Recombinant Expression and Molecular Elucidation of the Dual Functional Properties of a Truncated Pentatricopeptide Repeat Protein from *Arabidopsis thaliana*

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Submitted in fulfilment of the requirements for the degree of Doctor of Philosophy (PhD) in Biology in the Department of Biological Science, North-West University Mafikeng Campus, South Africa

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February 2017

Declaration

I, Tshegofatso Bridget Dikobe, declare that the thesis entitled "**Recombinant expression and molecular elucidation of the dual functional properties of a truncated Pentatricopeptide repeat protein from *Arabidopsis thaliana***" is my own work, which has not been submitted at this university or in any other institution elsewhere, and all the sources used or quoted have been indicated and acknowledged.

Name: TSHEGOFATSO DIKOBE

Signature: Dikobe **Date:** 09/10/2017

Supervisor: PROF. O. AJAYI

Signature: **Date:** 09/10/2017

Dedication

I dedicate this work with love to my late parents, Boitumelo and Tholo Dikobe, who always served as my inspiration.

Acknowledgements

This PhD study has been an eye-opener and an eventful part of my life journey, where a number of friends, family and institutions have been supportive, assisting and guiding me towards the right direction for the past three years. I am really enlightened from this journey.

First and foremost, I would like to thank Almighty **God** for His mercy, protection, guidance and strength to complete this research project. Secondly, my most humble gratitude and appreciation goes to my supervisor and mentor, Professor Oziniel Ruzvidzo, for his extraordinary supervision during the past five years of my studies. Thank you for allowing me to join your lab to pursue my postgraduate studies without prior research experience in plant biotechnology and for having faith in my ability. Under your mentorship, I learnt to think and work independently; you always encouraged and reminded me of the long-term goals of my PhD research, on what we have established and what we still have to answer. I appreciate your drive, hard work and guidance during the course of my studies. I will always remember your exceptional inputs and values that you have instilled in me as an aspiring scientist.

I would like to extend my sincere gratitude to Doctor Lusisizwe Kwezi for his words of encouragement, advice on research and for providing training in most applications of molecular biology, biochemistry and plant biotechnology laboratory techniques that allowed me to accomplish the set objectives of my research. With such skills I believe I can stand toe-to-toe with any scientist in the world. I am grateful to Doctor Takalani

Mulaudzi-Masuku for her words of encouragement and advice on research. I would also like to thank all the members of the Plant Biotechnology Research Group for their time, appreciation, and their spirit of companionship in sharing our research frustrations and achievements, which made my studies so memorable. To all of my colleagues in the Department of Biological Sciences, North-West University (Mafikeng campus), thank you for your support. Special thanks to Ms. Madira Manganyi and Mr. Johannes Morapedi, in particular, for always encouraging and providing me with moral support through their words of wisdom.

I would like to thank my family for their love, patience and support. To my loving aunt, Mrs. Keitumetse Tsatsimpe, and her husband for their unwavering parenting role they played after the passing-on of my parents, you kept on encouraging me to pursue my dreams with your full support. To my aunt, Ms. Thoredi Choabi, thank you mom for your love, support and cheering. Also to my brother Kagiso Dikobe and my best friend Adam Motsamai for your understanding when I devoted most of my time and attention, even during holidays to this research work when you really needed me. To all of my cousins, aunts, uncles, and friends, you have all played a role in my life. Without you guys, I'm nothing; I thank **God** for having you in my life because you gave me strength to move forward.

Finally, I wish to thank the North-West University (NWU)–Mafikeng Campus for awarding me a postgraduate bursary and the National Research Foundation (NRF) for their financial assistance and support towards the completion of my studies.

