion channel receptors, receptors with intrinsic enzymatic activity, and the serine/threonine kinase receptors (Lodish *et al.*, 2000).

In the G protein associated system, there are three main structures involved and being the receptor (R) which receives the signal, the heterotrimeric G protein, which conveys the information through, and the adenylate cyclase. There are also two sets of receptors associated with a single AC catalytic subunit in the cell membrane: one set is stimulatory and is designated R_s while the other one is inhibitory and designated R_i (Gilman, 1984). The binding of a ligand to a specific receptor induces a conformational change in the receptor, enabling it to interact with a GTP-binding protein (G-protein) and influencing its activity. Two forms of G-protein are also present: the G_s, which stimulates the AC, and the G_i, which inhibits it. Both forms undergo a cycle in which they exist as heterotrimers, to which either GDP or no guanosine nucleotide is bound. These heterotrimers dissociate on binding GTP and the free $G_{s\alpha}$ subunit undergoes a conformational change enabling it to interact with and stimulate the catalytic unit of the AC. The dissociation of the G_s heterotrimer is transient; after the hydrolysis of GTP to GDP, re-association takes place and a further dissociation occurs only after the GDP has been replaced by GTP. On the other hand, the dissociated $G_{i\alpha}$ subunit exerts an inhibitory effect onto the AC (Taussig *et al.*, 1993, Taussig and Gilman, 1995) however, some types of ACs are not sensitive to $G_{i\alpha}$. For instance, adenylate cyclase 1 (AC1) is inhibited by both $G_{i\alpha}$ and $G_{\beta\gamma}$; while others (AC 5 and AC 6) are stimulated by the $\beta\gamma$ subunit of G_i and catalyse the conversion of ATP to cAMP, which in turn is then released into the interior of the cell. The cAMP signal is switched off by another set of enzymes, the phosphodiesterases, which hydrolyse the cAMP to AMP. On release into the cytosol, cAMP elicits a response in two main ways. The first established mechanism is via the stimulation of two isoforms of a cAMP-dependent protein kinase. Binding of cAMP to this kinase, which is composed of two types of subunits, causes the kinase to dissociate into a regulatory dimer, to which four molecules of cAMP are bound, and two catalytic monomers, which are then capable of phosphorylating a wide range of protein substrates. Phosphorylation alters the

activity of the substrate as a result of a change in surface charge and the subsequent change in conformation (Newton *et al.*, 1999). In the second mechanism, cAMP mediates the cellular responses through the activation of ion channels in the plasma membrane in a way that is very similar to the action of cGMP, i.e. through a direct interaction with an ion channel.

In the ion-channel receptor system, the binding of a ligand brings about some specific conformational changes of the receptor system such that specific ions may flow through it, and the resultant ion movements alter the electric potential across the cell membrane (Lodish et al., 2000). The acetylcholine receptor at the nerve-muscle junction is an example. In Tyrosine kinase–linked receptors, these receptors lack the intrinsic AC catalytic activity, but the binding of a ligand stimulates formation of a dimeric receptor, which then interacts with and activates one or more cytosolic protein-tyrosine kinases. The receptors for many cytokines, interferons, and human growth factor, are of this type. These tyrosine kinase–linked receptors are sometimes referred to as the cytokine-receptors in animals or the receptor-like kinase (RLK) superfamily in plants (Lodish et al., 2000).

For receptors with intrinsic enzymatic activity, their function is activated by the binding of a ligand (Lodish *et al.*, 2000). For instance, some activated receptors catalyze the conversion of ATP to cAMP while others act as protein phosphatases, removing phosphate groups from phospho-tyrosine residues in substrate proteins, and thereby modifying their activity (Lodish et al., 2000). The receptors for insulin and many growth factors are ligand-triggered protein kinases; in most cases, the ligand binds as a dimer, leading to dimerization of the receptor and activation of its kinase activity. These receptors - often referred to as receptor serine/threonine kinases or receptor tyrosine kinases - auto-phosphorylate residues in their own cytosolic domains and also can trans-phosphorylate other various substrate proteins (Lodish *et al.*, 2000). Naturally, many enzymes are regulated by the covalent attachment or removal of phosphate group, in ester linkage to the side-chain hydroxyl group of a particular

amino acid residue; usually serine, threonine or tyrosine, and this is commonly termed the covalent functional modification of a protein (Widmaier *et al.*, 2008).

For the serine/threonine kinase receptors (commonly known as the receptor-like kinases (RLKs)), which form a very large gene family in plants, most contain the Ser/Thr kinase as a cytosolic domain while having structural elements similar to animal receptor tyrosine kinases (RTKs). The RLKs mostly convey signals to their target proteins in the cytoplasm by catalytic processes of protein kinases. In *Arabidopsis thaliana*, the RLK family includes >600 members, with the leucine-rich repeat RLKs (LRR-RLKs) constituting the largest group among this RLK diverse family of proteins that physically link the cell wall to the cytoplasm, and making them ideal candidates for cell wall sensors (Shiu and Bleecker, 2001, Shiu and Bleecker, 2003, Gish and Clark, 2011). Typically, all RLKs are situated at the plasma membrane and contain an extracellular domain, a trans-membrane domain, and an intracellular Ser/Thr kinase domain (Steinwand and Kieber, 2010). In most higher plant systems, RLKs have been implicated in various signalling pathways, including meristem function, brassinosteroid perception, floral abscission, ovule development, embryogenesis, plant defense, and overall plant morphogenesis (Steinwand and Kieber, 2010).

1.2.4 The Adenylate Cyclase Effector System

There are three main effectors of the second messenger cAMP: protein kinase A (PKA), the guanine-nucleotide-exchange factor (GEF), exchange protein activated by cyclic AMP (EPAC), and the cyclic nucleotide-gated ion channels (Berridge, 2008, Sassone-Corsi, 2012). Protein kinase A (PKA) is a symmetrical complex of two regulatory (R) subunits and two catalytic (C) subunits (there are several isoforms of both subunits). It is activated by the binding of cAMP to two sites on each of the R subunits, which causes their dissociation from the C subunits (Taylor *et al.*, 1992). The catalytic activity of the C subunit is decreased by a

protein kinase inhibitor (PKI), which can also act as a chaperone and promote nuclear export of the C subunit, and thereby decreasing nuclear functions of PKA (Sassone-Corsi, 2012). When activated, protein kinase A phosphorylates an array of other cellular targets including other kinases, phosphatases, gene transcription factors and an ever-growing list of ion channels (Trautwein and Hescheler, 1990, Montminy, 1997, Gray *et al.*, 1998, Shipston, 2001). While the activity of some ion channels can be enhanced by cAMP and/or cAMP-dependent PKA treatment, the activity of others can actually be down-regulated following the same treatment (Osterrieder *et al.*, 1982, Siegelbaum *et al.*, 1982, Shuster *et al.*, 1985, Milhaud *et al.*, 1998).

Of the two types of PKA, protein kinase A (PKA) I is found mainly free in the cytoplasm and has a high affinity for cAMP, whereas protein kinase A (PKA) II has a much more precise location by being coupled to the A-kinase-anchoring proteins (AKAPs). The AKAPs are examples of the scaffolding proteins that function in the spatial organization of signalling pathways by bringing PKA into contact with its many substrates (Berridge, 2008). The A-kinase anchoring proteins, or AKAPs, are a large family of scaffold proteins that tether inactive PKAs to specific sites within the cell where they are readily available to phosphorylate local proteins (Glantz *et al.*, 1992, Rubin, 1994, Coghlan *et al.*, 1995, Klauck *et al.*, 1996, Malbon *et al.*, 2004, Mcconnachie *et al.*, 2006).

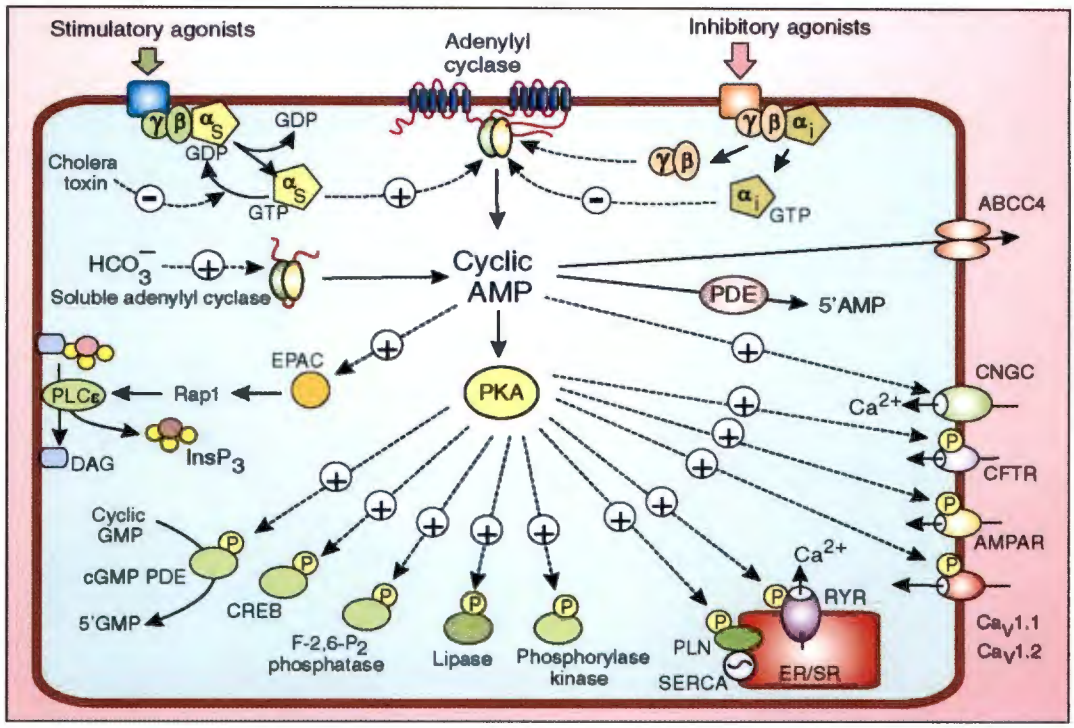

Figure 1.1: Organization and function of the cyclic AMP signalling pathway. Cyclic AMP is formed both by membrane-bound adenylate cyclase and by the bicarbonate-sensitive soluble adenylate cyclase. The former is regulated by both stimulatory agonists that act through the α_S subunit or through inhibitory agonists that act through either the α_i or the $\beta\gamma$ subunits. The increase in cyclic AMP then acts through three different effector systems. (a) It acts through the exchange protein activated by cyclic AMP (EPAC), which functions to activate Rap1. (b) It can open cyclic nucleotide-gated channels (CNGCs). (c) The main action of cyclic AMP is to activate protein kinase A (PKA) to phosphorylate a large number of downstream targets. Some of these effector systems drive specific processes such as gene transcription through phosphorylation of cAMP response element-binding protein (CREB), and activation of ion channels and various enzymes that control metabolism. Other downstream targets are components of other signalling pathways such as the cyclic GMP phosphodiesterase (cGMP PDE) and the Ca^{2+} channels $Ca_V1.1$ and $Ca_V1.2$ (adapted from Berridge, 2008).

A different important effector for cAMP is EPAC, a GEF that promotes activation of certain small GTPases (e.g., Rap1). A major function of Rap1 is to increase cell adhesion via integrin receptors (Bos *et al.*, 2003). Lastly, cAMP can bind to and modulate the function of a family of cyclic-nucleotide-gated ion-channels. These are relatively non selective cation channels that conduct calcium. Calcium stimulates CaM and CaM-dependent kinases and, in turn, modulates cAMP production by regulating the activity of ACs and PDEs (Zaccolo and Pozzan, 2003, Sassone-Corsi, 2012).

1.2.5 Forms of Adenylate Cyclases

Following the purification, partial sequencing, and subsequent cloning of the first adenylate cyclase (AC) in 1989, a structure was revealed that comprised of 12 transmembrane-spanning (TM) domains, two homologous apparent ATP-binding regions and extensive NH_2 and COOH termini (Figure 1.2). Tentatively, the AC family is composed of ten isoforms: of which nine are membrane-bound (AC1-AC9; tmACs); while one is soluble (AC10; sAC) (Berridge, 2008).

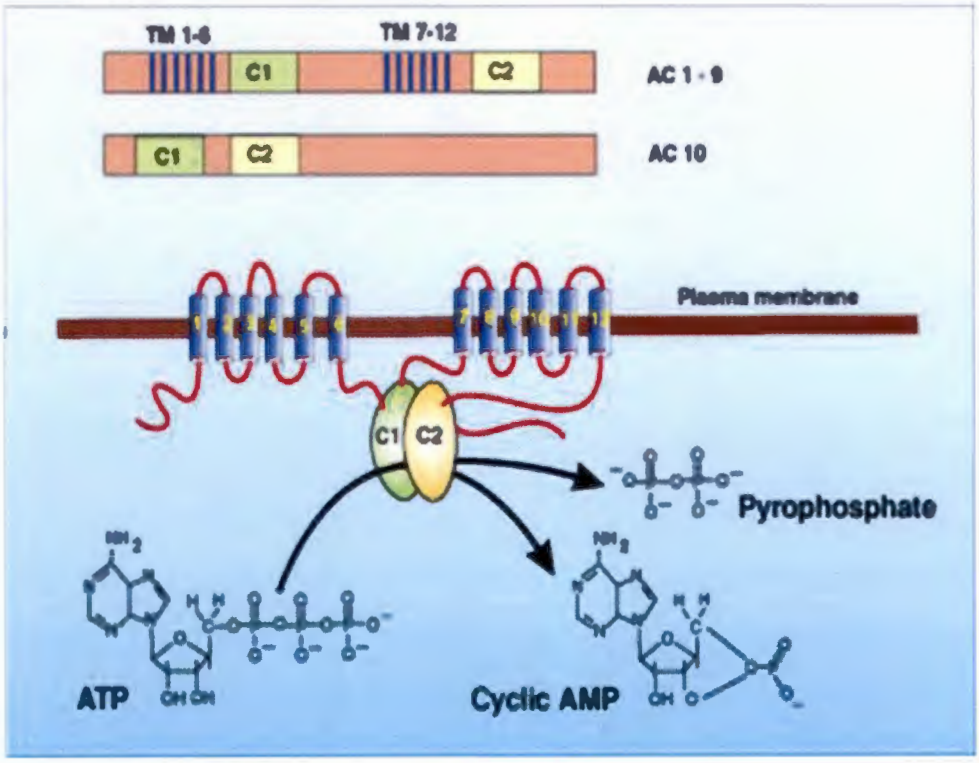

Figure 1.2: Domain structural organization of adenylate cyclases. The nine membrane-bound adenylate cyclases (AC1-AC9) have a similar domain structure. The single polypeptide has a tandem repeat of six trans-membrane domains (TM) with TM1-TM6 in one repeat and TM7-TM12 in the other. Each TM cassette is followed by large cytoplasmic domains (C1 and C2), which contain the catalytic regions that convert ATP into cAMP. As is shown in the lower segment of the panel, the C1 and C2 domains come together to form a heterodimer. The ATP-binding site is located at the interface between these two domains. The soluble (AC10) isoform lacks the trans-membrane regions, but however retains the C1 and C2 domains that are responsible for enzymatic catalysis (Adapted from Berridge, 2008).

To date, at least nine closely related isoforms of tmACs (AC1-AC9), and two splice variants of AC8, have been cloned and functionally characterized in mammals (Iyengar, 1993, Sunahara *et al.*, 1996, Hanoune *et al.*, 1997). All of them share a large sequence homology in the primary structure of their catalytic sites and the same predicted three-dimensional structure. Each of them consists of two hydrophobic domains (with 6 trans-membrane spans) and two cytoplasmic domains, resulting in a pseudo-symmetrical protein. It is only the cytoplasmic domains (C1 and C2), which constitute the catalytic site, that is subject to intracellular regulations specific for each subtype. In particular, the catalytic activity as well as the sites for interaction with forskolin and $G_s\alpha$, require both cytoplasmic moieties (Defer *et al.*, 2000). Apparently, a remarkable and puzzling feature of the ACs, noted upon their first structural and functional characterization, was their possession of two ATP binding domains (Abrams *et al.*, 1991). These domains are highly homologous and complementary both within a single AC and among different mammalian AC isoforms. When these domains were separately expressed in *Escherichia coli* and purified, they had to dimerize first for full AC activity; and upon this recombination, an AC functional activity was revealed, which could be regulated by both $G_s\alpha$ and forskolin (Tesmer *et al.*, 1997).

Generally, the cAMP generated by tmACs acts locally (Rich *et al.*, 2000, Zaccolo and Pozzan, 2002), and most likely being restricted by phosphodiesterase "firewalls" (Zaccolo and Pozzan, 2002), which define the limits of these cAMP signalling micro-domains (Zippin *et al.*, 2004). However, targets of cAMP do not solely reside at the plasma membrane; EPAC is localized to the nuclear membrane and mitochondria (Qiao *et al.*, 2002), while PKA is tethered throughout the cell by a class of proteins termed AKAP (A-kinase–anchoring proteins) (Michel and Scott, 2002). The observation that cAMP does not diffuse far from tmACs (Bacskai et al., 1993; Zaccolo and Pozzan, 2002) reveals that there must be another source of cAMP modulating the activity of distally situated targets.

Conceivably, a functional soluble AC (sAC) was then subsequently identified in seminiferous tubules and sperms of various animals (Braun and Dods, 1975). This type of enzyme preferentially utilizes MnATP, rather than MgATP, as substrate. The enzyme was then cloned and purified from rat testis, and its catalytic domain found to resemble that of ACs expressed in various microorganisms, such as cyanobacteria and yeasts (Buck *et al.*, 1999). Uniquely among ACs, the sAC enzyme is notably activated by bicarbonate ions *in vivo* and *in vitro* in a pH-independent manner (Chen *et al.*, 2000); and because carbonic anhydrase is ubiquitous in all cells and being able to instantaneously equilibrate HCO_3 with CO_2 at pH*i*, the sAC thus can function as a physiological CO_2/HCO_3/pH*i* sensor (Tresguerres *et al.*, 2010). Furthermore, since CO_2 is the end product of all energy-producing metabolic processes, the sAC is poised to function as a cell's intrinsic sensor of metabolic activity (Zippin *et al.*, 2001). Apparently, the sAC possesses no trans-membrane spanning domains (Buck *et al.*, 1999) and is distributed to subcellular compartments containing cAMP targets (Zippin *et al.*, 2003) that are physically distant from the plasma membrane.

Localization of sAC inside the nucleus, in close proximity to the CREB family proteins, and its regulation by calcium and bicarbonate suggested that sAC might be responsible for modulating CREB activity in response to intracellular signals. It has recently been demonstrated that the sAC is localized at multiple, subcellular compartments throughout the cell including mitochondria, centrioles, mitotic spindles, mid-bodies, and nuclei (Zippin *et al.*, 2003), each of which contains targets of cAMP. These data suggest that the cell may contain multiple, cAMP-independently modulated signalling micro-domains; whose targets near the plasma membrane would solely depend on tmACs for second messenger generation, whereas targets inside the cell would be modulated by sAC-generated cAMP (Wuttke *et al.*, 2001, Zippin *et al.*, 2003). A bicarbonate treatment of whole cells lead to an activation of the

CREB family of transcription factors, which in turn revealed that bicarbonate itself could induce a signal transduction cascade (Wuttke *et al.*, 2001, Zippin *et al.*, 2003).

1.2.6 The Adenylate Cyclase and Cyclic AMP Systems in Plants

Although cyclic nucleotides have been shown to have key regulatory roles in animals and bacteria, most investigations with higher plants in the 1970s and early 1980s were seriously criticized on the basis of (i) a lack of specificity of effects apparently elicited by cyclic nucleotides, (ii) the equivocal identification of putative endogenous cyclic nucleotides and (iii) the ambiguity in the identification of enzymes connected with cyclic nucleotides (Newton and Smith, 2004). Most recent evidence based on more rigorous identification procedures has conclusively demonstrated the presence of cyclic nucleotides, nucleotide cyclases and cyclic nucleotide phosphodiesterases in higher plants, and has identified plant processes subject to regulation by these molecules (Steer, 1975, Newton *et al.*, 1999, Gehring, 2010).

The early reports on the existence of cAMP in plants were criticized on the basis that they were either plausible deductions from the observed physiological effects of endogenously supplied cAMP or cAMP analogues, or conclusions based solely on insufficiently rigorous chromatographic identifications. As an example of the former, a report that cAMP could delay petiole abscission in *Coleus*, but could not demonstrate this *in vivo*, and hence this was therefore suspected to be a mere duplication of the action of auxin by this cyclic nucleotide (Salomon and Mascarenhas, 1971). In the latter category, a radiolabelled product from the incubation of [8-^{14}C] adenine with germinating barley seeds that was chromatographed together with cAMP in ten chromatographic systems was obtained (Pollard, 1970) but this outcome was criticized on the basis that the chromatographic systems obtained could not resolve the putative secondary messenger cAMP from the RNA catabolism intermediate,

adenosine 2′,3′-cyclic monophosphate. Apparently and in order to overcome such forms of criticism, the putative radiolabelled cAMP was then hydrolysed to AMP by a cAMP phosphodiesterase, which was then determined enzymatically (Narayanan *et al.*, 1970); however this expedient was once again and furthermore criticized in that the used phosphodiesterase was not of any demonstrated absolute specificity for cAMP.