List of Abbreviations

AC	:	Adenylate cyclase
ANOVA	:	Analysis of variance
AtCNGC	:	*Arabidopsis thaliana* cyclic nucleotide-gated channel
ATP	:	3′,5′-Adenosine 5′-triphosphate
AtPPR-AC/K	:	*Arabidopsis thaliana* pentatricopeptide repeat adenylate cyclase and kinase domain fragments
BLAST	:	Basic Local Alignment Searching Tool
Bp	:	Base pairs
cAMP	:	Cyclic 3′,5′-adenosine monophosphate
cDNA	:	(Copy DNA) DNA complementary to RNA
cGMP	:	Cyclic 3′,5′-guanosine monophosphate
CMS	:	Cytoplasmic male sterility
CRP	:	cAMP receptor protein
C-terminal	:	Carboxyl terminal
Cya	:	Adenylate cyclase gene
E value	:	Expectation value
EIA	:	Enzyme immunoassay
EIIA^{Glc}	:	Glucose-specific enzyme IIA
EIIC	:	Enzyme IIC
GC	:	Guanylate cyclase
GPCR	:	G-protein-coupled receptor
G-protein	:	Guanine nucleotide-binding protein

GTP	:	3′,5′-Guanosine 5′-triphosphate
HpAC1	:	*Hippeastrum hybridum* adenylate cyclase 1
HR	:	Hypersensitive response
IBMX	:	3-Isobutyl-1-methyl xanthine
IPTG	:	Isopropyl-β-D-thiogalactopyranoside
kDa	:	kiloDalton
K_m	:	Michaelis constant
LB	:	Luria-Bertani
MOPS	:	3-(N-morpholino) propanesulfonic acid
MSMO	:	Murashige and Skoog basal salt with minimum organics
NASC	:	Nottingham Arabidopsis Stock Centre
NbAC	:	*Nicotiana benthamiana* adenylate cyclase
NC	:	Nucleotide cyclase
NCBI	:	National Centre for Biotechnology Information
Ni-NTA	:	Nickel-nitrilotriacetic acid
N-terminus	:	Amino-terminus
OD	:	Optical density
OmpT	:	Outer membrane protease
ORF	:	Open reading frame
P value	:	Probability value
PDEs	:	Phosphodiesterases
PEG	:	Poly-ethyl glycol

PMSF	:	Phenylmethylsulfonyl fluoride
PPR	:	Pentatricopeptide repeat
PSiP	:	Pollen signaling protein
PSKR1	:	Phytosulfokine receptor 1
PTS	:	Phosphotransferase system
Rf	:	Restorer of fertility
RLK	:	Receptor like kinase
RT-PCR	:	Reverse transcriptase polymerase chain reaction
sAC	:	Soluble adenylyl cyclase
SDS-PAGE	:	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SNK	:	Student Newman Kuehls
SOC	:	Super optimal broth with catabolite repression
STAND	:	Signal transduction ATPases with numerous domains
TAIR	:	The Arabidopsis Information Resource
TBE	:	Tris/borate/EDTA
TIR-NBS-LRR	:	Toll interleukin receptor nucleotide-binding site leucine rich repeat protein
tmAC	:	Transmembrane adenylyl cyclase
TPR	:	Tetratricopeptide repeat
V_{max}	:	Maximum reaction velocity
YT	:	Yeast-tryptone

Definition of Terms

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

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

Cell signalling: The transmission of molecular signals from a cell's exterior to its interior for appropriate responses to effectively occur.

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

Domain: A distinct functional or structural unit in a protein that is usually responsible for a particular function or interaction, contributing to the overall role of a protein.

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

Gene annotation: The process of identifying the locations of genes and all of the coding regions in a genome and determining their functional roles.

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

***In vitro*:** A process that is made to occur in a laboratory vessel "test-tube", or other experimentally-controlled environments rather than within a living organism or their normal biological settings.

***In vivo*:** A biological process that is tested on whole or parts of living organisms as opposed to the dead systems.

Kinase: An enzyme that catalyzes the transfer of phosphate groups from high-energy phosphate-donating molecules to specific substrates and at times, including itself.

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

Motif: A short, conserved group of amino acids or nucleotides which share structural and functional similarities in a protein.

Primer: A short synthetic nucleic acid sequence capable of forming base pairs with a complementary template RNA/DNA strand and facilitating its specific amplification.

Refolding: A conformational process used to restore the biological activity or function of an un-folded or mis-folded protein.

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.

RIP-chip: A technique used (for RNA co-immunoprecipitation and chip hybridization) to pinpoint the *in vivo* RNA ligands of the maize (*Zea mays*) PPR protein CRP1.