Therefore, in a desperate attempt to try and conclusively identify cAMP as an endogenous component of plant cells, a sequential chromatographic and electrophoretic procedure for the extraction and isolation of cAMP was developed (Brown and Newton, 1973). The identity of the putative cAMP was then verified by a co-chromatographic system with an authentic sample in five paper- and three thin-layer chromatography systems and by high-voltage electrophoresis in three different buffers. Collectively, these steps were capable of separating cAMP from all the then known naturally occurring adenine nucleotides, including 2′,3′-cyclic AMP. During and after this initial phase of investigation in this area, a considerable number of reports quantifying cAMP in various plant species were made, and they were all with concentrations of a similar order ranging from 2.1-3.5 pmol cAMP g^{-1} wet weight in Zea (Tarantowicz-Marek and Kleczkowski, 1978) to 220-280 pmol g^{-1} wet weight in Lactuca (Kessler and Levenstein, 1974). Nevertheless, some authors still objected that the reported concentrations of cAMP were too close to and/or below the sensitivity of the used methods (Niles and Mount, 1973, Amrhein, 1974, Bressan and Ross, 1976, Amrhein, 1977) and as a consequence, several reviews at the time concluded that cAMP was not present in plants (Keates, 1973, Lin, 1974, Amrhein, 1977), while others suggested that any cAMP present was a result of bacterial infection (Bonnafous *et al.*, 1975). Notably, the contamination concept was subsequently refuted by Ashton & Polya (1978), who calculated that less than 0.1% was contributed by bacteria and demonstrated the presence of cAMP in the axenic cell cultures of rye grass (Ashton and Polya, 1978), further supporting earlier reports of cAMP

presence in axenic cultures of soybean callus tissue (Brewin and Northcote, 1973) and tobacco cell lines (Lundeen *et al.*, 1973).

However, even although most reviews in this initial phase had expressed the opinion that cAMP did not, or was unlikely to, function in higher plants (Keates, 1973, Amrhein, 1974, Lin, 1974, Amrhein, 1977), these opinions were still being superseded by commentaries suggesting its potential functions (Brown and Newton, 1981, Newton and Brown, 1986, Assmann, 1995, Trewavas, 1997). Apparently, the main problem was that the various extraction, purification and detection techniques used during those times were exactly or similar to those described for animal tissues without considering the inherent problems peculiar to plants, such as high chemical contaminations and low chemical concentration of the targeted cAMP (Newton and Smith, 2004). Subsequently, it was only with the use of mass spectrometric analysis that cAMP was finally and unequivocally established as being endogenous to plant tissues (Newton *et al.*, 1980, Janistyn, 1983).

Probably the most convincing data towards directly establishing a specific function for cAMP in plants came from whole-cell patch-clamp current recordings in *Vicia faba* mesophyll protoplasts that revealed that the outward K^+-current increased in a dose-dependent fashion following intracellular application of cAMP and not AMP, cGMP or GMP, and an indirect evidence indicating that this modulation was occurring via a cAMP-regulated protein kinase system (Li *et al.*, 1994). Furthermore, some cAMP-dependent up-regulation of a calcium-permeable conductance activated by hyperpolarization was also reported in guard cells as well as mesophyll cells of *Arabidopsis thaliana* and *Vicia faba* (Lemtiri-Chlieh and Berkowitz, 2004). Even despite this compelling evidence, the history of cAMP function in plants has not been free of controversy and much more is still needed before we may have a clear picture of the generation and modes of action of this molecule in plant physiology generally and plant stress responses in particular.

Notably, since the mid-1980s, more reports discussing the presence and potential functions of cyclic nucleotides in plants have been published. The potential roles of cAMP in plants include regulation of ion channels (Bolwell, 1995) and ion transport in *Arabidopsis thaliana* (Anderson *et al.*, 1992, Trewavas, 1997); activation of phenylalanine ammonia lyase (Bolwell, 1992), cAMP-dependent signal transduction pathways and cell cycle progression in tobacco BY-2 cells (Ehsan *et al.*, 1998). Cyclic AMP has also been shown to play a role in stimulating protein kinase activity in rice (*Oryza sativa*) leaves (Komatsu *et al.*, 1993), and more recently, to eliciting stress responses and plant defence in the same plant (Choi and Xu, 2010). For example, increased levels of cAMP coincide with the early stages of the response to phytoalexins and mediate the production of 6-methoxymellein and the activation of calcium uptake into cultured carrot (*Daucus carota*) cells (Kurosaki *et al.*, 1987, Kurosaki *et al.*, 1993).

In summary, the existence of cAMP in higher plants has now been established using advanced analytical tools and in addition to the presence of cAMP, there is also some conclusive evidence for the presence of PDEs and cAMP-binding proteins and a number of cAMP-dependent physiological responses in higher plants (Anderson *et al.*, 1992, Meier and Gehring, 2006, Choi and Xu, 2010). Overall, this shows that regardless of the low and seemingly un-physiological levels of cAMP in plants as compared to animals, the perception that plants also have a functional cAMP-dependent signalling system remains alive and awaits detailed elucidation.

Notably, AC activity was demonstrated in higher-plant material by the use of both histochemical and biochemical procedures. Using the former techniques, early indications of the presence of AC activity were found in the plasma membrane, the endoplasmic reticulum and the nuclear membranes of *Zea mays* root tips (Al-Azzawi and Hall, 1976), on internal

membranes of cytoplasmatic vacuoles of *Pisum sativum* (Hilton and Nesius, 1978) and on the external side of the host plasma membrane and membranes surrounding the endophyte of root nodules of *Alnus glutinosa* (Gardner *et al.*, 1979) and on the external side of the plasma membrane of *Pisum sativum* (Nougare'de *et al.*, 1984). Additionally, a sedimentable AC activity was also identified in *Pisum sativum* (Pacini *et al.*, 1993) using mass spectrometric techniques for the first time, and producing the unambiguous identification of the reaction product. This enzyme extract utilized Mg^{2+}-ATP as a substrate and was stimulated by GTP at 100 nM. Higher concentrations of GTP (110 μM) inhibited the activity, probably owing to competition with ATP. Sadly, the first paper reporting a plant gene sequence showing high homology with that of mammalian AC and detailing the aspects of its regulation was unfortunately withdrawn (Ichikawa *et al.*, 1998).

Subsequently, when some *Medicago sativa* cell cultures were exposed to the elicitor of the phytopathogenic fungus *Verticillium alboatrum*, they responded with an increased AC activity (Cooke *et al.*, 1994). Again and in this same study, cAMP formation was also confirmed unequivocally by mass spectrometric analysis, where the associated AC activity was found to be highly dependent on Mg^{2+} and was stimulated by Ca^{2+}. Basal activity of this same experiment was very low (maximum 400 fmol min^{-1} mg^{-1} protein) but increased by a 300% factor within a time span of 4 min on application of the elicitor. Such a transient rise in AC activity was accompanied by an increase in intracellular cAMP concentration and was also followed by a transient increase in phosphodiesterase activity.

Phosphodiesterase activity had been demonstrated in diverse sources such as tobacco tissues (Wood *et al.*, 1972), barley seeds (Vandepeute *et al.*, 1972), carrot leaves (Venere, 1972), potato tissues (Shimoyama *et al.*, 1972, Ashton and Polya, 1975, Ashton and Polya, 1978) and the *Jerusalem artichoke* tubers (Giannattasio *et al.*, 1974). The occurrence of

phosphodiesterase activity in these plants was interpreted to mean that the substrate of phosphodiesterase, cAMP, must be an endogenous component of the tissue and that it would possess functions analogous to those of cAMP in other organisms. However, a conflicting view was expressed by a report that unlike its mammalian counterpart, the phosphodiesterase from pea seedlings had an acidic pH optimum, was insensitive to methylxanthines, yielded 3′-AMP rather than 5′-AMP as the major hydrolytic product, and most significantly, had markedly greater activity with the RNA breakdown intermediate 2′,3′-cyclic AMP as a substrate rather than with the putative secondary-messenger isomer 3′,5′-cAMP (Lin and Varner, 1972) (at that time, all mammalian phosphodiesterases functioning in the cAMP secondary-messenger cascades produced only the 5′- mononucleotide product and would not significantly hydrolyse 2′,3′-cyclic AMP). On account of this, it was then concluded that the pea phosphodiesterase was functioning not in a plant signal transduction system but as part of a catabolic sequence of RNA.

However, further examination of the more purified plant phosphodiesterases then indicated that more than one form was actually present (Brown *et al.*, 1975, Brown *et al.*, 1977). In some work that involved some French dwarf bean seedlings, it was found that such seedlings did contain a phosphodiesterase form that possessed properties that were even more similar to those of mammalian phosphodiesterases than the earlier ones (Brown *et al.*, 1975, Brown *et al.*, 1977). Other phosphodiesterases are activated by Fe^{3+} while some can hydrolyse both cAMP and cGMP, others have an optimum pH that is acidic while others have an optimum pH that is alkaline and yet others can hydrolyse 3′,5′- and/or 2′,3′-cyclic nucleotides (Chiatante *et al.*, 1987, Newton *et al.*, 1990).

Thus in an attempt to try and conclusively identify any physiological role(s) for cAMP in higher plants it was even necessary to establish specific and particular cellular targets for its

functional actions. In mammals, cAMP regulates a considerable number of physiological processes by interfering with gene expression. Induction is generally fast and independent of intermediate protein synthesis *de novo*. Hence in a search for *cis*-acting sequences governing the sensitivity of somatostatin gene expression to cAMP, a short palindromic sequence motif (5′-TGACGTCA-3′) was found that was highly conserved in the promoter region of many cAMP-induced genes (Montminy *et al.*, 1986). However, as has been stated earlier on, cAMP does not act solely through the phosphorylation of proteins. Olfactory perception, for example, is governed by a direct binding of cAMP to cyclic-nucleotide-gated channels. In addition, a search for cAMP-binding activity in plants also readily yielded some cAMP-specific binding activities without any accompanying protein kinase activity. For instance, an identified Helianthus binding protein was very specific for cAMP and 8-bromo-cAMP yet the 5′-AMP and other 3′,5′-cyclic nucleotides could not bind to this same protein (Giannattasio *et al.*, 1974). Apparently, there was also some strong evidence suggesting that cAMP could be involved in plant defence responses that produce phytoalexins. This form of a stress response system has clear analogies to the mammalian secondary-messenger system, involving an extracellular signal, a receptor, a signal transduction system and a metabolic response (Smith, 1996). However, in the absence of any as yet undetermined alternative pathway of biosynthesis, then the presence of cAMP in plant tissues meant that ACs could also have been present (Newton and Smith, 2004).

Up until recently, such evidence of AC activity in higher plants had been treated with some scepticism, because of the failure to identify DNA sequences in plant genomes with significant homology to those of established cyclases. However, there are now reports of cloning and activity determination of some putative AC candidates in higher plants. One from *Zea mays*, the PsiP (pollen-signalling protein), representing a functional AC molecule (Moutinho *et al.*, 2001), while the other is from *Arabidopsis thaliana*, the pentatricopeptide

protein (Ruzvidzo *et al.*, 2013), the HpAc1 invloved in stress signalling in *Hippeastrum hybridum* (Świeżawska et al., 2014) and the NbAC (*Nicotiana benthamiana* adenylyl cyclase (Ito et al., 2014). In both cases, the cloned fragment domain confirmed some evident AC activity (Ruzvidzo *et al.*, 2013).

Apparently, in *Arabidopsis thaliana*, functionally tested GCs have been identified with a 14 amino acid long search term (Ludidi and Gehring, 2003) deduced from an alignment of conserved and functionally assigned amino acids in the catalytic centre of annotated GCs (Liu *et al.*, 1997, Mccue *et al.*, 2000) from lower and higher eukaryotes ([RK][YFW][CTGH][VIL][FV]X[DNA]X[VIL]X(4)[KR]X(1,3)[DE]). For this same reason, it was also anticipated and expected that a similar approach could lead to the discovery of novel ACs in the same genome. Hence in order to achieve this, the previously identified 14 amino acid long GC catalytic centre search motif was used but after its modification for specificity to ATP rather than GTP binding and with the C-terminal metal binding residue ([RK][YFW][DE][VIL][FV]X(8)[KR]X(1,3)[DE]). Subsequently, this motif successfully retrieved nine putative AC protein candidates (Table 1) of which the maternal effect embryo arrest 22 (AtMEE22) was one of them (Gehring, 2010). Apparently, while one of these putative candidates, the pentatricopeptide protein, has already been experimentally tested and confirmed as a functional AC (Ruzvidzo *et al.*, 2013), the rest including the AtMEE22 are yet to be practically tested and confirmed. Therefore, this study was thus specifically set to experimentally test the AtMEE22 protein (which is also known as EMB1611: EMBRYO DEFECTIVE 1611) (Meinke *et al.*, 2008); F19I3.1; and F19I3_1 (http://www.ncbi.nlm.nih.gov/gene/818043) if it could operate as a functional AC, and particularly with respect to the critical plant processes like growth, development and stress response mechanisms.

Table 1: The nine 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 homology1
At1g62590	-KFDVVISLGEKMQR–LE-	Pentatricopeptide (PPR) protein
At1g68110	-KWEIFEDDYRCFDR–KD-	Epsin N-terminal homology2
***At2g34780**	**-KFEIVRARNEELKK-EME-**	**Maternal effect embryo arrest 22***
At3g02930	-KFEVVEAGIEAVQR–KE-	Chloroplast protein
At3g04220	-KYDVFPSFRGEDVR–KD-	TIR-NBS-LRR class
At3g18035	-KFDIFQEKVKEIVKVLKD-	Linker histone-like protein-HNO4
At3g28223	-KWEIVSEISPACIKSGLD-	F-box protein
At4g39756	-KWDVVASSFMIERK–CE-	F-box protein

ATG represents the assigned *Arabidopsis thaliana* gene bank numbers for the nine putative AC proteins, followed by their amino acid sequences suspected to be their AC catalytic centres, and the names to which each protein was bioinformatically inferred (annotations).

*The AtMEE22 protein that was cloned and functionally characterized in this study.

1.3 The Prospects of Maternal Effect Embryo Arrest Protein as a Functional Adenylate Cyclase

The name maternal effect embryo arrest (MEE22) is derived from the description of mutant screens (*mee*) of Ds transposon insertion lines with defects in embryogenesis due to mutations in the female gametophyte (Pagnussat *et al.*, 2009). The same mutants have differently been named embryo-defective (*emb*) mutants by McElver et al. (2001). While the *mee* mutants were determined using the Ds transposons (Pagnussat *et al.*, 2009), the *emb* defects were determined using T-DNA (Mcelver *et al.*, 2001) even though both are just two different methods of mutational analysis. In Arabidopsis plants, the MEE22 protein (referred to as the AtMEE22) has previously been shown to be an essential molecule with physiological roles in the maintenance of shoot-apical meristems (SAM) (Leasure *et al.*, 2009). Apparently all plant leaves, stems, and floral parts develop from the SAM, a

conserved stem cell niche that resides at the top of the apical-basal axis of the plant (Figure 1.3) (Miwa *et al.*, 2008, Murphy *et al.*, 2012).

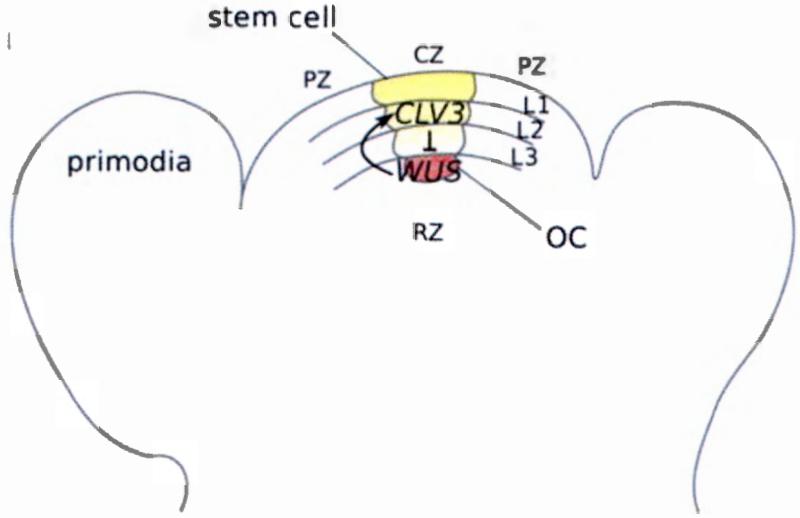

Figure 1.3: Structure of SAM and the expression of its regulatory genes. The SAM is divided into three zones, the peripheral zone (PZ), the central zone (CZ) and the rib zone (RZ) and three layers, L1, L2 and L3. CLV is expressed in the stem cell (yellow) whereas WUS is expressed is expressed in the organizing centre (OC red). The coordinated SAM structure is regulated by WUS-CLV3 negative feedback loop (adapted from Miwa *et al.*, 2008).

Besides its involvement in the maintenance of shoot-apical meristems (SAM) (Leasure *et al.*, 2009), the AtMEE22 protein has also been previously reviewed by various computational analyses and was inferred to have a central role in the following critical and specific biological processes: regulation of flower development; primary shoot apical meristem specification; mRNA export from the nucleus; photomorphogenesis; sister chromatid cohesion; seed dormancy process; lipid storage, cotyledon development, leaf development, determination of bilateral symmetry, seed maturation, sugar mediated signalling pathway, covalent chromatin modification, embryonic pattern specification, embryo sac egg cell differentiation, regulation of cell cycle process, seed germination, response to freezing, regulation of cell differentiation, protein ubiquitination, mitotic recombination, cell division, meristem initiation, meristem maintenance and vegetative to reproductive phase transition

meristem, (Heyndrickx and Vandepoele, 2012). In addition to this, when the protein was also analyzed by mutant phenotyping, the AtMEE22 was further inferred to be involved in the maintenance of meristem identity and meristem structural organization (Leasure *et al.*, 2009) and also in embryo development ending in seed dormancy (Meinke *et al.*, 2008).

Furthermore, when the AtMEE protein was analyzed based on its expression pattern, it was realized that it is also expressed during the following essential growth stages: the F mature embryo stage, the petal differentiation and expansion stages, the 4 leaf senescence stage, the C globular stage, the 4 anthesis stage, the L.P. 02 two leaves visible stage, the L.P. 04 four leaves visible stage, the L.P.06 six leaves stage, the L.P. 08 eight leaves visible stage, the L.P. 12 twelve leaves visible stage, the E expanded cotyledon stage and the D bilateral stage (Schmid *et al.*, 2005) Again and based on its expression patterns, the AtMEE22 protein was further found to be differentially expressed in the following different anatomical structures: the shoot apex, the vascular leaves, the petiole, the leaf lamina base, the inflorescence meristem, the flower, the stamen, the hypocotyl, the root, the shoot system, the stem, the cotyledon, the cauline leaf, the carpel, the sepal, the collective leaf structure, the petal, the pedicel, the guard cell, the leaf apex, the plant embryo and the seed (Schmid *et al.*, 2005).

Apparently, by considering the fact that the AtMEE protein has previouly been implicated in various physiological processes listed above (Schmid *et al.*, 2005, Meinke *et al.*, 2008, Leasure *et al.*, 2009, Heyndrickx and Vandepoele, 2012), and whose signalling systems typically involve mediation by the second messenger, cAMP and yet again, the same protein has recently been bioinformatically annotated as a putative AC candidate (Gehring, 2010). The folliwing set of objectives were set to be met: 1. To isolate and clone the Arabidopsis AtMEE-AC gene into a stable and viable heterologous prokaryotic expression system; 2. To optimize strategies for the expression and purification regimes of the recombinant AtMEE-AC protein; 3. To determine the biological/enzymatic activity of the annotated candidate AtMEE-AC protein; and 4. To further characterize the biological/enzymatic activities of the candidate AtMEE-AC protein with the intention of establishing its exact physiological roles

in plants, particularly in biotic and abiotic environmental stress response and adaptation mechanisms.

CHAPTER 2

Molecular Cloning and Partial Expression of the Arabidopsis Maternal Effect Embryo Arrest Gene Fragment

Abstract

Second messengers have a key role in linking environmental stimuli to cellular responses. One such messenger, adenosine 3′,5′-cyclic monophosphate (cAMP) generated by the enzyme, adenylate cyclase (AC), has long been known to be an essential signalling molecule in many cellular processes including growth, development and responses to stressful factors. However, while extensive detail about these important molecules is widely available in animals and lower eukaryotes, the presence of ACs in higher plants has up to date largely remained obscure and elusive. Thus, in an effort to search for a functional higher plant AC, we cloned and partially expressed an AC-containing fragment domain of the maternal effect embryo arrest protein from *Arabidopsis thaliana* (AtMEE-AC) in competent BL21 (DE3) *Escherichia coli* cells and demonstrated its ability to induce the generation of endogenous cAMP in these prokaryotic systems.