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

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

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Introductory Research Summary

Plants play essential roles in the general life systems of mankind even though they also tend to experience constant challenges during their own life cycles from periodic exposures to various environmental stimuli (e.g. light, hormones, pathogens, sugars, and wounding), which affect their productivity and developmental systems. While, mechanisms by which these plants use to detect and transduce such external signals into their internal cellular environments have not been elucidated. However, there is a need for them to be clearly understood so that they can then be manipulated for the ultimate benefit of mankind. Currently, a special group of plant molecules termed adenylate cyclases (ACs) have been the main focus. These are enzymes capable of catalyzing the conversion of adenosine 5′-triphosphate (ATP) to the second messenger, 3′,5′-adenosine cyclic monophosphate (cAMP), which in turn is involved in a variety of physiological and developmental processes in a number of organisms. Despite the fact that the roles of both the AC and its product, cAMP have been extensively studied and documented in animals and lower eukaryotes, not much is known about ACs in plants even though the cAMP has been widely implicated in a number of cellular processes such as the cell cycle, responses to stressful environmental factors, defense responses, and activation of the protein kinases. To date, only five higher plant ACs are known and these are the PSiP protein from *Zea mays*; the AtPPR-AC protein from *Arabidopsis thaliana*; the NbAC protein from *Nicotiana benthamiana*; the HpAC1 protein from *Hippeastrum hybridum*; and the AtKUP7 protein from *Arabidopsis thaliana*.

Apparently, each of these five identified plant ACs bear a single catalytic domain in the form of the characterized AC domain but a recent study has further identified, from *Arabidopsis thaliana*, a related protein molecule with two annotated catalytic domains; the AC and kinase domains. This protein molecule is termed a pentatricopeptide repeat protein (AtPPR) coded

for by the At1g62590 gene. Therefore, in an attempt to identify yet another additional functional AC in higher plants and also to elucidate the possible functionality of twin-domain proteins in plants, we targeted the AtPPR protein in this study. In this plan, we cloned and partially expressed its AC/kinase-containing domain fragment (AtPPR-AC/K) in competent *E. cloni* EXPRESS BL21 (DE3) pLysS cells and demonstrated its ability to induce the generation of endogenous cAMP in these prokaryotic host cells. In addition, we also demonstrated a complementation of the mutant non-lactose fermenting *cyaA* SP850 *E. coli* cells by this recombinant protein to apparently ferment lactose, and as a result of this AtPPR-AC/K's ability to generate the most needed cAMP necessary for this process. Furthermore, we also managed to chemically purify this recombinant AtPPR-AC/K protein and determined its AC activity *in vitro*, and during which it was also firmly established that the recombinant AtPPR-AC/K was indeed a *bona fide* soluble AC (sAC), whose functional activities in plants are mediated by cAMP via a calmodulin-dependent signalling system.

Lastly, the possible kinase activity of the recombinant AtPPR-AC/K was also assessed resulting in this protein being established as a *bona fide* functional kinase with the intrinsic trans-phosphorylation and auto-phosphorylation activities. In line with this, we thus managed to establish the AtPPR-AC/K as a *bona fide* bi-functional plant molecule, having both the AC and kinase activities. More so, this work also, undoubtedly, established that there is a cross-talking scenario between the two catalytic domains of the AtPPR-AC/K - an aspect that partly explains how this putative protein is functionally modulated in higher plants. Finally, this study also managed to establish the AtPPR protein as the sixth ever AC molecule to be identified and experimentally confirmed in higher plants, while at the same time, it becomes the first ever higher plant molecule to possess a dual catalytic function of being both an AC and a kinase.

CHAPTER ONE

General Introduction and Literature Review

1.1 General Introduction

1.1.1 Overview

Plants play key roles in the general life systems on earth in which organisms such as humans, animals and microorganisms becomes dependent on them for food, oxygen, medicines and habitat. Even though they play such crucial roles, throughout their lifecycles, plants tend to experience undergo constant suffering by being exposed to continuous stress stimuli, including pathogen infections, droughts and salinity. In all cases, these adverse environmental conditions always affect the plant's productivity and developmental systems, and therefore, plants have to develop some coping mechanisms against such conditions, mostly through cell signalling and molecular transduction systems (Tuteja, 2007; Ning *et al.*, 2010).

Notably, since environmental fluctuations and climatic changes are likely to continue to occur, then we can also expect some increasing difficulties in the growing of crops in many parts of the world, South Africa included (White *et al.*, 2004; Vinocur and Altman, 2005). In this regard, food security is heavily dependent on the development of crop plants with increased resistance to both biotic and abiotic stress factors like pathogens and droughts, respectively. Thus, the urgent need to use rational approaches to develop crop plants with increased stress tolerance has recently 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 could possibly deliver effective biotechnological solutions (Stuhmer *et al.*, 1989). Since proteins that systemically affect homeostasis in plants are a target candidate group for biotechnology, one such molecule termed the pentatricopeptide

repeat protein (encoded by the At1g62590 gene) from *Arabidopsis thaliana* was hereby extensively studied in this project in order to elucidate its role in plant stress response and adaptation mechanisms. Findings from this study may be used to support efforts to improve crop yields and, consequently, food and nutrition security in South Africa.

1.1.2 Problem Statement

Despite the fact that some previous studies on mutational analysis have convincingly demonstrated a sole involvement of the AtPPR protein in important cellular processes such as RNA processing and the restoration of cytoplasmic male sterility, all of which are strictly dependent on the enzymatic activities of adenylate cyclases and kinases (Bentolila *et al.*, 2002; Desloire *et al.*, 2003), yet none of these two enzymatic activities has ever been fully characterized in this protein molecule (Ruzvidzo *et al.*, 2013), it is rather still very surprising. Furthermore, it is also in spite of the fact that a recent bioinformatic study in the Arabidopsis genome has firmly reported a physical co-existence of the adenylate cyclase and kinase domains within the structural architecture of AtPPR (Gehring, 2010). This study was, therefore, set to determine the possible dual catalytic function of this putative protein as well as to further ascertain and elucidate a potential cross-talking scenario between these two co-existing catalytic activities, particularly with respect to their possible involvement in plant stress response and adaptation mechanisms.

1.1.3 Research Aim

The major research question of this work was to find out if the putative AtPPR protein from *Arabidopsis thaliana* does possess any possible dual catalytic function as a result of its possession of the adenylate cyclase and kinase domains within its architectural structure. If so, whether such a function has any form of a cross-talking scenario between the two inherent

activities, and particularly with respect to the key plant cellular processes of stress response and adaptation mechanisms.

1.1.4 Research Objectives

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

1. To isolate and clone the annotated Arabidopsis PPR gene fragment harbouring the adenylate cyclase and kinase catalytic domains as a dual gene fragment (AtPPR-AC/K) into a stable and viable heterologous prokaryotic expression system.
2. To optimize the expression strategies of this twin-domain gene fragment into its respective twin domain recombinant protein (AtPPR-AC/K).
3. To optimize the affinity purification regimes of this AtPPR-AC/K recombinant protein.
4. To determine the inherent adenylate cyclase activity of this twin-domain recombinant AtPPR-AC/K protein.
5. To determine the inherent kinase activity of this twin-domain recombinant AtPPR-AC/K protein.
6. To further characterize the two inherent catalytic activities of this recombinant AtPPR-AC/K protein, particularly with respect to their possible cross-talking scenario and probable involvement in stress response and adaptation mechanisms.

1.1.5 Significance of the Research Project

This study is significant in that a complete functional characterization of the AtPPR-AC/K (At1g62590) gene would clearly elaborate on the interactive aspect by which adenylate cyclase and kinase enzymes collectively function in plant systems to facilitate responses and adaptation mechanisms to stress. In addition, this study would advance our scientific knowledge on adenylate cyclases and kinases in higher plants, enlighten our current understanding of the

trends through which environmental stress affects plants as well as assisting in the possible integrated management of both biotic and abiotic stress conditions of agronomically important crops in South Africa. Potentially, a possible horizontal transfer of the AtPPR-AC/K into South African crop cultivars through genetic engineering would enhance crop yields and ultimately, improve food security both in the country and the sub-Saharan region.