2.1 Introduction

Expression of proteins in bacterial systems such as *Escherichia coli*, plays a very important role in the efficient production of genetically-engineered proteins particularly when their biological functions do not depend on post-translational modifications such as glycosylation, methylation and phosphorylation (Allocco *et al.*, 2004, Abahssain *et al.*, 2010). These systems remain the most attractive ones due to their low cost, high productivity, and the availability of a large number of plasmid vectors and host strains that have specifically been developed to maximize and perfect the expression of heterologous proteins (Applebaum and Shatzman, 1999, Rai and Padh, 2001, Terpe, 2006). The gram-negative bacterium *E. coli,* which usually is either *E. coli* BL21 or *E. coli* K12, is the most commonly used prokaryotic organism for the heterologous production of recombinant proteins (Applebaum and Shatzman, 1999). Notably, All high-level expression vectors have the following structural and functional features that make them most suitable and ideal for heterologous protein expression in *E. coli*: (a) regulatory elements which control transcription and translation; (b) restriction endonuclease cleavage sites for convenient (directional) insertion and cloning of coding sequences; (c) a region encoding vector replication functions; and (d) a selectable marker (usually an antibiotic resistance gene) for maintaining selection pressure of cells carrying the vector (Applebaum and Shatzman, 1999, Rai and Padh, 2001). Many vectors encode additional optional components such as signal sequences to direct secretion, or peptide tags that are added to the N- or C- terminus of the protein for its detection and subsequent purification processes (Applebaum and Shatzman, 1999, Rai and Padh, 2001).

Some of the vector systems that have so far been developed and are currently in common use include; pET, pGEX, pQE, pTOPO and pAS, all whose promoters may be the T7-based that require IPTG for expression induction. The critical key to all pTOPO cloning systems is the enzyme DNA topoisomerase I, which functions both as a restriction enzyme and as a ligase.

Its biological role is to cleave and rejoin DNA during replication. It cleaves one DNA strand, enabling the DNA to unwind and it then re-ligates the ends of the cleaved strand before releasing itself from the DNA (Technologies, 2010). New and recent technologies like the Gateway and pDEST have been developed and established from the pTOPO design (Technologies, 2010). In this chapter, we detail the recombinant expression of an AC-containing fragment domain of the maternal effect embryo arrest protein from *Arabidopsis thaliana* (AtMEE-AC) using a pCRT7/NTTOPO prokaryotic expression system (Invitrogen) followed by an explorative assessment of this protein's potential endogenous AC activity by enzyme immunoassay (Sigma-Aldrich, Missouri, USA).

2.2 Materials and Methods

2.2.1 Regeneration of Arabidopsis Plants and Plant Growth Conditions

The recombinant AtMEE-AC protein used in this study was generated from 4-week-old *Arabidopsis thaliana* (Columbia-0) plants which were generated and maintained in a growth chamber (Labcon Design Engineering, Johannesburg, RSA) under the general plant cell culture conditions (16 hrs light/8 hrs dark cycles; day/night temperatures of 23°C; average day/night humidity of 30/70%; average range of day-time irradiances of 150-650 $\mu mol \cdot s^{-1}m^{-2}$ and daily CO_2 concentrations of 380-390 ppm). The four specific steps listed below were then sequentially and specifically followed in order to regenerate the desired Arabidopsis plants.

2.2.1.1 Seed Sterilization

Under laminar flow conditions, approximately 150-200 (~4 mg) (Cotter, 2005) Arabidopsis (Columbia-0) seeds were surface-sterilized by collecting them in a 1.5 ml Eppendorf tube followed by the addition of 0.5 ml 70% (v/v) ethanol (EtOH) and vortexing for 30 sec. The EtOH was discarded and 0.5 ml sterilization buffer (5% (v/v) bleach and 0.1% (w/v) SDS and 0.1% (v/v) Triton-X-100) was then added and the mixture was vortexed for a minute. This process was repeated three times over. The sterilization buffer was then discarded and 1 ml of distilled H_2O added, followed by a further vortexing for 30 sec (washing step). This washing step was then repeated 5 more times before the sterilization process was completed.

2.2.1.2 Seed Stratification

The surface-sterilized seeds were then stratified by adding to them 1.0 ml a 0.01% (w/v) Murashige and Skoog (MS) agar (Sigma-Aldrich, Missouri, USA) followed by an incubation at 4°C for 3 days in the dark (Yamauchi *et al.*, 2004). This process is employed to break the dormancy as well as to ensure synchronous germination among the treated plant seeds (Olmez *et al.*, 2006).

2.2.1.3 Seed Germination

The surface-sterilized and stratified seeds were subsequently placed into petri plates containing MS growth medium (0.43% (w/v) of MS salt (Sigma-Aldrich, Missouri, USA), 3% (w/v) sucrose (Sigma-Aldrich, Missouri, USA), and 0.8% (w/v) type M agar (Sigma-Aldrich, Missouri, USA), 0.01 mg/ml kinetin (Sigma-Aldrich, Missouri, USA) and 0.01 mg/ml napthalene acetic acid (Sigma-Aldrich, Missouri, USA), pH: 5.8 (with 1M KOH)). The seeded plates were then sealed off with parafilm (to minimise desiccation and contamination) before being incubated for a period of 14 days in a GC-300TL plant growth chamber (Jeio Tech, Seoul, South Korea) under plant cell culture growth conditions. This 2-week incubation period then allowed the synchronized germination of the surface-sterilized seeds into sterile and healthy seedlings.

2.2.1.4 Seedling Transplantation

After the 14 days of seed germination, the generated seedlings were then transplanted to sterile healthy potting soil (33.3% peat-based soil, 33.3% humus and 33.3% vermiculite) supplemented with a commercial plant fungicide (0.01% (w/v) Gaucho). The seedlings were allowed to grow for a further 4 weeks before being used as experimental material for this study.

2.2.2 Designing and Acquisition of AtMEE-AC Sequence-specific Primers

The genomic sequence of the At2g34780 gene (Fig 2.1), which encodes the putative AtMEE22 protein (Fig 2.7) and whose fragment domain (AtMEE-AC) was cloned and utilized in this study was bioinformatically retrieved from The Arabidopsis Information Resource (TAIR) web page (http://arabidopsis.org). Since in this study, the AtMEE-AC gene fragment was going to be cloned into a pCRT7/NT-TOPO expression vector (Fig 2.3), restriction sites with nucleotide sequences complementary to those of the pCRT7/NT-TOPO expression vector were included within the specific primers for the specific directional cloning and expression of the targeted truncated AtMEE22 protein. The forward primer carried the restriction site for *Bam* HI (bold and underlined) (GCC CGG AA**G GAT CC**A ATG TCG GAG TTG GAG GTG) while the reverse primer carried the restriction site for *Eco* RI (bold and underlined) (GCG CCG **GAA TTC** CGA GAC TAA TTG CGC TTC TTG). These sequence-specific primers were manually designed to strategically amplify about 50 amino acids from the N and C terminal sites of the probable adenylate cyclase catalytic centre of the AtMEE22 protein. After their design, the primers were then chemically synthesized at the University of Cape Town, and acquired as freeze-dried samples. The primers were then reconstituted in sterile distilled H_2O to working concentration stock solutions of 10 µM, and for the subsequent polymerase chain reaction (PCR) work.

ATGGCGACGACCAATGCTCCACCTGAGCTAGCTTCAGGAAACCCTTGCTGCCTCGCTTGGCAAGGGAA
GTACATAGGAATGAAGAAGAGGCGAGATGCTTTTAAAGAAGGAGTAACTCTTCTCCAGAAGGCTATAG
AAAATGTTAATGCTGAAAAATCCAATCTCGAGAGAAAATTTGGGGAGATGGCGACTGACGGAGATACT
AAGGAAAATGGTTCAACTGTGAAAGCATCTTTGGAGAAAGAAATATCTCGTTTGAAGTTTGAGATAGT
GTCTTTGCAACAAAAACTGGAGCGGAACCTTAAAGAAAAAAGTGAAGAAACAAAGCTTCTTCAGGATC
AAGCTTCTGGTCGAGAAAAGGAGATCAATGAACTGAGGGATCTCCTTAAGAAAGAAACATTAAGGGCA
GATAGTTCTGTCGGAGAAGAAGAGAGGGAACATGCTTTTAAGGAACTGAACAAGGCAAAGGCTCTGAT
AGTAAAAGATGAGGAAATCGAGCAAGATATTCCTGAAGTAAAAAGGGAAATAAGTCTGGTTAAAAATC
TTCTTGCTAGTGAGAGACAGAAGACAGAGTCAGAGAGGAAGAAAGCAGAATCAGAGAAAAAGAAAGCT
GATAAGTAT**CTTTTGGAGGTGTTA**AGAAATTCAGCTCATAAGACTAGTTCTGATTTACTTACTTTGAC
CTCCAATCTTGAGACTGTAAAAAAGCAGCTTGAGCTTGAAAAACAGAAGACACTGAAAGAGAAAAAAC
GTGCAGATATGGAGAGTGCAAAAGCTAGGGACCAGATGAAGCTGGCAGAAGATGTGTCTAAG**AAGTTT**
GAAATCGTTAGAGCAAGAAATGAAGAGCTCAAGAAAGAAATGGAATCGCAGACCGCTAGTAGCCAAGT
GAAGTTTGCTGAAAACTCGGAAAAGCTGGAAGAGAAGATAAGGCTCCTTGAGATGAATAAAAAAACAG
CCATGGACTGGAAATCTCGTACTGATGATCTGACTCAACAGTTA**CAAGAAGCGCAATTAGTCGCT**GAA
GGTTTGAAGAAACAAGTGCATGAGCTTTCACTTTCCCAGAAATCCATCAAGACTCACTCTATTTCTCC
TCAGAAAGTCAGAGACCTGGAAAAGGCTGAAATGAGGCTTTTGAAAAAAAAGATGAAGTTTGAGAGAA
ATTGTGCAAAACACTCCCAAACAGTGGCTAAATTTGAAAAGTTTCGAAGGGAATTTCAGTGCGAAGAG
CTAGGTCGGTTGAAACTGGAGTTCGGTAGTCTTACGAATCGCATGAATCTACTGGATGAGTATTTTTC

Figure 2.1: Nucleotide sequences of the AtMEE22 gene. The probable AC catalytic centre is marked red and bold, while the forward priming site is highlighted green and bold, and the reverse priming site highlighted blue and bold. The sequences between the forward and reverse priming sites represent the truncated AtMEE-AC gene fragment that was cloned and used in this study.

The subsequent cloning of the putative AtMEE-AC gene fragment before its recombinant expression first involved its molecular linkage to the pCRT7/NT-TOPO expression vector whose physical (Figure 2.2a) and sequence (Figure 2.2b) maps are shown and briefly described below.

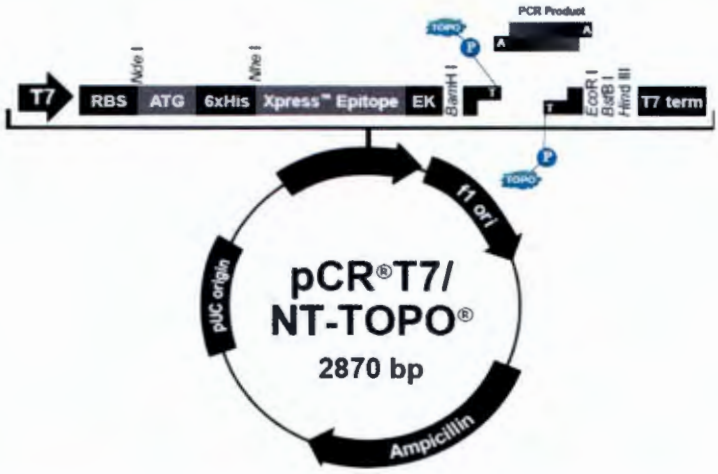

Figure 2.2a: Physical features of a commercially acquired pCRT7/NT-TOPO expression vector. Features are ***Eco* RI** and ***Bam* HI sites:** flank the restriction cloning site, **T7:** promoter bases, **RBS:** ribosome binding site, **ATG:** start codon, **6xHis:** His tag sequence, **Xpress™ Epitope:** bases, **T7 term:** transcription termination region, **pUC origin:** origin of replication, **f1ori:** expression origin, **Ampicillin:** ampicillin resistance gene site (adapted from the http/www.fermentas.com).

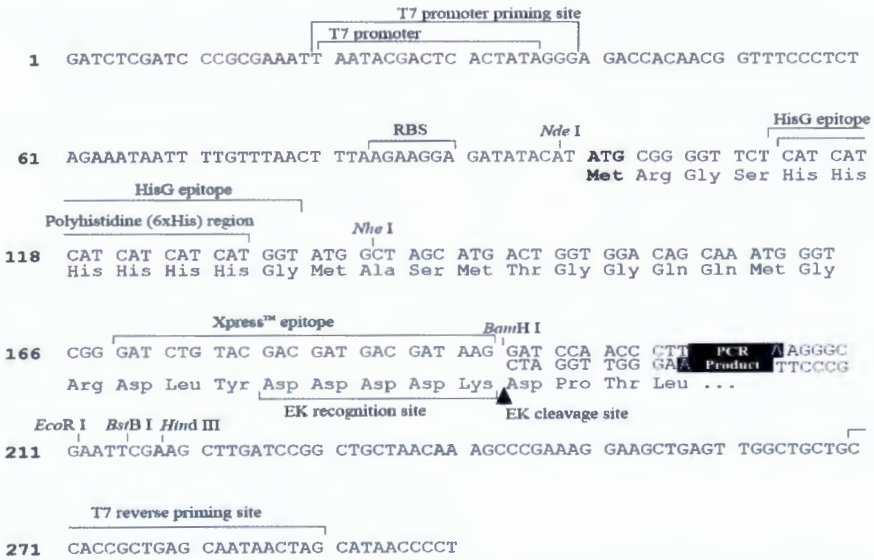

Figure 2.2b: The sequence map of a commercially acquired pCRT7/NT-TOPO expression plasmid vector. The illustration shows the expression and purification features of the expression plasmid vector, which include the T7 promoter for high levels of protein expression. There is also a region of forward and reverse priming sites for both *Bam* HI and *Eco* RI restriction enzymes within a multiple cloning site. For purification purposes, the vector expresses a recombinant 6-Histidine fusion protein that can be affinity purified on positively-charged chromatographic columns (adapted from http/www.fermentas.com).

2.2.3 Extraction and Purification of Arabidopsis Total RNA

In order to isolate and clone the targeted AtMEE-AC gene fragment, total RNA from the generated 4-week-old *Arabidopsis thaliana* plants (section 2.2.1) was isolated from the leaf tissue using the RNeasy Plant Mini Kit (QIAGEN Science, Maryland, USA). Following the manufacturer's instructions, an estimated amount of around 100 mg fresh leaf material was weighed and flash-frozen in liquid nitrogen before being thoroughly ground into a fine powder within an ice-cold mortar and pestle. The ground tissue powder and liquid nitrogen were then decanted into a liquid nitrogen-cooled RNase-free, 2 ml microfuge tube. The liquid nitrogen was allowed to evaporate, but the tissue was not allowed to thaw. Working as quickly as possible, 450 µl RLT buffer was added to the tissue powder and incubated at 56°C for 3 min to increase the disruption of the tissue, and the mixture was vortexed vigorously. The lysate was then transferred to a QIAshredder spin column placed in a 2 ml collection tube, and centrifuged for 2 min at 16 300*g* (full speed) using a Corning LSE High Speed Microcentrifuge (Corning Inc., Amsterdam, The Netherlands). The lysate was homogenized as it passed through the spin column (Qiagen, 2012). The supernatant of the flow-through was carefully transferred to a new microfuge tube without disturbing the cell debris pellet in the collection tube. Only the supernatant was then used in subsequent steps.

Absolute (100%) ethanol equal to half the volume of the supernatant (~200 µl) was added to the cleared lysate, and the sample then immediately mixed by pipetting. The sample, including any precipitate that might have formed, was transferred to an RNeasy spin column placed in a 2 ml collection tube. The lid was closed gently and the sample centrifuged for 15 sec at 10 000*g* using a Corning LSE High Speed Microcentrifuge (Corning Inc., Amsterdam, The Netherlands). The flow-through was discarded. Then, 700 µl RW1 buffer was added to the RNeasy spin column, the lid was closed gently and the sample then centrifuged for 15 sec at 10 000*g*, and in order to wash the spin column membrane. The flow-through was

discarded while 500 µl of RPE buffer was then added to the RNeasy spin column. The lid was closed gently and the sample centrifuged for 1 min at 10 000*g* (Corning Inc., Amsterdam, The Netherlands), and in order to wash the spin column membrane. The RNeasy spin column was placed into a 1.5 ml collection tube and a total volume of 50 µl RNase-free water was then added directly onto the spin column membrane and the sample centrifuged for 1 min at 10 000*g* to elute the RNA (Qiagen, 2012).

The actual concentration of the total RNA isolated from the above procedures was then determined with a Nanodrop 2000 spectrophotometer (Thermo Scientific, Massachesetts, USA) and further resolved by electrophoresis on a 0.8% (w/v) agarose gel (Biocom Ltd, Bridge of Weir, UK) for 50 min at 80 V, and to confirm its quality and integrity.

2.2.4 Isolation of the AtMEE-AC Gene Fragment

In order to specifically isolate the targeted AtMEE-AC gene fragment for its subsequent cloning and expression in the study, three different steps were undertaken and these involved its amplification by reverse transcription polymerase chain reaction (RT-PCR), its resolution by gel electrophoresis and its final cleaning and purification.

2.2.4.1 Reverse Transcription-Polymerase Chain Reaction

For us to be able to amplify the targeted AtMEE-AC gene fragment, firstly, the total Arabidopsis RNA isolated in section 2.2.3 above was used as a template for the generation of copy DNA (cDNA) and using a specialized reverse transcription polymerase chain reaction (RT-PCR) system (Verso 1-Step RT-PCR ReddyMix), (Thermo Fisher Scientific Inc., Massachusetts, U.S.A.). Secondly, the generated cDNA was then used as a template in the

same RT-PCR system for both the manually prepared forward and reverse sequence specific primers (section 2.2.2) to specifically amplify the targeted AtMEE-AC gene fragment. The RT-PCR system was prepared in a final reaction volume of 50 µl and in accordance with the manufacturers' instructions (Thermo Fisher Scientific Inc., Massachusetts, USA) (Table 2.1 below).

Table 2.1: Reaction components of the 1-Step RT-PCR system used for the amplification of the targeted AtMEE-AC gene fragment.

Component	Volume	Final Concentration
Verso Enzyme Mix	1 µl	
1-Step PCR ReddyMix (2x)	25 µl	1x
Forward Primer (10 µM)	1 µl	200 nM
Reverse Primer (10 µM)	1 µl	200 nM
RT Enhancers	2.5 µl	
Water (PCR grade)	19.5 µl	
Template (RNA)	1 µl	1 ng
Total Volume	50 µl	

After preparing the above reaction mixtures, the mixed reaction components were then subjected to the thermo-cycling profile indicated in Table 2.2 below and in accordance with the manufacturer's instructions (Thermo Fisher Scientific Inc, Massachusetts, USA). A Bio Rad C-1000 thermo-cycler (Bio-Rad Laboratories Inc., California, USA) was used to carry out this specific amplification of the targeted AtMEE-AC gene fragment.

Table 2.2: Reaction conditions for the 1-Step RT-PCR thermal cycling program used for amplification of the targeted AtMEE-AC gene fragment.

Component	Temperature	Time	Number of Cycles
cDNA Synthesis	50°C	15 min	1 cycle
RT Inactivation	95°C	15 min	1 cycle
Denaturation	95°C	20 sec	↕ 40 cycles
Annealing	63.5°C	30 sec	
Extension	72°C	1 min	
Final Extension	72°C	5 min	1 cycle

2.2.4.2 Agarose Gel Electrophoresis

After the RT-PCR process, the amplified AtMEE-AC gene fragment was resolved on a 1% (w/v) agarose gel stained with 0.1 µg/µl of ethidium bromide at 80 V for 50 min and against a 100 bp GeneRuler DNA Ladder (Thermo Fisher Scientific Inc, Massachusetts, USA). Visualization of the gel was done under short wavelength of the UV illumination using a Chemidoc gel imaging system (Bio-Rad Laboratories, California, USA) and in order to confirm and verify the presence of an RT-PCR product corresponding to the anticipated size of the AtMEE-AC gene fragment.

2.2.4.3 Cleaning of the Amplified Gene Product

After confirming amplification of the anticipated AtMEE-AC gene fragment by gel electrophoresis, the amplified gene product was then cleaned and purified from the whole PCR solution using a DNA Clean & Concentrator-5 kit and in accordance with the manufacturer's instructions (Zymo Research Corp, California, USA). Briefly, about 100 µl of the amplified AtMEE-AC gene product solution was briefly mixed (by vortexing) with 100 µl of DNA binding buffer in a 1.5 ml microfuge tube. The mixture was then transferred into a Zymo-Spin Column mounted into a collection tube and centrifuged at 10 000*g* in a Corning LSE High Speed Microcentrifuge, (Corning Inc., Amsterdam, The Netherlands) for 30 sec and the flow-through was discarded as waste. For two times, 200 µl of wash buffer was added to the column and centrifuged at 10 000*g* (Corning® LSE™ High Speed Microcentrifuge, Corning Inc., Amsterdam, The Netherlands) for 30 sec. In order to elute the purified DNA product, 6 µl of warm sterile distilled water was directly loaded onto the column matrix and allowed to stand for 1 min. The column was then transferred to a 1.5 ml microfuge tube and centrifuged at 10 000*g* (Corning Inc., Amsterdam, The Netherlands) for

30 sec. The eluent containing the cleaned AtMEE-AC gene fragment was then collected and quantified by using Nanodrop ND 2000 spectrophotometer (Thermo Scientific, Massachusetts, USA).