1.2 Literature Review

1.2.1 Cellular Signalling and Second Messengers

Intracellular signalling molecules play key roles as intermediates in many physiological and biochemical responses of both prokaryotes and eukaryotes. Those signalling molecules are termed "transduction molecules" or "second messengers" include Ca^{2+}, lipid-based compounds, kinases and cyclic nucleotides such as 3′,5′-cyclic adenosine monophosphate (cAMP) and 3′,5′-cyclic guanosine monophosphate (cGMP) (Martinez-Atienza *et al.*, 2007). Naturally, adenylate cyclases (ACs) are enzymes capable of converting the adenosine 5′-triphosphate (ATP) molecule into cAMP and pyrophosphate (PPi) (Cassel and Selinger, 1976; Codina *et al.*, 1983; Gilman, 1987). In animals and lower eukaryotes, cAMP has firmly been established as an important signalling molecule and acting as a second messenger in several cellular signal transduction pathways (Donaldson *et al.*, 2004). Most of the information on second messengers have been well elucidated from animal studies since the first second messenger was discovered in liver tissue years ago (Rall *et al.*, 1957; Sutherland and Rall, 1958).

Nonetheless, not much is known in plants about ACs and their enzymatic product cAMP as is in animals, prokaryotes and lower eukaryotes (Gehring, 2010.). Currently, a few ACs in higher plants have been experimentally confirmed to fill up the gap on ACs in the plant kingdom.

Such molecules include the *Zea mays* pollen signalling protein responsible for the polarized pollen tube growth (Moutinho *et al.*, 2001). The disease resistance protein with an *in vitro* Mn^{2+}-dependent AC activity and whose gene expression analysis supports a role in plant defense (Hussein, MSc thesis, KAUST 2012). The *Arabidopsis thaliana* pentatricopeptide repeat protein responsible for pathogen responses and gene expressions (Ruzvidzo *et al.*, 2013). The *Nicotiana benthamiana* adenylyl cyclase protein responsible for the tabtoxinine-β-lactam-induced cell deaths during wildfire diseases (Ito *et al.*, 2014). Furthermore, the *Hippeastrum hybridum* adenylyl cyclase protein involved in stress signalling (Swiezawska *et al.*, 2014). Lastly, the *Arabidopsis thaliana* K^+-uptake permease 7 (AtKUP7) capable of fermenting lactose in an AC-deficient mutant *E. coli cya*A host and its recombinant AtKUP7 generating cAMP *in vitro* (Al-Younis *et al.*, 2015).

Most recent studies have since shown the capability of ACs to generate cAMP from ATP (Moutinho *et al.*, 2001; Ruzvidzo *et al.*, 2013). Various AC activities have also been demonstrated in several plant species such as alfalfa, tobacco and *Arabidopsis thaliana* (Carricarte *et al.*, 1988; Witters *et al.*, 2004; Ruzvidzo *et al.*, 2013). This second messenger signalling molecule (cAMP) has also been reported to be involved in stress response (Choi and Xu, 2010; Thomas *et al.*, 2013), primarily through the cyclic nucleotide-gated channels (CNGCs) (Zelman *et al.*, 2012). Recent studies in which genetic and/or molecular signalling of enzymes involved in the synthesis of ACs or their product cAMP have also further helped in elucidating their role in higher plants .

By the mid-1970s, the molecule 3′,5′-cyclic adenosine monophosphate (cAMP) had been firmly established as an important signalling chemical and a second messenger in both animals and lower eukaryotes (Robison *et al.*, 1968; Goodman *et al.*, 1970; Gerisch *et al.*, 1975;

Wiegant, 1978). It was also understood that ACs are the enzymes responsible for the generation of this cAMP from ATP hydrolysis, and that the generated cAMP can affect many different physiological and biochemical processes including the activity of kinases (Robison *et al.*, 1968). Given such a growing realization of the importance of ACs and cAMP, it was not surprising that plant scientists were also keen to learn if such a signalling system was universal and, therefore, operational in plants too. The major reasons why AC and/or cAMP information was not readily available in plants as in animals and lower eukaryotes were, firstly, that the levels of cAMP detected in plants seemed to be very low (<20 pmol/g fresh weight) (Ashton and Polya, 1978) compared to those found in animals (>250 pmol/g wet weight) (Butcher *et al.*, 1968) and, secondly, that the vagaries of assays conducted in plants were not conducive to reach firm conclusions (Amrhein, 1977).

These lower levels of cAMP were speculated to be due to the higher activity of phosphodiesterase (Assmann, 1995) and probably bacterial contaminants (Ashton and Polya, 1978). However, the fact that signalling in plants at lower molecular levels is feasible is not an uncommon scenario because, incidentally, low levels of yet another cyclic nucleotide, cGMP (<0.4 pmol/g fresh weight) (Meier *et al.*, 2009), were already being reported in plants where the molecule plays a physiological role in specific responses to avirulent pathogens and defense mechanisms. In addition, the availability of highly modernized and most advanced analytical tools has also dramatically improved the assaying systems in plants and thus, the eventual affirmation of solid conclusions.

Apparently, given the basis that cAMP plays an important role in signalling in higher plants, it is not surprising that many research groups have put considerable efforts into the search for ACs in plants and particularly, in *Arabidopsis thaliana*. Incidentally, the first ever AC

molecule to be identified in higher plants is the *Zea mays* pollen signalling protein (PsiP) responsible for the polarized growth of pollen tubes (Moutinho *et al.*, 2001). Its Arabidopsis orthologue (At3g14460) is annotated as a disease resistance protein belonging to the nucleotide-binding site-leucine-rich repeat (NBS-LRR) family used for pathogen sensing and with a role in defense responses and apoptosis (DeYoung and Innes, 2006). The NBS-LRR proteins directly bind pathogen proteins and associate with either a modified host protein or a pathogen protein leading to conformational changes in the amino-terminal and LRR domains of the NBS-LRR proteins which are thought to promote the exchange of ADP for ATP by the NBS domain. It is thus conceivable that NBS-LRR downstream signalling (DeYoung and Innes, 2006), and possibly the AtPPR signalling, may be enabled by cAMP.

1.2.2 Cyclic Nucleotides as Second Messengers

The cyclic nucleotides monophosphates (cNMP), adenosine 3',5'-cyclic monophosphate (cAMP) and guanosine 3',5'-cyclic monophosphate (cGMP) are well-known cyclic catalytic products derived from the hydrolytic activities of the enzymes adenylyl cyclases (ACs) and guanylate cyclases (GCs), respectively. These cyclic nucleotides play significant roles in stimuli response, cellular signalling, growth and developmental processes in all kingdoms of life (Goodman *et al.*, 1970; Gerisch *et al.*, 1975; Wiegant, 1978), and they also act as second messengers. It has also been noted that the cNMPs do not always have the same functions in different organisms (Gancedo *et al.*, 1985).

1.2.3 Adenylyl Cyclase Classes

The adenylyl cyclase group is made up of six different classes, which are extensively distributed across all kingdoms of life and all of them having a common sequence motif within their catalytic site regions that appear to be unrelated phylogenetically, but producing cAMP

due to convergent evolution (Danchin, 1993; Linder and Schultz, 2008). Classes I, II and IV are found in bacteria where they play different roles such as the catabolite repression of sugars in *Escherichia coli* (Botsford, 1981) and the extracellular secretion of toxins from pathogenic bacteria such as *Bacillus anthracis* and *Bordetella pertussis*. Ideally, during their virulence infection, the class II ACs translocate a highly toxic AC to disrupt the intracellular signalling system by flooding host cells with relatively high amounts of cAMP (Petersen and Young, 2002).

Most interesting are the class III adenylyl cyclases, which have been comprehensively studied and are known to be closely related to guanylyl cyclase phylogenetically, also found in prokaryotes and eukaryotes. All known eukaryotic adenylyl cyclases, including the soluble adenylyl cyclases (sACs) and transmembrane adenylyl cyclases (tmACs) from animals, belong to this class. Most bacterial ACs belonging to this class are involved in processes such as osmoregulation, chemotaxis, phototaxis or pH regulation (Linder and Schultz, 2003). Up to date, class IV ACs in bacteria such as *Aeromonas hydrophila* (Sismeiro *et al.*, 1998) and/or *Yersinia pestis* (Gallagher *et al.*, 2006) have not yet been assigned a clear functional role. As for the last two classes (V and VI), there is very limited data on them and are currently represented by single members each from an anaerobic bacteria *Prevotella ruminicola* (Cotta *et al.*, 1998) and a *cya*C isoenzyme from *Rhizobium etli* (Tellez-Sosa *et al.*, 2002), respectively.