2.2.5 Restriction Double Digestion of the AtMEE-AC Amplicon and the pCRT7/NT-TOPO Vector

In order to prepare the purified AtMEE-AC gene amplicon (insert) for ligation into the pCRT7/NT-TOPO plasmid vector, similar restriction double digestion processes of both the AtMEE-AC insert and its corresponding pCRT7/NT-TOPO plasmid vector were simultaneously conducted following the manufacturer's protocol (Thermo Scientific, Inc., Massachusetts, USA). A 50 µl reaction mixture containing 10 units of *Bam* HI, 10 units of *Eco* RI, 2X Tango buffer (Thermo scientific Inc., Massachusetts, USA) and either 15.0 ng/µl plasmid DNA (pCRT7/NT-TOPO plasmid vector) or 10.0 ng/µl insert DNA (AtMEE-AC gene fragment) was prepared in a 1,5 ml Eppendorf tube and incubated on a D1100 AccuBlock Digital dry bath (Labnet International Inc., New Jersey, USA) to digest at 37°C for 4 hrs. The reaction was finally halted by heating for 20 min at 80°C. The digested DNA fragments were then cleaned with a DNA Clean & Concentrator-5 kit, and in accordance with the manufacturer's instructions (Zymo Research Corp, California, USA) as has already been outlined in section 2.2.4.3. The digested products were then stored at -20°C for further use.

2.2.6 Ligation of the AtMEE-AC Insert into the pCRT7/NT-TOPO Plasmid Vector

The restriction double digested AtMEE-AC gene insert was correspondingly ligated into the cloning site of its complementary pCRT7/NT-TOPO plasmid vector which was also equally double digested to produce a pCRT7/NT-TOPO:AtMEE-AC fusion expression construct with

an N-terminus His purification tag. Restriction fragments generated from both the insert and the plamid during the double digestion process produced sticky (palindromic) ends, which were then taken advantage of so as to ligate them at a molar ratio of 1:3 vector:insert. In this regard, a 20 µl reaction mixture containing 20.0 ng/µl pCRT7/NT-TOPO plasmid vector, 60.0 ng/µl AtMEE-AC gene insert, 1 unit T4 DNA ligase enzyme, and 1X T4 DNA ligase buffer was prepared in a 0.2 ml microfuge tube. The reaction mixture was incubated at 22°C on a C1000 Touch Thermal Cycler (Bio-Rad Laboratories Inc., California, USA) for 1 hr and then held at 4°C overnight. The ultimate ligated mixture was then stored at -20°C for further use.

2.2.7 Preparation of Competent BL21 (DE3) Cells

Escherichia coli BL21 (DE3) cells (Life Technologies, California, USA) were prepared to become chemically competent before being transformed with the pCRT7/NT-TOPO:AtMEE-AC fusion expression construct for the subsequent expression of the targeted recombinant AtMEE-AC protein. A loopful of *E. coli* BL21 (DE3) cells from a plated colony was inoculated into 10 ml of Luria Bertani (LB) broth (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) sodium chloride and 0.4% (w/v) glucose) supplemented with 34 µg/ml chloramphenicol and incubated at 37°C in an SI-600 shaking incubator (Lab Companion, Seoul, South Korea) at 200 rpm. The following morning, a further 100 ml of freshly prepared LB broth was aerated under similar conditions for 2 hrs. The aerated broth was then inoculated with 1 ml of the overnight culture supplemented with 34 µg/ml of chloramphenicol and incubated at 37°C in a shaking incubator at 200 rpm and until an OD_{600} of 0.5 was reached as measured by a Spectronic Helios Epsilon spectrophotometer (Thermo Scientific Instruments LLC, Wisconsin, USA). The culture was then divided into 50 ml

portions (in sterile 50 mL Falcon tubes) and the cells were harvested by centrifuging at a low speed of 4 000*g* for 5 min at 4°C using a Hermle centrifuge (Labortechnik, Wehingen, Germany). The supernatant was discarded and the cells re-suspended into 15 ml of ice-cold Transformation Buffer 1 (TFB 1) (30 mM potassium acetate, 50 mM manganese chloride, 100 mM rubidium chloride, 10 mM calcium chloride, 15% (v/v) sterile glycerol, pH: 5.8) and incubated on ice for an additional 90 min. The cells were once again harvested by centrifuging at a low speed of 4 000*g* for 5 min at 4°C using a Hermle centrifuge (Labortechnik, Wehingen, Germany). The supernatant was discarded and the harvested cells re-suspended in 4 ml of ice-cold Transformation Buffer 2 (TFB 2) (10 mM MOPS [3-(N-morpholino)propanesulfonic acid], 75 mM calcium chloride, 10 mM rubidium chloride, 15% (v/v) glycerol, pH: 6.8). Several volumes of 200 µl aliquots of the competent cells were then dispensed into 1.5 ml microcentrifuge tubes and snap-frozen with liquid nitrogen before being stored at -80°C for further use.

2.2.8 Transformation of the Competent BL21 (DE3) Cells with the pCRT7/NT-TOPO:AtMEE-AC Fusion Construct

A total 10 µl of the ligation mix (pCRT7/NT-TOPO:AtMEE-AC Fusion Expression Construct) was transferred into a clean ice-cold Eppendorf tube and kept on ice. An aliquot of competent cells was thawed on ice and its 100 µl portion was then added to the ligation mix. The mixture was gently mixed by swirling with a pipette tip and then kept on ice for 20 min. The mixture was heat-shocked in a water bath at 42°C for 90 sec. The mixture was immediately placed on ice and 500 µl of the Super Optimal Broth with Catabolite Repression (SOC) broth (2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10 mM sodium chloride, 2.5 mM potassium chloride, 10 mM magnesium chloride, 10 mM magnesium sulphate, 20 mM glucose) was added. The mixture was incubated for 90 min at 37°C in an SI-600 orbital

shaker at 200 rpm (Lab Companion, Seoul, South Korea). Using the spread plate technique, aliquots of 50, 100 and 200 µl of the transformation mixture were then inoculated onto LB agar plates supplemented with both 100 µg/ml ampicillin and 34 µg/ml chloramphenicol (Bioline, London, United Kingdom) and then left to grow overnight at 37°C.

2.2.9 Screening and Verification of the AtMEE-AC Recombinants by Colony PCR

So as to screen for the BL21 (DE3) cells that had successfully picked up the intended pCRT7/NT-TOPO:AtMEE-AC Fusion Expression Construct, several replica colonies from the LB plates above were picked and used as templates for a modified RT-PCR cycling system previously outlined in section 2.2.4.1. Briefly, a 50 µl reaction mixture (Table 2.1) in which the total RNA template was replaced by a bacterial colony was prepared. The reaction mixture was then subjected to a cycling system shown in Table 2.2 but with the initial step (cDNA synthesis step) omitted. The resultant and amplified RT-PCR fragment was then analysed by SDS-PAGE to verify if it was representing the cloned AtMEE-AC gene fragment.

2.2.10 Partial Expression of the Recombinant AtMEE-AC Protein

In order to validate the cloning success of the targeted AtMEE-AC gene fragment that was earlier on verified by colony PCR (section 2.2.9 above), a replica set of the confirmed colony was picked from the LB plate and used to inoculate 10 ml of a double strength yeast-tryptone (2YT) medium (1.6% (w/v) tryptone, 1% (w/v) yeast extract, 0.5% (w/v) sodium chloride, 0.4% (w/v) glucose) supplemented with 100 µg/ml ampicillin and 34 µg/ml chloramphenicol. The inoculated culture was then incubated overnight at 37°C in an SI-600 shaking incubator at 200 rpm (Lab Companion, Seoul, South Korea). The next morning, 20 ml of 2YT medium

supplemented with both ampicillin and chloramphenicol were then inoculated with 200 µl of the overnight culture followed by an incubation at 37°C at 200 rpm and up until the optical density (OD_{600}) had reached 0.5. At this optical density, the culture was split into two equal portions. One culture was then induced with 1 mM IPTG to express the targeted AtMEE-AC recombinant protein (induced culture) while the other one was left un-induced (non-induced culture). The two cultures were then allowed to grow for a further 1 hr at 37°C in an orbital shaker shaking at 200 rpm. Cells of both cultures were then harvested by centrifugation at 4 000*g* for 5 min before protein expression was finally analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Successful expression of the targeted recombinant AtMEE-AC protein was then validated by the presence of a protein band of the size of its approximate molecular weight in the induced culture but not in the non-induced culture.

2.2.11 Determination of the Endogenous AC Activity of AtMEE-AC

After confirming the partial expression of the targeted and desired recombinant AtMEE-AC protein, and in to determine its possible endogenous adenylate cyclase (AC) activity, a bacterial culture (10 ml) of the BL21 (DE3) cells harbouring the pCRT7/NT-TOPO:AtMEE-AC fusion expression construct was grown overnight at 37°C in an orbital shaker at 200 rpm in 2YT medium supplemented with 100 µg/ml ampicillin and 34 µg/ml chloramphenicol. The next morning, 20 ml of 2YT medium supplemented with both ampicillin and chloramphenicol were then inoculated with 200 µl of the overnight culture followed by an incubation at 37°C at 200 rpm and up until the optical density (OD_{600}) had been reached 0.5. The culture was immediately placed on ice and split into four portions of 5 ml each. Protein expression was then induced in three cultures through the addition of 1 mM IPTG, while the other fourth culture was left un-induced. From the three induced cultures, one culture was

further supplemented with 100 µM forskolin (Sigma-Aldrich, Missouri, USA) and the other culture also further supplemented with 100 µM 2′,5′-Dideoxyadenosine (Sigma-Aldrich, Missouri, USA). The cultures were then all allowed to grow for a further 2 hrs at 37°C in an orbital shaker at 200 rpm before their cells were harvested by centrifugation at 4 000*g* for 5 min.

To each culture of the harvested cells, 1 ml of Lysis Assay Buffer 1 (Amersham Healthcare, New Jersey, USA) supplemented with 2 mM IBMX (to inhibit phosphodiesterases) (Sigma-Aldrich, Missouri, USA) was added. The sample was then swirled at 100 rpm on an AGITADOR orbital shaker (Comecta, Pretoria, South Africa) for 30 min at 37°C, and to intensify the cell lysis process. The samples were then centrifuged at 16 300*g* for 5 minutes and the lysate transferred into a fresh Eppendorf tube, where 200 µl of the Lysis Assay buffer 2 (Amersham Healthcare, New Jersey USA) was added and mixed. The mixture was then centrifuged at 16 300*g* for 5 minutes before the resultant lysate was transferred to a fresh Eppendorf tube. The endogenous cAMP levels of each lysates were then measured by a CA201 cAMP enzyme immunoassay kit (Sigma-Aldrich, Missouri, USA), following the acetylation protocol and as is described by the manufacturer's manual. Measurements were taken in triplicates using a Labtech microplate reader (Labtech, East Sussex, UK) at 405 nm, and the resultant readings then subjected to statistical analysis.

2.2.12 Statistical Analysis

Results of all the undertaken enzyme immunoassays were based on the means of three replicates where outcomes of each assay were subjected to analysis of variance (ANOVA) (Super-Anova, Statsgraphics Version 7, 1993, Statsgraphics Corporation, USA). Where the

ANOVA revealed significant differences between outcomes, the means (n = 3) were separated using a *post hoc* Student Newman Kuehls (SNK), multiple range test ($p \leq 0.05$).

2.3 Results

In *Arabidopsis thaliana*, functionally tested and experimentally confirmed guanylate cyclases (GCs) have previously been identified with a 14-mer search motif (Figure 2.3) deduced from an alignment of conserved and functionally assigned amino acids in the catalytic centre of annotated nucleotide cyclases from lower and higher eukaryotes (Ludidi and Gehring, 2003, Gehring, 2010). This previously identified 14-mer GC catalytic search motif was later modified for specificity for ATP rather than GTP binding and with the C-terminal metal binding residues (Figure 2.3) and then used to query the Arabidopsis genome, resulting in the retrieval of nine putative AC candidates, which included the AtMEE22 (At2g34780) gene (Figure 2.1) that encodes for the putative AtMEE22 protein (Figure 2.6) (Gehring, 2010).

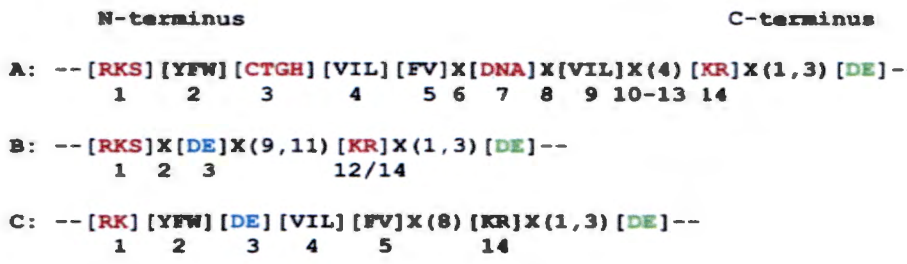

Figure 2.3: The catalytic centre motifs of nucleotide cyclases. (A) Centre motif of experimentally tested GCs in plants. The residue (red) in position 1 does the hydrogen bonding with the guanine, the amino acid in position 3 confers substrate specificity and the residue in position 14 stabilises the transition (GTP/cGMP). The Mg^{2+}/Mn^{2+}-binding site is C-terminal (green). In the derived motifs (B and C) specific for ACs, position 3 (blue) has been substituted to [DE] to allow for ATP binding (Adapted from Gehring, 2010).

Following the establishment of an AC catalytic motif in the Arabidopsis MEE22 gene, some sequence-specific AtMEE22 primers were then manually designed (section 2.2.2) to attempt and isolate an AtMEE22 truncated gene fragment containing the annotated AC catalytic motif (AtMEE-AC). The targeted truncated AtMEE-AC gene fragment was then successfully isolated from the Arabidopsis total RNA through RT-PCR, and as is shown in Figure 2.4 below.

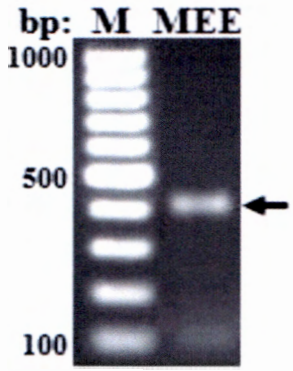

Figure 2.4: Isolation of the AtMEE-AC gene fragment from *Arabidopsis thaliana*. The AtMEE-AC gene fragment was isolated via an RT-PCR system of the total RNA from Arabidopsis using the two manually designed AtMEE22 sequence-specific primers. The amplified AtMEE-AC was then resolved on a 1.0% (w/v) agarose gel at 80 V for 60 min. M represents the 100 bp ladder (Thermo Scientific International Inc., Burlington, Canada) while MEE represents the amplified AtMEE-AC gene fragment. The arrow marks the resolved AtMEE-AC band.

After the successful isolation of the targeted AtMEE-AC gene fragment from the total Arabidopsis RNA, the AtMEE-AC gene fragment was then cloned into a pCRT7/NT-TOPO plasmid vector (Figure 2.2) to produce a pCRT7/NT-TOPO:AtMEE-AC fusion expression construct. This expression construct was then used to transform competent BL21 (DE3) *E. coli* cells, and in preparation for the recombinant expression of the desired AtMEE-AC protein. However, before any expression regime was initiated, both the cloning efficiency and transformation success of the AtMEE-AC gene fragment were first verified by colony PCR as is shown in Figure 2.5 below.

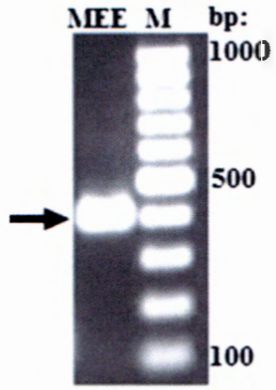

Figure 2.5: Verification of the cloning efficiency and transformation success of the AtMEE gene fragment. Some BL21 (DE3) *E. coli* cells harbouring the pCRT7/NT-TOPO-AtMEE-AC fusion construct were used as templates in an RT-PCR system, where the manually designed MEE sequence-specific primers were also used in order to target and amplify the AtMEE-AC gene fragment. The resultant AtMEE-AC gene amplicon was then resolved on a 1.0% (w/v) agarose gel at 80 V for 60 min. M represents the 100 bp ladder (Thermo Scientific International Inc., Burlington, Canada) while MEE represents the amplified AtMEE-AC gene fragment. The arrow marks the resolved AtMEE-AC band.

Following the verification of both the cloning efficiency of the AtMEE-AC gene fragment into the pCRT7/NT-TOPO plasmid vector and the transformation success of the pCRT7/NT-TOPO:AtMEE-AC fusion construct into the BL21 (DE3) *E. coli* cells, the transformed BL21 (DE3) *E. coli* cells were then induced with 1 mM IPTG to partially express the desired AtMEE-AC recombinant protein as a His-tagged fusion product (Crowe *et al.*, 1994) with an approximate size of 17.630 kDa (Figure 2.6).

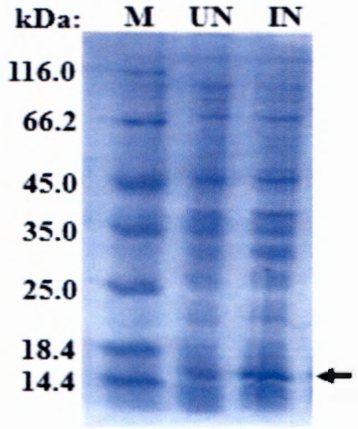

Figure 2.6: Partial expression of the recombinant AtMEE-AC protein. An SDS-PAGE of protein fractions expressed in BL21 (DE3) *E. coli* cells harbouring the pCRT7/NT-TOPO:AtMEE-AC fusion construct where lane 1 (M) represents the unstained low molecular weight marker (Thermo scientific International Inc., Burlington, Canada), lane 2 (UN) represents the non-induced culture and lane 3 (IN) representing the culture induced with 1 mM IPTG. The arrow marks the expressed recombinant AtMEE-AC protein.

The full amino acid sequences of the AtMEE22 protein plus its cloned and partially expressed truncated AtMEE-AC recombinant are shown below (Figure 2.7).

LSELEVLRNS AHKTSSDLLT LTSNLETVKK QLELEKQKTL
KEKKRADMES AKARDQMKLA EDVSK**KFEIV** **RARNEELKK**E
MESQTASSQV KFAENSEKLE EKIRLLEMNK KTAMDWKSRT
DDLTQQL**QEA QLV**

Figure 2.7: The full amino acid sequences of the truncated AtMEE-AC recombinant. The residues marked in red, bold and underlined represent the annotated AC catalytic center, while those highlighted in green and bold (green arrow) represent the forward priming site and the ones in blue and bold (blue arrow) represent the reverse priming site.

The physico-chemical parameters of this truncated recombinant protein were obtained from Expasy (ProtParam) and are indicated below as:

Number of amino acids: 133

Molecular weight: 15454.6

Theoretical pI: 8.06

Formula: $C_{662}H_{1123}N_{191}O_{221}S_5$

Total number of atoms: 2202

Extinction coefficients: 5500 in M^{-1} cm^{-1} at 280 nm measured in water

Aliphatic index: 79.25

After the successful expression of the recombinant AtMEE-AC protein, its possible ability to induce and/or generate cAMP within the transformed BL21 (DE3) cells, under various conditions, was then assessed and determined by enzyme immunoassay (Sigma-Aldrich, Missouri, USA, Catalogue # CA201) as is shown in Figure 2.7 below.

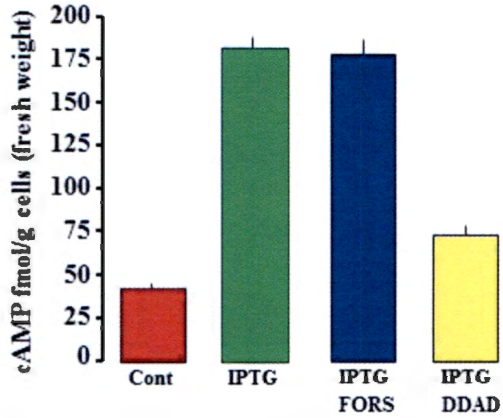

Figure 2.8: Determination of the endogenous adenylate cyclase activity of the recombinant AtMEE-AC protein. Cyclic AMP levels generated by uninduced (Control) and induced (IPTG) BL21 (DE3) *E. coli* cells harbouring the AtMEE-AC gene fragment and also from induced cell cultures in the presence of either forskolin or dideoxyadenosine. All cAMP levels were determined using the cAMP enzyme immunoassaying system (Sigma-Aldrich, Missouri, USA, Catalogue Number CA201,), where error bars represent the standard errors of the mean readings ($n = 3$; $p < 0.05$; determined by ANOVA).

2.4 Discussion

Arabidopsis maternal embryo arrest protein (MEE22) has previously been described as one of the most essential genes that are critical for the plant life cycles (Meinke *et al.*, 2008). Its essential roles include its involvement in processes like shoot apical meristem fate (Leasure *et al.*, 2009); embryo development (Pagnussat *et al.*, 2005); embryo sac egg differentiation (Capron *et al.*, 2009); embryonic pattern speciation and maintenance of meristem identity (Leasure *et al.*, 2009); meristem initiation, maintenance, structural organization and specification (Leasure *et al.*, 2009); protein ubiquitination, regulation of flower development, seed dormancy processes, seed germination, seed maturation, sugar mediated signaling pathways and vegetative to reproductive phase transition of meristems (Leasure *et al.*, 2009). However and despite the fact that the AtMEE22 protein has extensively been implicated in various cellular processes that involve or may be mediated by the second messenger, cAMP, this protein has to date, not yet been practically demonstrated to generate cAMP from ATP. This is also in spite of the fact that the same protein has recently been annotated to harbour a putative adenylate cyclase (AC) catalytic motif (Fig 2.3) within its domain structure (Gehring, 2010). Therefore and in this study, the AtMEE22 domain segment harbouring the AC catalytic motif (AtMEE-AC) (Fig 2.7) was targeted and cloned (Fig 2,4 and Fig 2.5) for functional analysis. The cloned gene fragment was then heterologously expressed in a prokaryotic system (*E. coli*) to yield a fusion protein product of approximately 17.630 kDa (Fig 2.6).

In order to determine if the cloned and expressed AtMEE-AC fusion protein could generate cAMP from ATP, *E. coli* cells harbouring this recombinant were assessed by enzyme-immunoassay; firstly, before induction; secondly, with induction; and lastly, with induction in the presence of either the inducer, forskolin or the potent inhibitor, 2′,5′-dideoxyadenosine. From this assessment, the induced cells showed a robust increase in cAMP levels (>2.5-fold)

as compared to cells that were not induced but carrying the competent AtMEE-AC cDNA (Figure 2.8), while on the other hand, the addition of forskolin (Ehsan *et al.*, 1998) to the AtMEE-AC-expressing bacteria had no detectable effects on the production of cAMP (Figure 2.8). Nevertheless, when the AtMEE-AC expressing bacteria were exposed to dideoxyadenosine (Desaubry *et al.*, 1996, Volotovski *et al.*, 1998) the cells consistently and transiently reduced their cAMP levels by a factor of around 1.6-fold (Figure 2.8).

All these observed outcomes positively and consistently correlate with some previous findings obtained in related experiments. When a PSiP coding region of a pollen-specific putative AC from *Zea mays* was cloned into bacterial cells, followed by the treatment of cells with 1 mM IPTG, the treated cells enhanced their cAMP levels by a factor of 3.0 (Moutinho *et al.*, 2001). In addition, application of 100 μM 2′,5′-dideoxyadenosine to the growing pollen tubes of *Agapanthus umbellatus* (*Liliaceae*), transiently caused a temporary arrest in growth accompanied by a reduction in cAMP concentration of a factor of 1.8-fold (Moutinho *et al.*, 2001). Furthermore, the failure of the recombinant AtMEE-AC to respond to forskolin is not unusual as it is physiologically typical of all cytoplasmic and soluble adenylate cyclases (sACs) that are non-inducible by this compound (Zippin *et al.*, 2004). In line with this last point, the AtMEE22 protein has also recently been bioinformatically localized to the cell cytoplasm, and specifically in the chloroplast (Andersson and Sandelius, 2004).

By summing up all these findings, it can be plausibly speculated that the recombinant AtMEE-AC protein that was cloned and partially expressed in this study is either itself a *bona fide* sAC that is capable of generating cAMP from ATP or is simply another functional plant molecule capable of stimulating other resident sACs (*E. coli* sACs in this case) to produce cAMP.

CHAPTER 3

Determination of the Adenylate Cyclase Activity of the Recombinant AtMEE-AC through Complementation System

Abstract

Recent bioinformatic and proteomic studies have revealed the presence of a novel class of higher plant cell signaling and cell communication molecules termed adenylate cyclases (ACs). These molecules are understood to generate the second messenger 3′,5′-cyclic adenosine monophosphate, cAMP which in turn is involved in the mediation and regulation of various cellular physiological and biochemical processes including the activity of kinases. Surprisingly and as of to date, only two functional ACs have yet been identified in higher plants and experimentally confirmed to generate cAMP. Thus, in a zeal and quest to identify additional higher plant ACs, we cloned an AC-containing fragment domain of the maternal effect embryo arrest protein from *Arabidopsis thaliana* (AtMEE-AC) into a non-lactose fermenting mutant strain of *E. coli* (*cyaA* SP 850 cells) and confirmed its ability to generate cAMP, which in turn complemented the mutant cells to behave as if they were wild type cells capable of fermenting lactose.

3.1 Introduction

Cyclic AMP is probably one of the most widely studied second messenger in bacteria, lower eukaryotes and animals, where it has critical roles in signaling the metabolic status (Lemtiri-Chlieh *et al.*, 2011). In bacterial systems like *Escherichia coli*, cAMP is involved in the positive regulation of the lac operon, which is a cluster of three structural genes that comprise the *lac*Z (which encodes β-galactosidase), the *lac*Y (which encodes galactose permease), and the *lac*A (which encodes thiogalactoside transacetylase), preceded by an operator site (O_{lac}) and a promoter (P_{lac}) (Matthews *et al.*, 2000, Nelson and Cox, 2004, Kuhlman *et al.*, 2007). In this regulation, cAMP operates via a transcription activator protein termed the catabolite activator protein, (CAP) or simply, the cAMP receptor protein, (CRP), where in an environment of a low glucose, cAMP accumulates and binds to the allosteric site of the CRP to form a CRP-cAMP complex, which then binds to a *cis*-element of the P_{lac} and activating transcription (Meiklejohn and Gralla, 1985). Activation of the *lac* operon transcription results in the consequent metabolism of lactose sugar of which lactic acid is one of the sole products (lactose fermentation).

In nature and basically, in all living systems, cAMP is generated by the action of a group of enzymes termed adenylate cyclases (ACs) that are capable of hydrolysing ATP into cAMP (Robison *et al.*, 1968, Goodman *et al.*, 1970, Gerisch *et al.*, 1975). However, while most other organisms have succeeded to keep several isoforms of their ACs across the evolutionary trend, *E. coli* has only managed to retain one that is characteristically expressed by a *cis-trans* gene system or simply a complementation system. In genetics, the *cis-trans* gene or complementation system is virtually a situation whereby two forms of an allele exist in two different genes yet the heterozygous condition in one gene can fully rescue the function of the other gene which then could have been lost in the homozygous recessive state.

Hence, the term complementation test is principally used to describe the process to test for gene function in recessive allelism (Britannica, 2014).

From this perspective, molecular biology has succeeded to develop an assay system (complementation test) that tests for the presence of a trait/gene that may otherwise represent a deletion mutation in an *Escherichia coli* cell line termed the *cya*A mutant (Shah and Peterkofsky, 1991, Karimova *et al.*, 1998). This mutant lacks the *cis-trans* gene system for the expression of the only and only one AC in *E. coli* and is therefore unable to ferment lactose. In the developed assay system, the phenotypic characteristics of this mutant is easily identified and differentiated from those of the wild type lactose fermenters (Karimova *et al.*, 1998). Therefore in this study, we hereby describe the utilization of the complementation test to determine the inherent enzymatic activity of a recombinant AC-containing fragment domain of the maternal effect embryo arrest protein from *Arabidopsis thaliana* (AtMEE-AC).

3.2 Materials and Methods

3.2.1 Isolation and Purification of the pCRT7/NT-TOPO:AtMEE-AC Fusion Construct from BL21 (DE3) Cells

The pCRT7/NT-TOPO:AtMEE-AC fusion expression construct generated in chapter 2 (section 2.2.6) and maintained in BL21 (DE3) *E. coli* cells (section 2.2.8) was extracted using the Zyppy miniprep kit and following the manufacturer's instructions (Zymo-Research, California, USA). Briefly, 100 µl of the BL21 (DE3) cells harbouring the pCRT7/NT-TOPO:AtMEE-AC fusion construct were used to inoculate 5 ml of the 2YT medium supplemented with 100 µg/ml ampicillin and 34 µg/ml chloramphenicol and the culture allowed to grow overnight at 37°C in an SI-600 orbital shaker at 200 rpm (Lab Companion, Seoul, South Korea). The next morning, 600 µl of the overnight bacterial culture was then added into a 1.5 ml Eppendorf tube and centrifuged for 30 sec at 9 200*g*. The pellet was re-suspended into 600 µl of water before 100 µl of the 7X Lysis Buffer was added to the suspension and mixed by inversion (2-3 times). A total amount of 350 µl ice-cold Neutralization Buffer was added to the suspension and mixed thoroughly by inversion (4-6 times). The sample was then centrifuged at 11 000*g* for 4 min. Approximately 900 µl of the supernatant was then transferred into a Zymo-Spin column which was placed into a collection tube and centrifuged for 15 sec at the same speed. The flow-through was discarded and 200 µl of the Endo Wash Buffer was added to the column and centrifuged at 11 000*g* for 30 sec. About 400 µl of the Zyppy Wash Buffer was added to the column and centrifuged for 1 min at the same speed. The Zymo-Spin column was then transferred into a fresh 1.5 ml Eppendorf tube where 30 µl of the Zyppy Elution Buffer was directly added onto the column matrix and allowed to stand for 1 mine. The column was then centrifuged at 11 000*g* for 30 sec so as to elute the extracted plasmid DNA (pCRT7/NT-TOPO:AtMEE-AC fusion construct). The eluted plasmid DNA was then stored at -20°C for further use.

3.2.2 Preparation of Competent SP850 *cya*A Cells

Some SP850 *cya*A *E. coli* cells obtained from the Coli Genetic Stock Centre (Yale University, Connecticut, USA), were prepared to become chemically competent as is already described in chapter 2 (section 2.2.7) but replacing the 34 µg/ml chloramphenicol with 15 µg/ml kanamycin.

3.2.3 Sorting Out of the Competent SP850 *cya*A Cells for Complementation Testing

The chemically competent SP850 *cya*A *E. coli* cells prepared above (section 3.2.3) were divided into three portions. The first portion was transformed with the pCRT7/NT-TOPO:AtMEE-AC fusion construct previously extracted from the BL21 (DE3) cells (section 3.2.2). The second portion was transformed with an empty pCRT7/NT-TOPO plasmid vector that was commercially acquired for this study (http/www.fermentas.com). The third and last portion was left un-transformed. All transformation regimes were undertaken as previously detailed (chapter 2, section 2.2.8).

3.2.4 Culturing of the Sorted SP850 *cya*A Cells and Phenotypic Scoring

A MacConkey agar plate (Merck, Darmstadt, Germany) supplemented with 15 µg/ml kanamycin and 0.1 mM IPTG (Sigma-Aldrich, Missouri, USA) was prepared and then sub-divided into four quadrants using a permanent marker. The fourth quadrant was streaked with the mutant c*ya*A cells harbouring the pCRT7/NT-TOPO:AtMEE-AC fusion construct, the third quadrant was streaked with the mutant *cya*A cells transformed with the empty pCRT7/NT-TOPO plasmid vector, the second quadrant was streaked with the non-transformed mutant *cya*A cells, and the first quadrant was left un-streaked. The cultured

plate was then inverted and incubated at 37°C for 40 - 48 hrs. After the incubation, all quadrants were then visually inspected for the different scorable phenotypic characteristics.

3.3 Results

The plate below (Figure 3.1) represents the different scorable phenotypes displayed by the various forms of SP850 *cya*A *E. coli* cells when assessed for their ability to ferment lactose in a complementation assay system.

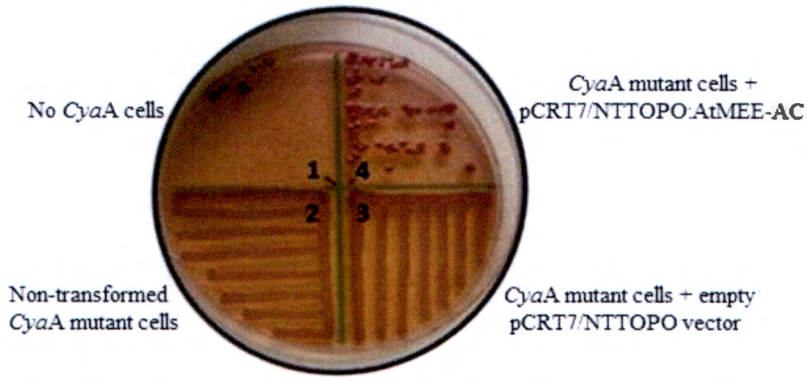

Figure 3.1: Determination of the recombinant AtMEE-AC's ability to complement mutant *cya*A cells. Different forms of the *cya*A *E. coli* cells were plated onto MacConkey agar supplemented with 0.1 mM IPTG and incubated at 37°C for 40 - 48 hrs. Quadrant 1 contains no cells, quadrant 2 contains non-transformed *cya*A cells, quadrant 3 contains *cya*A cells transformed with the empty pCRT7/NT-TOPO plasmid vector, and quadrant 4 contains *cya*A cells transformed with the pCRT7/NT-TOPO:AtMEE-AC fusion construct. Cells in quadrants 2 and 3 are both non-lactose fermenters and produce white or yellowish colonies while cells in quadrant 4 have picked a deep purple phenotype -a characteristic significant of lactose fermentation.

3.4 Discussion

The fundamental principle behind the complementation test is that the developed *cya*A mutant cells lack the *cis-trans* adenylate cyclase (AC) gene responsible for generation of the second messenger, cAMP (Karimova *et al.*, 1998), which ultimately activates the *lac* operon to ferment lactose (Meiklejohn and Gralla, 1985). In other words, the *cya*A mutants are non-lactose fermenters (Roy *et al.*, 1983) and when grown on MacConkey agar, they display a colourless or yellowish phenotype as compared to the magenta red or deep purple phenotype produced by wild type cells (wild type cells ferment lactose, generating lactic acid which then reacts with the indole red indicator in the media) (Allen, 2005, Bridson, 2006). This differential growth aspect of these two types of *E. coli* cells on MacConkey agar can be technically taken advantage of to assess and establish the ability of any suspected AC candidate to generate cAMP *in vivo*. Copy DNA (cDNA) of the suspected gene is cloned into the cyaA mutant cells followed by an assessment of the phenotype of these cells on MacConkey agar supplemented with an inducing agent. The shifting of the mutant phenotype (colourless or yellowish) to the wild phenotype (magenta red or deep purple) will typically signify the biological functionality (as an adenylate cyclase) of the gene under assessment (Britannica, 2014).

Since in chapter 2 of this thesis, the cloned and partially expressed AtMEE-AC recombinant protein could not be conclusively established as a *bona fide* AC, the complementation test was then undertaken in this chapter to attempt and establish its possible biological function. When its cDNA was cloned into the SP850 *cya*A *E. coli* cells via the pCRT7/NT-TOPO plasmid vector, followed by the growth of these cells on MacConkey agar supplemented with 0.1 mM IPTG, the phenotype of these cells shifted from yellowish to deep purple (Figure 3.1), thus signifying the AtMEE-AC's ability to generate cAMP from ATP. This elegant finding therefore firmly establishes the recombinant AtMEE-AC as a biologically functional

AC and thereby becoming the fifth such molecule to be identified in higher plants after the pollen protein from *Zea mays* (Moutinho *et al.*, 2001), the pentatricopeptide protein from *Arabidopsis thaliana* (Ruzvidzo *et al.*, 2013), the HpAc1 invloved in stress signalling in *Hippeastrum hybridum* (Świeżawska *et al.*, 2014) and the NbAC (*Nicotiana benthamiana* adenylyl cyclase (Ito *et al.*, 2014).

CHAPTER 4:

Purification and Functional Characterization of the Recombinant AtMEE-AC Protein

Abstract

Several lines of evidence have revealed the identification of adenylate cyclase (AC) and 3′,5′-cyclic adenosine monophosphate (cAMP) dependent processes in plant cells mainly by biochemical methods. Cyclic AMP is the prototypical second messenger and AC is its sole source. However, while most of these methods have typically been undertaken using either intact cells or their crude and purified extracts, none has to date been practically demonstrated to have used a purified form of an AC system. Hence in an effort to attempt and achieve this, we successfully prepared a pure and biologically active recombinant AC protein by empirically optimizing the expression and purification strategies of an AC-containing fragment domain of the maternal effect embryo arrest protein from *Arabidopsis thaliana* (AtMEE-AC) followed by the demonstration of its *in vitro* enzymatic activity. Furthermore, additional functional characterization of this purified recombinant protein exhibited it as a manganese-depended enzyme whose activity is positively modulated by both calcium and carbonate ions, and thus confirming it as a soluble AC whose physiological roles may specifically be mediated by the second messenger, cAMP and probably through control mechanisms involving the calcium-binding protein, calmodulin.

4.1 Introduction

Molecular biology and/or biotechnology has brought about elegant and well established recombinant DNA methodologies for the production of heterologous proteins in microbial hosts (Lilie *et al.*, 1998). In recombinant bacterial systems such as *Escherichia coli*, the over-expression of plasmid-encoded genes usually triggers the transcription of heat-shock genes and other stress responses, which often results in the aggregation of the encoded proteins into inclusion bodies (Villaverde and Carrio, 2003). Over-expression refers to the directed synthesis of very large amounts of desired proteins (Rai and Padh, 2001) while inclusion bodies are insoluble, non-functional protein aggregates of foreign origin that are refractile and protease-resistant (Guise *et al.*, 1996, Villaverde and Carrio, 2003), and often occurring in recombinant bacterial systems upon their deliberate and gratuitous induction to overexpress cloned genes (Carrio and Villaverde, 2001).

While protein expression in the form of inclusion bodies is often considered undesirable, their formation can also be advantageous, as their isolation from cell homogenate is a convenient and effective way of purifying the protein of interest (Singh and Panda, 2005, Yang *et al.*, 2011). The actual advantages associated with the formation of inclusion bodies are firstly, expression of a very high level of protein (more than 30% of the cellular protein in some cases) (Ventura and Villaverde, 2006); secondly, easy isolation of the inclusion bodies from cells due to differences in their sizes and densities when compared to other cellular molecules (Lilie *et al.*, 1998, Villaverde and Carrio, 2003); thirdly, lower degradation of the expressed protein which furthermore, can be easily purified under denaturing conditions (Ventura and Villaverde, 2006); fourthly, resistance to proteolytic attack by cellular proteases; fifthly, homogeneity of the protein of interest in inclusion bodies (lesser contaminants) which helps in reducing the number of purification steps to recover pure proteins; and finally, the expression of recombinant proteins as inclusion bodies can be directly observed and

monitored through the use of phase contrast microscopy thereby avoiding a requirement for their initial identification by such methods like gel electrophoresis and/or Western blotting (Singh and Panda, 2005).

However, despite all these stated advantages, there are often several challenges and difficulties in attempting to convert inclusion body proteins from their form into soluble, fully refolded and functional native states. Fortunately and perhaps luckily, molecular biology and/or biotechnology has since developed and fully described several methods which all primarily involve the two key aspects of solubilization and refolding (Tsumoto *et al.*, 2003, Singh and Panda, 2005). Solubilization involves the dissolution of the insoluble protein aggregates while refolding involves the conversion of those solubilized protein aggregates into bioactive molecules (Villaverde and Carrio, 2003, Singh and Panda, 2005). Often and always, the solubilization and refolding processes of relatively pure inclusion body proteins drastically reduce the number of chromatographic steps required for the final purification and production of all expressed recombinant proteins (Singh and Panda, 2005).

Briefly, cells containing inclusion bodies are usually disrupted by high-pressure homogenisation and/or a combination of mechanical, chemical and enzymatic methods. Inclusion bodies are then separated from the cell debris after cell lysis using low-speed centrifugation as they are denser than many of the cellular components. Semi-pure protein aggregates alongside other cellular contaminants are then solubilized using high concentrations (6 - 8 M) of chaotropic reagents such as urea or guanidine hydrochloride (Lilie *et al.*, 1998). Additional reducing agents like β-mercaptoethanol, dithiothreitol or cysteine are also often used for the solubilization of inclusion body proteins. This helps to maintain cysteine residues in a reduced state and thus preventing the formation of undesired non-native intra- and/or inter-disulfide bonds in highly concentrated protein solutions at alkaline pHs (Fischer *et al.*, 1993).

Typically, refolding is a change in protein conformation from an unfolded to a folded state, and inclusion protein bodies can only be solubilized by strong denaturants, whereby the proteins are unfolded - the extent of which solely depends onto the proteins and type of the denaturants used. Renaturation of the solubilized inclusion bodies is then initiated by the removal of the denaturant either by dialysis or dilution and of which the efficiency of such a renaturation process is solely dependable onto the competition between correct folding, mis-folding and aggregation (Tsumoto *et al.*, 2003). If proteins do contain several disulfide bonds, then the renaturation buffer has to be appropriately supplemented with a viable redox system. Usually, the addition of a mixture of the reduced and oxidized forms of low molecular weight thiol reagents, such as glutathione, cysteine and cysteamine (molar ratios of reduced to oxidized compounds 5:1 to 10:1, respectively) provides the appropriate redox potential to allow for the formation and reshuffling of disulfide bridges in the refolding proteins (Lilie *et al.*, 1998).

Another possibility for suppressing non-specific and un-directed intermolecular interactions is the coupling of the denatured protein to a solid matrix such as the nickel-nitrilotriacetic acid (Ni-NTA) affinity matrix, originally developed for an efficient protein purification system (Crowe *et al.*, 1994). After binding the denatured protein onto the matrix via a histidine tail (His-tag), the column is then equilibrated with a renaturation buffer and the refolded protein can then be finally eluted with a high concentration (250 mM) imidazole or a pH gradient. Here we report the preparation of a biologically active recombinant AC-containing fragment domain of the maternal effect embryo arrest protein from *Arabidopsis thaliana* (AtMEE-AC) using a pCRT7/NTTOPO expression system (Invitrogen) and a QIAexpress purification/refolding system (Qiagen, 2003) followed by an extensive analysis and evaluation of its functional properties by enzyme immunoassay. These three systems are

specifically designed to allow for high expression levels of recombinant proteins, their rapid purification and refolding as well as their precise *in vitro* functional characterization.

4.2 Materials and Methods

4.2.1 Overexpression of the Recombinant AtMEE-AC Protein

In order to yield high levels of the recombinant AtMEE-AC protein, the recombinant protein was overexpressed by modifying its partial expression profile previously outlined in chapter 2 (section 2.2.10), whereby the induced cells were this time around allowed to grow for a further 3 hrs instead of 1 hr at 37°C in an SI600 shaking incubator (Lab Companion, Seoul, South Korea) at 200 rpm.

4.2.2 Determination of the Solubility/Insolubility Nature of the Recombinant AtMEE-AC Protein

The solubility nature of the recombinant AtMEE-AC was determined following an amendment of protocol 6 of the QIA expressionist manual (Qiagen, 2003). Since the overexpression of heterologous proteins in prokaryotic systems commonly favours the production of inclusion bodies, the overexpressed AtMEE-AC recombinant was checked to determine if it was generated as either a soluble or insoluble product. Briefly, a pellet of the transformed and induced cells was re-suspended in phosphate buffered saline (PBS) solution in the ratio of 5:1 PBS:pellet weight, supplemented with lysozyme to a final concentration of 1 μg/ml, and incubated on ice for 30 min. The cells were lysed by vortexing at high speed 10 times with 10 sec pausing intervals on ice. The mixture was then centrifuged at 10 000*g* at 4°C for 30 min before the supernatant and pellet were separated as the soluble and insoluble fractions respectively. Both fractions were then analysed by SDS-PAGE to determine the presence and relative concentration of the expressed recombinant AtMEE-AC.

4.2.3 Preparation of the Cleared Lysate

After realizing that the recombinant AtMEE-AC protein was wholly overexpressed as inclusion bodies, its cleared lysate was then prepared following protocol 10 of the QIAexpressionist manual (Qiagen, 2003), whereby its insoluble fraction from section 4.2.2 was re-suspended in 5 ml of lysis buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl; pH: 8.0, 500 mM NaCl, 20 mM β-mercaptoethanol, 10 mM imidazole and 7.5% (v/v) glycerol) at a ratio of 5 ml buffer to every 1 g pellet weight. The cell pellet was then solubilized into a crude lysate by vigorously vortexing for 45 to 60 min. The produced crude lysate was then centrifuged at 10 000*g* for 30 min at room temperature to pellet out the cell debris and obtaining a cleared lysate (supernatant). The cleared lysate was then collected for further downstream processing.

4.2.4 Purification of the Recombinant AtMEE-AC Protein

The recombinant AtMEE-AC protein in the solubilized cell pellet from above (section 4.2.3) was then purified under non-native and denaturing conditions using a nickel-nitrotriacetic acid (Ni-NTA) (Sigma-Aldrich, Missouri, USA) column following protocol 17 of the QIAexpressionist manual (Qiagen, 2003) and as is briefly described below.

4.2.4.1 Binding of the Recombinant AtMEE-AC Protein

The His Select Ni-NTA (Sigma-Aldrich, Missouri, USA) resin was used to purify the recombinant AtMEE-AC protein from its cleared lysate. Since the recombinant AtMEE-AC protein was initially expressed with a 6xHis tag from the pCRT7/NT-TOPO vector fused to its N-terminus end, this tag specifically had a high affinity for the nickel ion resin and thus could totally exclude out all the other bacterial proteins during the purification process.

Briefly, an aliquot of 1 000 µl beads was first washed by mixing the beads with 2 000 µl filter-sterilized distilled water at room temperature in a 15 ml Falcon tube and in order to remove the ethanol solution that is normally used to preserve the beads. The mixture was then allowed to settle by gravity for 30 min before the supernatant was discarded. The washed beads were then equilibrated with 5 000 µl PBS lysis buffer by allowing them to rotate on a bench revolver (Labnet International, New Jersey, USA) at 30 rpm for 15 min at room temperature, after which the equilibration buffer was removed and discarded. A total of 5 ml cleared lysate was then mixed with the washed beads and allowed to rotate on a revolver at 30 rpm for 2 hrs at room temperature. The beads were then allowed to settle by gravity for 20 min at room temperature and the supernatant collected as flow-through and saved for analysis by SDS-PAGE.

4.2.4.2 Washing of the Recombinant AtMEE-Protein bound to the Ni-NTA beads

After binding the recombinant AtMEE-AC onto the Ni-NTA beads, unbound bacterial proteins were then washed off by re-suspending the bound beads into 10 column volumes (cvs) of wash buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl; pH: 8.0, 500 mM NaCl, 20 mM β-mercaptoethanol, 7.5% (v/v) glycerol, and 10 mM imidazole), and allowing the mixture to stand at room temperature for 5 min before spinning it at 12 000*g* for 1 min. The supernatant was discarded as a 'first wash' and its aliquot (1 ml) saved for analysis by SDS-PAGE. The washing step was repeated three more times with aliquots (1 ml) for each wash also saved for analysis by SDS-PAGE. Furthermore, an aliquot (20 µl) of the washed bound beads was also collected for analysis by SDS-PAGE.

4.2.5 Refolding of the Purified and Denatured Recombinant AtMEE-AC

In order to convert the purified recombinant AtMEE-AC protein from its denatured non-functional form into its native and functional form, the following refolding procedures were undertaken:

4.2.5.1 Preparation of a Refolding Column

The washed beads carrying the bound and purified recombinant AtMEE-AC protein were first re-suspended into 10 cvs of gradient buffer (8 M urea, 200 mM NaCl, 50 mM Tris-HCl; pH: 8.0, and 20 mM β-mercaptoethanol) before the whole slurry was carefully loaded into an empty XK16 column (Bio-Rad Laboratories, California, USA). Proper loading was achieved by pouring the slurry from the top part of the column while a small tap positioned at its bottom part was kept closed. The beads were then allowed to sediment by draining 8 column volumes of the buffer through the tap before the loaded column was connected to a BioLogic DuoFlow Chromatography System (Bio-Rad Laboratories, California, USA) for the initiation of a refolding gradient system.

4.2.5.2 The Refolding Gradient System

A very slow but linearized refolding gradient system was created and run on the BioLogic DuoFlow Chromatography System based on parameters listed in Table 4.1 below. In this system, the 8 M urea gradient buffer was slowly and linearly diluted to 0 M urea concentration with a refolding buffer (200 mM NaCl, 50 mM Tris-HCl; pH: 8.0, 500 mM glucose, 0.05% (w/v) poly-ethyl glycol, 4 mM reduced glutathione, 0.4 mM oxidized glutathione, 100 mM non-detergent sulfobateine, and 0.5 mM

Phenylmethanesulfonylfluoride). This process would then slowly convert the recombinant AtMEE-AC protein from its denatured non-functional form into a native and biologically functional molecule.

Table 4.1: Conditions for the refolding process of the recombinant AtMEE-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 with	2.00 cv
Length of gradient	600 min
Clean after gradient with	10.00 ml
Elute protein with	1.00 ml

4.2.5.3 Elution of the Refolded Recombinant AtMEE-AC Protein

In order to elute the refolded recombinant AtMEE-AC protein from the Ni-NTA beads, the bound beads in the column were first washed with 10 cvs of washing buffer (200 mM NaCl, 50 mM Tris-HCl; pH 8.0, 20% (v/v) glycerol, and 0.5 mM phenylmethanesulfonylfluoride (PMSF)). This washing step was carried out to remove most of the residual refolding agents from the refolded protein prior to its elution. The final product was then eluted in 1 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.

4.2.5.4 Concentration and De-salting of the Recombinant AtMEE-AC Protein

The eluted recombinant AtMEE-AC protein sample was both concentrated and de-salted by transferring it into a Spin-X UF concentrating and de-salting device with a molecular weight cut off point of 3.0 kDa (Corning, New York, USA) followed by spinning at 2540*g* (Hermle Labortechnik, Wehingen, Germany) at 4°C for 4 hrs or until the final volume had gone down to 200 µl. The concentrated and de-salted protein fraction was then removed from the device and transferred into a new Eppendorf tube. Protein concentration was then determined using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Massachusettes, USA). A fraction (20 µl) of this eluted protein was also saved aside for analysis by SDS-PAGE while the rest of the protein was stored at -20°C for further use.

4.2.6 Cyclic Nucleotide Assays

In order to determine the *in vitro* enzymatic activity of the recombinant AtMEE-AC protein and further characterize its functional properties, 5 µg of its purified form were added to 50 mM Tris-HCl; pH: 8.0, 2 mM isobutylmethylxanthine (IBMX) (phosphodiesterase inhibitor), 5 mM Mg^{2+} or 5 mM Mn^{2+}, and 1 mM ATP, in a final volume of 200 µl followed by incubation at room temperature (24°C) for 20 min. At the end of the 20 min, the reaction was terminated by adding 4 mM EDTA, heating at 100°C for 5 min and chilling rapidly at 4°C for 10 min. The terminated reaction was then clarified by centrifugation at 16 600*g* for 5 min. The supernatants were then collected and their cAMP contents measured in triplicates by enzyme immunoassay, following the acetylation protocol and as is described by the

manufacturer's manual (Sigma-Aldrich, Missouri, USA, code CA201). Other additives such as 1 mM GTP, 250 μM $CaCl_2$, 50 mM $NaHCO_3$, and 10 mM NaF + 30 μM $AlCl_3$ were also individually tested in the same reaction system for their possible modulation of the *in vitro* enzymatic activity of the purified recombinant AtMEE-AC. Background cAMP levels were measured in tubes containing all components of the reaction medium but no protein.

4.2.7 Statistical Analysis

The results of all the undertaken immunoassaying systems were based on the means of three replicates where outcomes of each assay were subjected to analysis of variance (ANOVA) (Super-Anova, Statsgraphics Version 7, 1993, Statsgraphics Corporation, USA). Where the ANOVA revealed significant differences between outcomes, the means (n = 3) were separated using a *post hoc* Student Newman Kuehls (SNK), multiple range test ($p \leq 0.05$).

4.3 Results

The overexpression conditions used in this study to target the generation of very large amounts of the recombinant AtMEE-AC protein, largely favoured the production and accumulation of insoluble inclusion bodies (Lilie *et al.*, 1998). Therefore, purification of this protein was then undertaken on an Ni-NTA affinity system (Stempfer *et al.*, 1996, Lindwall *et al.*, 2000) under denaturing conditions (Qiagen, 2003), where nearly a complete purification of the recombinant AtMEE-AC was achieved as is shown in Figure 4.1 below.

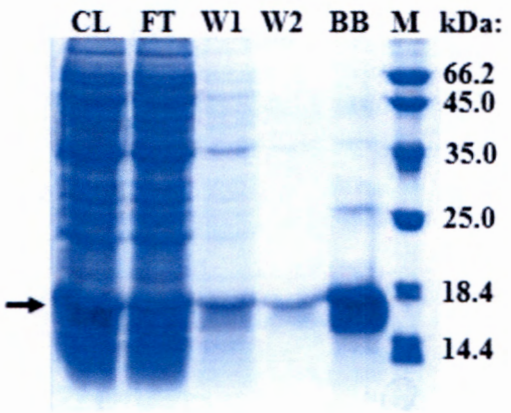

Figure 4.1: Purification of the recombinant AtMEE-AC protein under non-native denaturing conditions. An SDS PAGE of the recombinant AtMEE-AC fractions collected at different steps of its purification procedure. (CL) is the cleared lysate after solubilization of the inclusion bodies in 8 M urea buffer, (FT) is the flow-through of the cleared lysate after it was passed through the Ni-NTA resin, (W1) is the first wash of the bound AtMEE-AC with wash buffer, (W2) is the second wash, and (BB) is the purified and bound AtMEE-AC. The arrow marks the recombinant AtMEE-AC band while (M) represents the unstained low molecular weight marker (Fermentas International Inc., Burlington, Canada).

In order to facilitate a regaining of biological activity for the purified and denatured recombinant AtMEE-AC, the bound protein was passed through a refolding process that was directly linked to a DuoFlow system (Bio-Rad Laboratories, California, USA), and set to run at a flow rate of 0.5 ml/min for 600 min (Table 4.1). During this process, an 8 M urea buffer into which the recombinant AtMEE-AC was resuspended, was slowly diluted over a 600-minute time period with a non-denaturing refolding buffer to a final concentration of 0 M urea. In this regard, the recombinant protein was therefore slowly refolded into its native form while it was still bound to the Ni-NTA matrix. The refolded purified recombinant AtMEE-AC (Figure. 4.2) was then finally eluted off the Ni-NTA matrix with 250 mM imidazole using an elution process that was once again linked to the DuoFlow system.

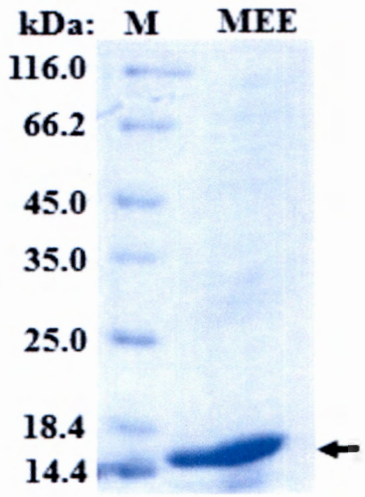

Figure 4.2: Elution of the refolded recombinant AtMEE-AC protein from the Ni-NTA resin. An SDS-PAGE of a purified refolded fraction of the recombinant AtMEE-AC protein eluted from the Ni-NTA matrix with 250 mM imidazole in an elution process that was carefully controlled by a DuoFlow system, where (M) is the low molecular weight marker while the arrow marks the refolded purified recombinant AtMEE-AC protein.

In order to test if the refolded recombinant AtMEE-AC had regained its AC enzymatic activity, its eluted fraction was assessed in an enzyme immunoassay system for the ability to generate cAMP from ATP. As is shown in Figure 4.3 below, the recombinant AtMEE-AC indeed demonstrated biological activity with a higher preference for manganese metal than magnesium as its co-factor ion.

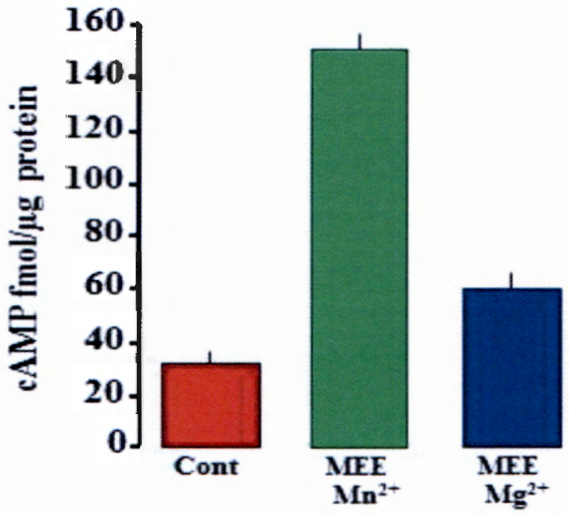

Figure 4.3: Determination of the *in vitro* AC activity of the recombinant AtMEE-AC protein and its associated co-factor preference. Cyclic AMP levels generated with 5 µg (purified) recombinant AtMEE-AC protein in a reaction system containing 50 mM Tris-HCl; pH: 8.0, 1 mM ATP, 2 mM IBMX and either 5 mM Mg^{2+} or 5 mM Mn^{2+}, and as is determined by enzyme immunoassay system following the acetylation protocol (Sigma-Aldrich, Missouri, USA, code CA201). Alongside this experiment, a reaction control system containing 50 mM Tris-HCl pH: 8.0, 1 mM ATP, 1 mM GTP, 2 mM IBMX, and 5 mM Mn^{2+} with no protein was also set and its residual cAMP levels also similarly measured. Error bars represent the standard errors of the mean readings (n=3; $p < 0.05$; determined by ANOVA)

Since the substrate (ATP) of the recombinant AtMEE-AC protein has a cellular structural analogue (GTP) that is capable of producing another second messenger (cGMP) like cAMP, it was therefore sought to find out if the recombinant AtMEE-AC had a particular substrate specific for ATP only or probably could utilize both ATP and GTP as its sole substrates. As is shown in Figure 4.4 below, the recombinant AtMEE-AC demonstrated an enhanced substrate specificity for ATP other than its analogue, GTP.

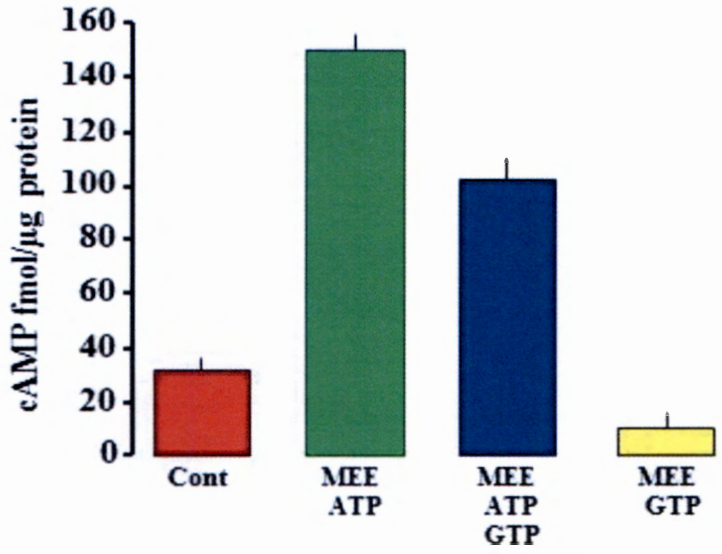

Figure 4.4: Determination of substrate specificity for the recombinant AtMEE-AC protein. Equimolar concentrations (1 mM) of ATP and GTP were added either individually or together to a reaction systems containing 50 mM Tris-HCl; pH: 8.0, 5 µg recombinant AtMEE-AC, 2 mM IBMX, and 5 mM Mn^{2+} followed by an incubation at room temperature for 20 min. Alongside this experiment, a reaction control system containing 50 mM Tris-HCl pH: 8.0, 1 mM ATP, 1 mM GTP, 2 mM IBMX, and 5 mM Mn^{2+} with no protein was also set. Cyclic AMP levels generated by each of the described reaction systems were then measured by enzyme immunoassay system following the acetylation protocol (Sigma-Aldrich, Missouri, USA, code CA201). Error bars represent the standard errors of the mean readings (n=3; $p < 0.05$; determined by ANOVA).

After determining the *in vitro* biological activity of the purified and refolded recombinant AtMEE-AC protein, cofactor preference and substrate specificity, its adenylate cyclase activity was further characterized by testing its responses to the various and commonly known AC modulators. As is shown in Figure 4.5 below, the recombinant AtMEE-AC could be positively modulated by calcium and bicarbonate ions but with no apparent responses to the fluoride ion.

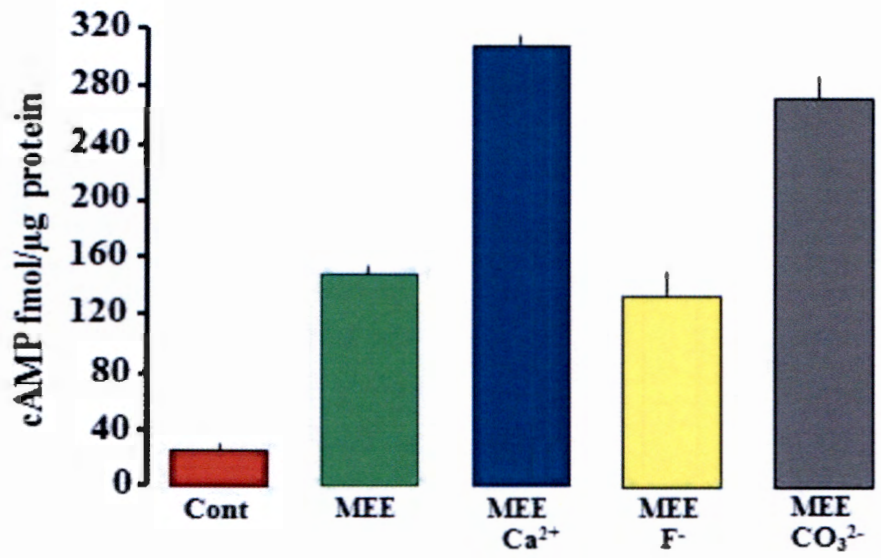

Figure 4.5: Effects of various enzymatic modulators onto the AC activity of the AtMEE-AC recombinant protein. A reaction system containing 50 mM Tris-HCl; pH: 8.0, 5 µg recombinant AtMEE-AC, 1 mM ATP, 2 mM IBMX, 5 mM Mn^{2+}, and 250 µM $CaCl_2$ or 50 mM $NaHCO_3$ or 10 mM NaF + 30 µM $AlCl_3$ followed by an incubation at room temperature for 20 min. Alongside this experiment, a reaction control system containing 50 mM Tris-HCl pH: 8.0, 1 mM ATP, 1 mM GTP, 2 mM IBMX, and 5 mM Mn^{2+} with no protein was also set. Cyclic AMP levels generated by each of the described reaction systems were then measured by enzyme immunoassay system following the acetylation protocol (Sigma-Aldrich, Missouri, USA, code CA201). Error bars represent the standard errors of the mean readings (n=3; $p < 0.05$; determined by ANOVA).

4.4 Discussion

A primary consideration for recombinant protein expression and purification is the experimental purpose for which the protein will be used. For biochemical and structural studies, it is important to optimize conditions for the expression of soluble and functionally active proteins, whereas for antigen production, where protein activity is not a primary consideration, the recombinant protein can be expressed either in its native or denatured form (Qiagen, 2003). However, when an overexpression of a recombinant protein required for biochemical or structural studies occurs, then its production in form of inclusion bodies may be inevitable (Lilie *et al.*, 1998). This means that such inclusion bodies need to be purified under denaturing conditions followed by a refolding process that allows the expressed recombinant protein to regain its biological function before being used for either biochemical or structural studies (Qiagen, 2003, Anant, 2008). A wide variety of heterologous expression systems for the expression of recombinant proteins as inclusion bodies and the subsequent refolding of such inclusion bodies into native and active forms of such proteins have already been developed and are currently in use (Rudolph and Lilie, 1996, Mukhopadhyay, 1997, Lilie *et al.*, 1998, Li *et al.*, 2003, Li *et al.*, 2004). What is only required when using these expression systems is to empirically optimize their expression conditions so that they suite the involved individual recombinant proteins (Rudolph and Lilie, 1996, Mukhopadhyay, 1997, Li *et al.*, 2003, Li *et al.*, 2004).

In this study, a biologically active recombinant AC-containing fragment domain of the maternal effect embryo arrest protein from *Arabidopsis thaliana* (AtMEE-AC) was prepared by empirically optimizing its overexpression, purification and refolding strategies. Optimization of the overexpression conditions resulted in generation and recovery of the targeted AtMEE-AC recombinant in form of inclusion bodies that literally are mere insoluble protein aggregates (Lilie *et al.*, 1998). For the purpose of purification, the recombinant

AtMEE-AC was then first solubilized in 8 M urea buffer supplemented with other solubilization agents such as 20 mM β-mercaptoethanol and 7.5% (v/v) glycerol followed by its purification on a Ni-NTA affinity matrix (Rudolph and Lilie, 1996, Mukhopadhyay, 1997, Lilie *et al.*, 1998, Li *et al.*, 2003, Li *et al.*, 2004). Purification of the recombinant AtMEE-AC on the Ni-NTA affinity matrix was made possible by the fact that the recombinant protein was expressed using a pCRT7/NT/TOPO expression system that adds a negatively-charged 6xHistidine tag (His tag) to its N-terminus end, and thereby facilitating its binding to the affinity matrix (Lilie *et al.*, 1998, Qiagen, 2003). Furthermore, solubilization of the recombinant AtMEE-AC into 8 M urea buffer resulted in the "stretching out" of the His-tags, and thereby enhancing the binding of the protein onto the immobilized nickel ions on the affinity matrix (Crowe *et al.*, 1994, Anant, 2008). The optimized purification conditions resulted in nearly complete purification of the recombinant AtMEE-AC as is shown in Figure 4.1.

The purified and denatured recombinant AtMEE-AC was then refolded while it was still bound to the Ni-NTA affinity matrix. The keeping of proteins onto a solid support during the refolding process (Stempfer *et al.*, 1996, Lindwall *et al.*, 2000, Tsumoto *et al.*, 2003) has an added advantage of preventing molecules from diffusing towards each other and forming aggregates or mis-folded products (Holzinger *et al.*, 1996, Li *et al.*, 2003, Tsumoto *et al.*, 2003, Li *et al.*, 2004). In addition, the refolding process was systematically linked to an automated BioLogic DuoFlow chromatographic system (Bio-Rad Laboratories, California, USA) that in turn enhanced both the process control as well as the overall product output (Allocco *et al.*, 2004, Abahssain *et al.*, 2010). Furthermore, the refolding buffer was supplemented with a number refolding agents that include glucose, NaCl, reduced and oxidized glutathione, non-detergent sulfobetaine, and poly-ethyl glycol that are reported to

tremendously improve the final outcome of the refolding process (Wingfield *et al.*, 1995, Lilie *et al.*, 1998, Tsumoto *et al.*, 2003, Umetsu *et al.*, 2003, Vincentelli *et al.*, 2004).

The biological activity of the refolded recombinant AtMEE-AC (Figure 4.2) was then tested in an *in vitro* enzyme immunoassay system for the ability of the refolded AtMEE-AC to generate cAMP from ATP. As is shown in Figure 4.3, a relatively high level of AC activity (>5 times than control) of the AtMEE-AC was demonstrated with higher preference for Mn^{2+} metal (>2.5-folds) than Mg^{2+} as its cofactor ion for activity. In addition, the refolded recombinant AtMEE-AC protein also demonstrated a sole substrate specificity that was strongly biased towards ATP than its structural analogue, GTP (Figure 4.4) even though the presence of GTP in an ATP containing reaction system could have had some binding inhibitory effects (~1.5-fold reduction effect). Furthermore, the recombinant AtMEE-AC protein also demonstrated that its inherent AC activity could positively be modulated by calcium (~2.0-folds) and bicarbonate ions (~1.8-folds) but with no apparent response to the fluoride ion (Figure 4.5). All these observations are neither unexpected nor unusual because in all living organisms, the adenylate cyclase (AC) system is represented by its two forms, the soluble AC (sAC) and the transmembrane AC (tmAC) versions (Kamenetsky *et al.*, 2006), both of which are well represented in plant systems (Lomovatskaya *et al.*, 2008). All sACs are functionally activated by the calcium and bicarbonate ions (Garty and Salomon, 1987, Carricarte *et al.*, 1988, Visconti *et al.*, 1990, Chen *et al.*, 2000) but not the fluoride ion and forskolin, which on the other hand are the main activators of all tmACs (Rall and Sutherland, 1958, Robison *et al.*, 1971, Wuttke *et al.*, 2001, Newton and Smith, 2004). Furthermore, the activity of all sACs is strictly dependent onto the Mn^{2+} metal ion as a co-factor (Braun and Dods, 1975) compared to tmACs that flexibly depend on both Mn^{2+} and Mg^{2+} metal ions (Zippin *et al.*, 2004). In addition, while all tmACs are mediated by the second messenger, cAMP via control mechanisms that are regulated by the GTP-binding proteins, all sACs are

mediated by the second messenger, cAMP via control mechanisms that are regulated by the calcium-binding protein, calmodulin (Kamenetsky *et al.*, 2006).

In this study, the observed functional characteristics of the recombinant AtMEE-AC protein firmly and unequivocally establishes it as a functional sAC and thereby becoming the first ever higher plant candidate to be cloned, expressed and practically confirmed. This elegant and novel finding also strongly concurs with the fact that the AtMEE gene (At2g34780) has previously been bioinformatically localized to cell chloroplast (Andersson and Sandelius, 2004) while on the other hand, several unidentified isoforms of the sAC system have also been detected (Lomovatskaya *et al.*, 2008) and further localized in potato (Romanenko *et al.*, 2008).

CHAPTER 5

Analysis of the Role of MEE in Plant Stress Response Mechanisms

Abstract

Adenylate cyclases (ACs) are enzymes capable of hydrolyzing adenosine 5′-triphosphate (ATP) into 3′,5′-cyclic adenosine monophosphate (cAMP), which in turn is involved in different physiological and developmental processes in a number of organisms. In higher plants, the first ever functional AC candidate to be identified (*Zea may* pollen protein) was implicated in pollen tube development while the second candidate (*Arabidopsis thaliana* pentatricopeptide protein) was implicated in chloroplast biogenesis as a result of its regulation of RNA processing and translation. Thus in an effort to attempt elucidating the exact physiological roles of our cloned recombinant AtMEE-AC protein, a combination of web-based computational tools commonly known as Genevestigator and Babelomics to bioinformatically assess the expressional profiles of its gene, *AtMEE22* (At2g34780). Results from this study show that this protein (AtMEE-AC) is specifically involved in embryogenesis and abiotic stress responses through its direct regulation of RNA transcript processing and protein post-translational ubiquitination.

5.1 INTRODUCTION

With the completion of the genome sequencing in *Arabidopsis thaliana*, it is logical to query the functional roles of all of its 25, 498 genes (Wright and Dyson, 1999, Arabidopsis Genome, 2000, Meier and Gehring, 2008). Apparently, the actual number of genes in Arabidopsis keeps on being reviewed as new and up to date sequencing and array-based technologies are providing more and more evidence for the many previously un-annotated genes, and the prediction which previously had been at 26,819 genes in 2008 (Swarbreck *et al.*, 2008) has since shifted to 27, 416 genes by 2009 (Tair, 2009).

In bioinformatics and microarray technology, it is generally thought that genes with similar patterns of mRNA expression and similar functions are likely to be regulated via the same mechanisms (Allocco *et al.*, 2004). Genes with strongly correlated mRNA expression profiles are more likely to have their promoter regions bound by a common transcription factor (TF) (Ideker *et al.*, 2001). From the co-expression of large sets of genes, it is evident that genes do not act alone in response to specific stimuli, and many cellular processes require the coherent participation of multiple gene products (Meier and Gehring, 2008). Furthermore, a number of studies have shown that genes that have been confirmed to be co-expressed in response to a range of conditions, presumably resulting from co-regulation, have correlated functional relationships including physical interactions between their proteins (Jansen *et al.*, 2002, Allocco *et al.*, 2004). As such, inferences to the function of an un-annotated gene can be made by observing conditions in which the gene is expressed and by analysing and interpreting other genes with which it is commonly co-expressed. Results from co-expression analyses are particularly telling if one or several of the genes studied have well-defined functions (Allocco *et al.*, 2004, Manfield *et al.*, 2006, Meier and Gehring, 2008). Also, co-expression of genes of known function with poorly characterized or novel

genes, such as plant ACs, may provide a simple means of gaining leads to the functions of many genes for which information is not yet available (Eisen *et al.*, 1998).

Analysis of microarray expression data by correlation analysis has the advantage that a single gene of interest (GOI) can be selected as a "reference gene" and its expressional correlation with all other genes within the genome determined (Eisen *et al.*, 1998, Meier and Gehring, 2008). Apparently, a co-expression in response to a particular condition in a microarray experiment does not necessarily imply co-regulation. A specific condition can co-activate multiple transcription regulatory pathways resulting in the co-expression of genes that are regulated by different mechanisms (Meier and Gehring, 2008). Following the establishment of an expression correlated gene group (ECGG), the combinational use of a number of web-based tools can then be used to integrate different data sets and provide a rapid global functional assessment of the biological processes in which the gene (GOI) functions (Meier *et al.*, 2010). Genes that are co-expressed over multiple data sets show functional relatedness (Schulze and Downward, 2001). For this reason, a gene ontology (GO) analysis of the ECGG is performed to identify any statistically enriched terms associated with the ECGG compared to the background (complete organism genome) frequency. If the expression of the GOI is found to be most highly correlated with genes involved in a specific type of biological response, we can then infer functional roles through association with other genes that have more defined roles.

In order to elucidate an ECGG co-regulation, computational (computer-based) tools are used to identify similarities in their regulatory promoter elements (*cis*-elements). The basis of this being that the regulation of eukaryotic gene expression is critically determined by the combinational presence of specific *cis*-regulatory motifs in the promoter regions of genes that bind specific TFs and modulate expression (Riechmann *et al.*, 2000, Allocco *et al.*, 2004). The identification of *cis*-elements that are thought to be causative for the observed

transcriptional responses of the ECGG is achieved by identifying motifs that are enriched in the promoters of the ECGG as compared to background frequencies in the rest of the genome (Kankainen and Holm, 2004). Transcription factors play a significant role in controlling cellular processes and the MYB TF family is very large and is involved in controlling various processes like responses to biotic and abiotic stress factors, development, differentiation, metabolism and defense (Ambawat *et al.*, 2013). Here we report the use of a number of computer-based programs to elucidate the possible physiological roles of a maternal embryo arrest 22 (AtMEE22) protein from *Arabidopsis thaliana*.

5.2 Materials and Methods

In order to carry out the computer-assisted analysis of the desired *AtMEE22* gene for the possible elucidation of its exact physiological roles in the Arabidopsis plant, a number of bioinformatic programs and softwares were utilized, as clearly outlined below.

5.2.1 The Expression Profile of *AtMEE22*

In order to reveal the expression patterns of *AtMEE22* in various tissues of the Arabidopsis plant, the anatomy analysis tool, Genevestigator (http://www.genevestigator.com) (Zimmermann *et al.*, 2004) was used, which calculates how strongly a gene is expressed in different anatomical parts of a plant. The data used for the expression analysis of *AtMEE22* was obtained from various microarray experiments and the average signal intensity values were then calculated for each type of the plant tissue.

5.2.2 Co-expressional Analysis of *AtMEE22*

In order to establish the co-expressional profile of the *AtMEE22* gene with the other related Arabidopsis genes, the Arabidopsis co-expression tool (http://www.arabidopsis.leeds.ac.uk) (Hruz *et al.*, 2008) was used. The tool analysis was performed across all available experiments using At2g34780 as the driver gene and leaving the gene list limit blank to obtain a full correlation list. This tool utilizes hybridization signal intensities from microarray experiments to calculate a Pearson correlation co-efficient (r-value), which is a scale-invariant measure of expression similarity that expresses the strength and direction of the linear relationship between the reference gene (GOI, *AtMEE22* in this case) and all other Arabidopsis genes represented on the selected chip. The tool calculates and returns both

negative and positive correlations (ranging from −1 to +1), associated probability (p), and expectation (e) values, which are a measure of the statistical significance.

5.2.3 Functional Enrichment of the *AtMEE22-ECGG50*

After establishing the co-expression group of the *AtMEE22*, the "Fatigoplus" (version 4.3) compare tool in the Babelomics suite (http://babelomics.bioinfo.cipf.es) (Al-Shahrour *et al.*, 2007) was then used to identify any significant enrichment in functional terms associated with the *AtMEE22-ECGG50*. In this analysis, all available Arabidopsis data bases were selected using default options, which included the GO categories of biological process, cellular component and metabolic function, the KEGG pathways and the Swissprot keywords. Enrichment significance was then determined using an adjusted p-value to correct for multiple hypothesis testing.

5.2.4 The Stimulus-specific Microarray Expression Profile of *AtMEE22-ECGG50*

The expression profiles of the *AtMEE22-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/pertubations tool (Zimmermann *et al.*, 2004). In order to obtain 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 were 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 were 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 (vesion 4.2.01) created by The Institute for Genomic Research (TIGR).

5.2.5 Promoter Analysis of the *AtMEE22-ECGG50*

The promoter regions of *AtMEE22* and *AtMEE500-ECGG50* were analyzed for any enrichments in potential transcription factor binding sites (TFBS) using the web-based Athena (http://www.bioinformatics2.wsu.edu/cgi-bin/Athena/cgi/visualize.pl) (O'connor *et al.*, 2005) and POBO (http://ekhidna.biocenter.helsinki.ft/poxo/pobo/pobo) (Kankainen and Holm, 2004) applications. The visualization tool in Athena performs an analysis of Arabidopsis promoter sequences and reports enrichments of known plant TFBS. The analysis of *AtMEE500-ECGG50* was performed using settings of 1000 bp upstream of the predicted transcription start site (TSS) and do not cut off at adjacent genes. The Athena results were subsequently confirmed in POBO by uploading promoter sequences 1 kb upstream of the coding regions of the *AtMEE500-ECGG50*. The analysis was run against an Arabidopsis background (clean) searching for the MYBCORE core motif (CNGTTR) using default settings. A two-tailed p-value was then calculated in the linked online GraphPad web-site using the generated *t*-value and degrees of freedom to determine the statistical differences between the input sequences and background.

5.3 Results

The Genevestigator (Zimmermann *et al.*, 2004) analysis of the *AtMEE22* gene using the anatomy tool showed that the AtMEE22 protein was expressed in many plant tissues which included petals, siliques, flowers, developing meristemoid zones, shoot apical meristems, pistils, suspensors and ovules. However, there was an immensely elevated expression of the AtMEE22 protein in the embryo of up to 3000 arbitrary fluorescence units as compared to most of the investigated plant tissues whose expression levels were well below 1500 fluorescence units (Figure 5.1).

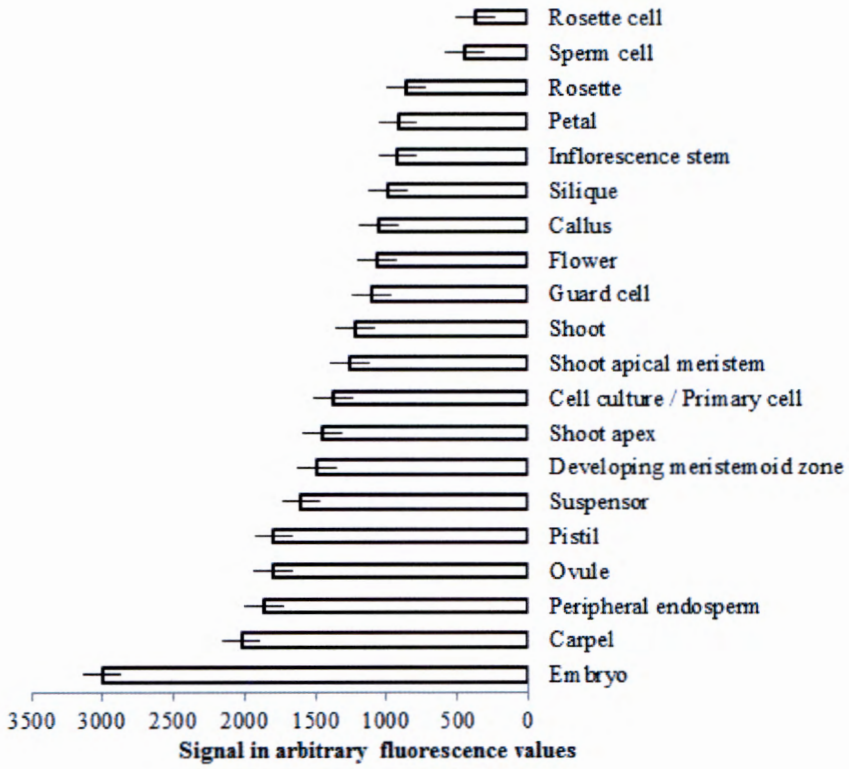

Figure 5.1: Expression profile of AtMEE22 in different tissues of *Arabidopsis thaliana*. The *AtMEE22* gene expression in various parts of the Arabidopsis plant showing considerably elevated expression levels in the embryo (data obtained from Genevestigator anatomy tool (Zimmermann *et al.*, 2004)).

In order to determine the co-expression levels of *AtMEE22* gene with the other Arabidopsis genes, an expression correlation analysis across a large number (322) of diverse microarray experiments represented on the ATH1-22K full genome microarray chip was performed. This analysis revealed that the *AtMEE22* is highly co-expressed with a number of genes in the Arabidopsis genome, with the top 50 genes having a Pearson correlation coefficient (r-value) ranging between 0.869 and 0.804 (Table 5.1). The top 50 most co-expressed genes (hereby referred to as the *AtMEE22*-Expression Correlated Gene Group (*AtMEE-ECGG-50*)) were considered here since their correlation values were high and their number being fair enough to be a good representative sample size for the subsequent functional enrichment and promoter analysis steps.

Table 5.1: List of top 50 genes that are co-expressed with *AtMEE22*, (At2g34780).

Locus	r-value	Annotation
AT2G34780	1	Maternal effect embryo arrest 22
AT3G66652	0.87	Fip1 motif-containing protein
AT1G79490	0.86	Pentatricopeptide repeat (PPR) superfamily protein
AT1G52160[RNAp, tRNAp]	0.85	tRNAse Z3
AT5G39960	0.85	GTP binding;GTP binding
AT4G04670[RNAp, tRNAp]	0.85	Met-10+ like family protein / kelch repeat-containing
AT5G46920[RNAp]	0.84	Intron maturase, type II family protein
AT1G30240	0.84	FUNCTIONS IN: binding.
AT4G22285	0.84	Ubiquitin C-terminal hydrolases superfamily protein
AT5G14050[RNAp, rRNAp]	0.84	Transducin/WD40 repeat-like superfamily protein
AT4G31120	0.83	SHK1 binding protein 1
AT1G72440[ESD, MG]	0.83	CCAAT-binding factor
AT5G64420	0.83	DNA polymerase V family

AT5G54910	0.83	DEA(D/H)-box RNA helicase family protein
AT3G10530	0.83	Transducin/WD40 repeat-like superfamily protein
AT5G18440	0.83	DOMAIN: Nuclear fragile X mental retard-interacting protein 1
AT5G06350	0.82	AT5G06350, ARM repeat superfamily protein
AT5G59980[RNAp, tRNAp]	0.82	Polymerase/histidinol phosphatase-like
AT3G49240	0.82	Pentatricopeptide repeat (PPR) superfamily protein
AT2G18220	0.82	Noc2p family
AT3G56990[ESD, MG]	0.82	Embryo sac development arrest 7
AT4G10620	0.82	P-loop containing nucleoside triphosphate hydrolases
AT5G22840	0.82	Protein kinase superfamily protein
AT1G15440	0.82	Periodic tryptophan protein 2
AT2G07750	0.82	DEA(D/H)-box RNA helicase family protein]
AT5G24840[RNAp, tRNAp]	0.81	tRNA (guanine-N-7) methyltransferase
AT1G71850	0.81	Ubiquitin carboxyl-terminal hydrolase family protein
AT5G38720	0.81	Unknown protein.
AT3G48250	0.81	Pentatricopeptide repeat (PPR) superfamily protein
AT2G05120	0.81	Nucleoporin, Nup133/Nup155-like
AT4G38890[RNAp, tRNAp]	0.81	FMN-linked oxidoreductases superfamily protein
AT2G01740	0.81	Tetratricopeptide repeat (TPR)-like superfamily protein
AT3G16840	0.81	P-loop containing nucleoside triphosphate hydrolases
AT1G17690	0.81	FUNCTIONS IN: molecular function unknown
AT3G13940	0.81	DNA binding;DNA-directed RNA polymerases
AT1G06220[ESD, MG]	0.81	Ribosomal protein S5/Elongation factor G/III/V family protein
AT4G19610	0.81	Nucleotide binding;nucleic acid binding; RNA binding
AT1G02370	0.81	Tetratricopeptide repeat (TPR)-like superfamily protein
AT4G02400[RNAp, rRNAp]	0.81	U3 ribonucleoprotein (Utp) family protein
AT3G16810	0.81	pumilio 24

AT4G04940[RNAp, rRNAp]	0.81	Transducin family protein / WD-40 repeat family protein
AT4G21170	0.81	Tetratricopeptide repeat (TPR)-like superfamily protein
AT5G39840	0.81	ATP-dependent RNA helicase, mitochondrial, putative
AT5G17930	0.81	MIF4G domain-containing / MA3 domain-containing
AT3G21540[RNAp, rRNAp]	0.80	Transducin family protein / WD-40 repeat family protein
AT1G08610	0.80	Pentatricopeptide repeat (PPR) superfamily protein
AT5G13680	0.80	IKI3 family protein
AT3G01160	0.80	FUNCTIONS IN: molecular function unknown.
AT2G18330	0.80	AAA-type ATPase family protein
AT3G11964[RNAp]	0.80	RNA binding;RNA binding
AT1G69070	0.80	FUNCTIONS IN: molecular function unknown

Abbreviations for the indicated GO terms:
RNAp = RNA processing (GO: GO: 0006396); tRNAp = tRNA processing (GO: 0008033); rRNA processing (GO: 0006364); ESC =Embryo Sac Development (GO: 0009553) and MG = Megagametogenesis (GO: 0009561).

After establishing the co-expressional profile of the *AtMEE22* with the *AtMEE22-ECGG50*, a "Fatigoplus" analysis of the *AtMEE22-ECGG50* was then undertaken to check if these two sets of genes may function in similar biological processes. This gene ontology (GO) analysis then succeeded to identify a number of statistically significant enrichments in the biological process categories that were generally related to the various post-transcriptional modifications like RNA processing and post-translational modifications like protein ubiquitination, all of which are essentially critical for embryo development and responses to stress factors (Fu *et al.*, 2010, Miura and Hasegawa, 2010, Trujillo and Shirasu, 2010) (Table 5.2). In this analysis, the significance was determined using an adjusted p-value that also corrected for multiple hypothesis testing. The program uses a Fisher's exact test and returns adjusted p-values (Family Wise Error Rate) accounting for multiple testing and to reduce the false-positive frequencies (Al-Shahrour *et al.*, 2007).

Table 5.2: The *AtMEE22:ECGG50* Gene Ontology output for Biological Process categories.

	AtMEE22-ECGG50		Genome		
GO Term	**No. of Genes**	**%**	**No. of Genes**	**%**	**Adj. *p* value**
Biological Process					
RNA processing (GO:0006396)	11 (40)	21.57	434 (30795)	1.39	$1.07E^{-07}$
tRNA processing (GO:0008033)	5 (46)	9.8	59 (31170)	0.19	$2.92E^{-05}$
rRNA processing (GO:0006364)	4 (47)	7.84	122 (31107)	0.39	$1.20E^{-02}$
Embryo sac development (GO:0009553)	3 (48)	5.88	74 (31155)	0.24	$4.80E^{-2}$
Megagametogenesis (GO:0009561)	3 (48)	5.88	42 (31187)	0.13	$1.20E^{-2}$
Ubiquitin ligase complex (GO:0000151)	5 (42)	10.64	248 (30978)	0.79	$1.2E^{-03}$
Cullin-RING ubiquitin ligase complex	5 (42)	10.64	157 (31059)	0.5	$1.8E^{-04}$
Cul4-RING E3 Ubiquitin ligase complex	5 (42)	10.64	117 (31109)	0.37	$6.7E^{-05}$
Small sub-unit proceosome	3 (44)	6.38	4 (31229)	0.01	1.3^{E-05}

After establishing the biological processes in which the *AtMEE22* and *AtMEE22-ECGG50* had similar functions, this pair set was further subjected to an *in-silico* global expression analysis in which specific experimental conditions that could induce differential expression of the both gene sets were identified. In accordance with the co-expression and GO analysis, the heat maps generated from the microarray expression analysis revealed that the transcriptional processes of *AtMEE22* and *AtMEE22-ECGG50* are generally collectively induced in response to a range of abiotic stress factors (Figure 5.2).

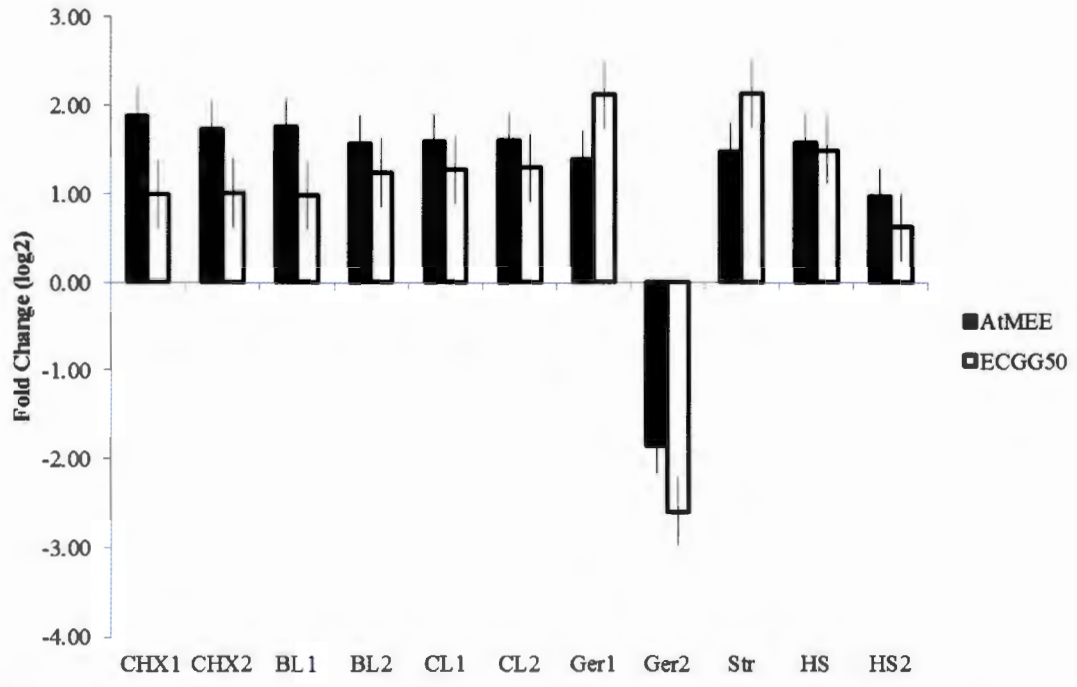

Figure 5.2: The expression profile of *AtMEE22* and *AtMEE22:ECGG50* correlated genes in response to selected abiotic treatments. The results presented illustrate the fold change (log2) in expression of *AtMEE22* and *AtMEE22:ECGG50* in response to 186 perturbations that fulfilled the filer criteria (the filter criteria being 'fold change' 2.5 and *p*-value 0.1), where ***AtMEE22*** = At2g34780 gene expression; ***ECGG50*** = average gene expression for the 50 Expression Correlated Group of Genes. **CHX1** = Cycloheximide/dexamethasone study 2 (pBeaconRFP_GR-ABI3); **CHX2** = Cycloheximide study 4 (pBeaconRFP_GR-ABI3); **BL 1** = BL/H3BO3 (10d)/untreated cell culture samples; **BL 2** = BL/H3BO3 (2d)/untreated cell culture samples; **CL 1** = Callus formation (48h); **CL 2** = Callus formation (96h); **Ger 1** = Germination 1h/seed desiccation; **Ger 2** = Germination 48h/Stratification; **Str** = Stratification (48h)/Seed desiccation; **HS** = Heat study 4 (Untreated samples); **HS2** = Heat study 6.

The highly correlated expressional profiles, biological processes and differential responses to specific abiotic stress stimuli of the *AtMEE22* and its *AtMEE22:ECGG50* counterpart strongly suggested that these genes could be under a common regulatory control and thus may share common *cis*-regulatory elements in their promoter regions. Therefore, promoter regions of the *AtMEE22:ECGG50* were analyzed for the presence of enriched *cis*-elements using the Athena (O'connor *et al.*, 2005) and POBO (Kankainen and Holm, 2004) promoter analysis programs. The Athena analysis identified a significant enrichment in the MYBCORE core elements (CNGTTR) which are known to bind MYB TFs, resulting in the activation of both development-related and stress-related gene transcriptions (Dubos *et al.*, 2010). The subsequent POBO analysis (Kankainen and Holm, 2004) of the *AtMEE22:ECGG50* further showed that the CNGTTR motif was indeed significantly enriched (*t*-test; p-value <0.0001), being present in 25/26 genes at an average of 4.8 copies/promoter compared to the average of 3.8 across all *A. thaliana* promoters (Figure 5.3).

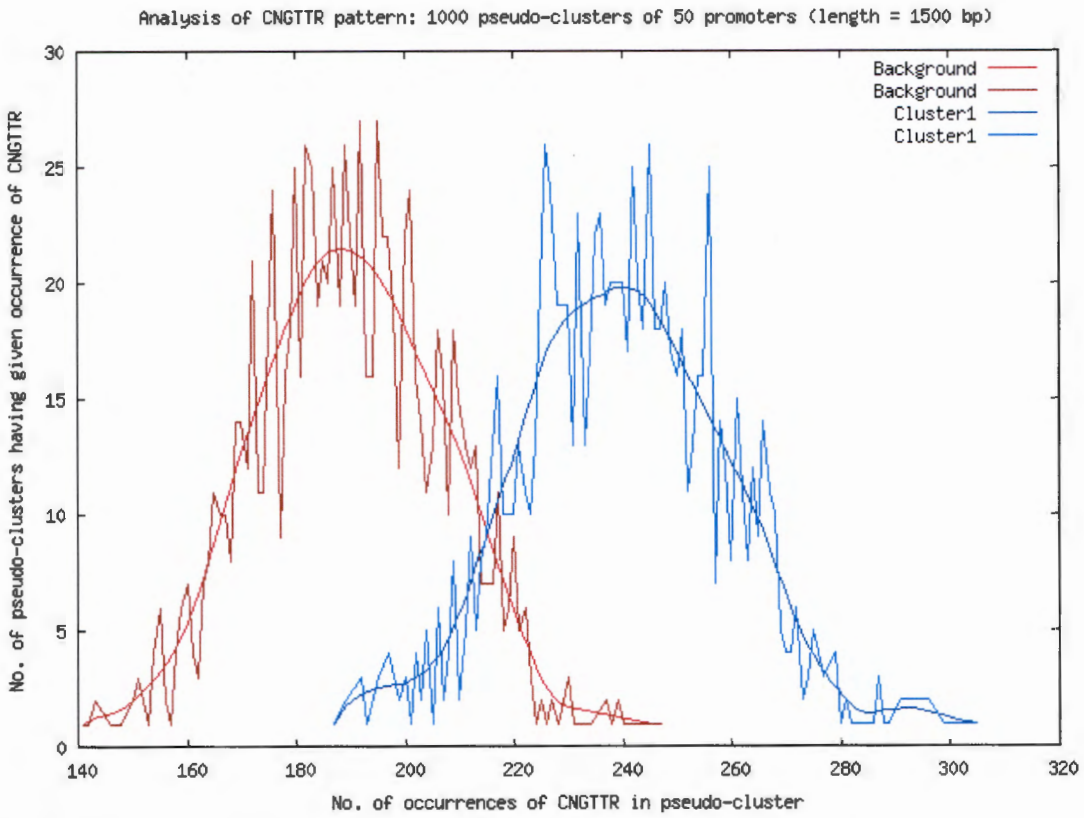

Figure 5.3: Illustration of the frequency occurrence of the MYBCORE (CNGTTR) motif in the promoters of the *AtMEE22:ECGG50* and in the background (genomic) *Arabidopsis thaliana* promoters. The 1-kb upstream promoter sequences of the AtMEE:ECGG50 were analyzed in Athena (O'connor *et al.*, 2005) and POBO (Kankainen and Holm, 2004) programs to determine the frequency of occurrence of the MYBCORE CNGTTR motif. The motif was then determined to be significantly enriched (*t*-test: p value >0.0001) in the AtMEE:ECGG50, being present in 96% of promoters at an average of 4.8 copies per promoter compared to 88% of promoters in the Arabidopsis genome at an average of 3.78 copies per promoter.

5.4 Discussion

Observations made from this study indicate that the *AtMEE22* gene is predominantly expressed in the embryo tissue of the Arabidopsis plant (Figure 5.1) alongside other genes (Table 5.1) that are specifically involved in embryo development and abiotic stress response (Table 5.2). In nature, stress can be recognized as a stimulus or influence that is outside the normal range of homeostatic control in a given organism: If a stress tolerance is exceeded, mechanisms are activated at molecular, biochemical, physiological and morphological levels; and once stress is controlled, a new physiological state is established, and homeostasis is re-established. When the stress is retired, the organism may return to the original state or a new physiological state (Fraire-Velázquez *et al.*, 2011).

While in nature, the *AtMEE22* gene does not seem to be up-regulated by biotic perturbations, the gene is highly responsive to abiotic stress stimuli (Figure 5.2) whose mode of action appears to involve MYB proteins as transcription factors (TFs) (Figure 5.3). As TFs, MYB proteins are strongly considered key regulatory factors that control development, metabolism, and responses to biotic and abiotic stresses in plants (Dubos *et al.*, 2010), and their mechanism of action in the Arabidopsis is typically believed to involve the binding onto the CNGTTR *cis* core element of *AtMEE22*, thereby activating its downstream transcriptional and translational processes (Lata *et al.*, 2011).

Meanwhile, by considering the fact that the two key processes of embryo development and abiotic stress response in which the *AtMEE22* gene is centrally involved, require the participation of second messengers like cAMP and/or cGMP yet the truncated form of AtMEE22 (AtMEE-AC) protein has since been practically demonstrated to possess functional adenylate cyclase properties (Chapters 2-4), it is thus conceivable to conclude that the AtMEE22 protein (studied in this work) is an important plant signalling molecule whose

mechanism of action involves the second messenger, cAMP and the MYB transcription factors.

General Discussion, Conclusion and Outlook

Since climate changes shall always continue to occur and extreme stresses likely to increase, we can therefore expect increasing difficulties in growing crop plants in many parts of the world (Munster *et al.*, 2004, Vinocur and Altman, 2005). Food security is therefore heavily dependent onto the development of crop plants with increased resistance to both biotic and abiotic stress factors such as pathogen infections and droughts respectively. Thus an urgent need to use rational approaches to develop crop plants with increased stress tolerance has led to an impressive body of work in the areas of plant genetics, plant physiology, plant biochemistry and plant molecular biology, and a realization that only an integrated and systems-based approach can possibly deliver effective biotechnological solutions (Denby and Gehring, 2005).

Hence since proteins that systemically affect homeostasis in plants are a target candidate group of molecules for biotechnology, one such molecule in form of an adenylate cyclase (AC)-containing fragment domain of the maternal effect embryo arrest protein from *Arabidopsis thaliana* (AtMEE-AC) was prepared in this study and its mode of action practically elucidated. Initially, when this recombinant protein was transiently expressed in competent BL21 (DE3) *Escherichia coli* cells, it induced a forskolin-insensitive (Zippin *et al.*, 2004)generation of the endogenous cAMP in these prokaryotic systems, and thereby proposing itself as either a *bona fide* soluble adenylate cyclase (sAC) that is capable of generating cAMP from ATP or is simply another functional plant molecule capable of stimulating other resident sACs (*E. coli* sACs in this case) to produce cAMP (Andersson and Sandelius, 2004) (Chapter 2). Secondly, when its cDNA was cloned into a non-lactose fermenting mutant strain of *E. coli* (*cyaA* SP 850 cells) via a pCRT7/NT-TOPO plasmid vector, followed by the growth of such cells on MacConkey agar supplemented with 0.1 mM IPTG, the recombinant AtMEE-AC protein succeeded to generate cAMP, which in turn

shifted the mutant cells to behave as if they were wild type cells and ultimately fermenting lactose. This immaculate finding thus therefore firmly established the recombinant AtMEE-AC protein as a biologically functional AC and thereby becoming a fifth ever such molecule to be identified in higher plants after the pollen protein from *Zea mays* (Moutinho *et al.*, 2001), the pentatricopeptide protein from *Arabidopsis thaliana* (Ruzvidzo, 2009), the HpAc1 invloved in stress signalling in *Hippeastrum hybridum* (Świeżawska et al., 2014) and the NbAC (*Nicotiana benthamiana*) adenylyl cyclase (Ito et al., 2014).

Furthermore, when the recombinant AtMEE-AC was purified followed by its rigorous *in vitro* functional characterization, it exhibited itself as a manganese-depended enzymatic protein whose functional activity was positively modulated by both calcium and carbonate ions, and thus confirming itself as a *bona fide* sAC (Garty and Salomon, 1987, Carricarte *et al.*, 1988, Okamura *et al.*, 1990, Visconti *et al.*, 1990, Chen *et al.*, 2000), whose physiological roles may specifically be mediated by the second messenger, cAMP and probably, through control mechanisms that involve the calcium-binding protein, calmodulin (Oh *et al.*, 2012). Lastly, when the expressional profiles of the *AtMEE22* (At2g34780) gene responsible for production of the AtMEE-AC protein fragment was bioinformatically assessed with a combination of web-based computational tools commonly known as Genevestigator and Babelomics (Zimmermann et al., 2004), its pattern revealed that it is specifically involved in embryogenesis and abiotic stress responses through its direct regulation of the RNA transcript processing and the post-translational protein ubiquitination.

All in all, the findings of this whole study conceivably established the recombinant AtMEE-AC protein as a functional sAC with specific roles in embryo development and responses to abiotic stress responses. This therefore firmly confirmed this protein as the third ever higher plants AC to be identified after the pollen protein from *Zea mays* (Moutinho *et al.*, 2001), the pentatricopeptide protein from *Arabidopsis thaliana* (Ruzvidzo *et al.*, 2013), the HpAc1

invloved in stress signalling in Hippeastrum hybridum (Świeżawska et al., 2014) and the NbAC (Nicotiana benthamiana adenylyl cyclase (Ito et al., 2014)..

As an outlook and since the *AtMEE22* (At2g34780) gene had previouly been bioinformatically detected together with other eight putative molecules (Gehring, 2010) and in this study, it has been practically confirmed to be a higher plant AC, it is therefore recommended that the other seven outstanding putative candidates be tested also so as to obtain a better understanding of their exact physiological roles in plants, and particularly in critical processes like growth, development and responses to stressful environmental factors.

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