1.2.4 Forms of Adenylyl Cyclases

In all cellular systems, adenylyl cyclases are represented by two forms/families; the transmembrane (tmAC) and soluble (sAC) (Kamenetsky *et al.*, 2006), both of which are as well present in plants (Lomovatskaya *et al.*, 2008). In mammals as is in other cellular systems, cAMP is derived from these two forms of ACs, which share features such as the conserved overall architectures and catalytic mechanisms, but then differing in their sub-cellular localizations and responses to various regulators (Kamenetsky *et al.*, 2006). This is better illustrated in the schematic diagram depicted in Figure 1.1 below showing how cAMP is activated by these two different forms of ACs.

1.2.4.1 Soluble Adenylyl Cyclases

It has been shown that the cellular localization of soluble ACs (sAC) is not only limited to soluble proteins as they are preferentially found in the cytosolic fraction, but are also specifically targeted to other well-defined intracellular compartments (Zippin *et al.*, 2003). Their (partial) activity has clearly been established in the cytosolic fraction as well as in some cellular membranes. Most biochemical and immunolocalization studies have actually shown that most sACs are found within the cell cytoplasm and specific organelles such as mitochondrion, chloroplast, and nucleus (Zippin *et al.*, 2003). Soluble ACs have also been noted to be directly regulated by intracellular signalling molecules such as the bicarbonate ion (HCO_3^-) which turns the (sACs) into physiological acid/base (A/B) sensors (Chen *et al.*, 2000), and also being activated by calcium ion (Jaiswal and Conti 2003; Litvin *et al.*, 2003). The sACs are also known to be insensitive to the heterotrimeric G protein and hormone regulation (Braun *et al.*, 1977). Soluble ACs have also been shown to be stimulated by manganese (Mn^{2+}) and resulting in an activity increase that is only detectable in the presence of ATP as a sole substrate (Braun and Dods, 1975).

1.2.4.2 Transmembrane Adenylyl Cyclases

Transmembrane adenylyl cyclases (tmACs) are located on the plasma membrane; studies have shown their possible role in controlling the virulence factor (cellulases and pectinases) activity in *Pseudomonas syringae* (Jimenez *et al.*, 2012) and *Rhizobium leguminosarum* (Robledo *et al.*, 2011). It has also been found that the tmACs are directly regulated by the heterotrimeric G proteins, which transduce physiological signals via the G protein coupled receptors (GPCRs). These ACs respond to hormones and neurotransmitters such as forskolin, which was found to play a role in the activation of all tmACs in mammals (Taussig and Gilman, 1995).

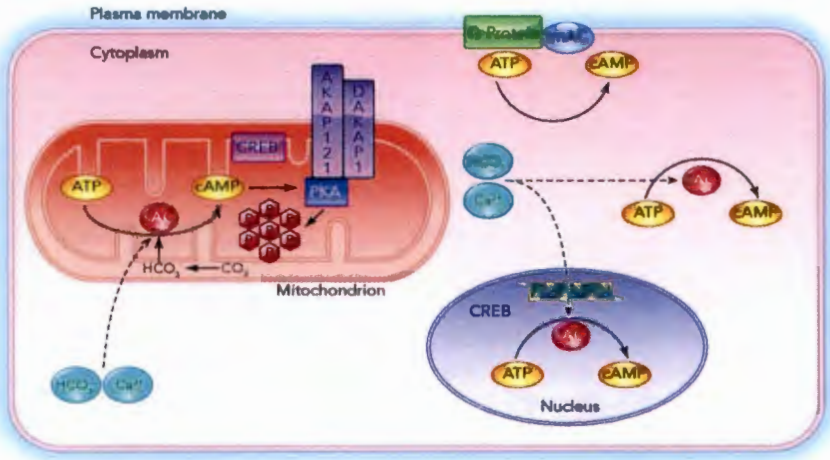

Figure 1.1: Cyclic AMP generation from ATP by soluble ACs (sAC) activated by the HCO_3^- and Ca^{2+} ions and by transmembrane ACs (tmACs) activated by hormones and neurotransmitters (Adapted from Valsecchi *et al.*, 2013).

1.2.5 Is cAMP Really Necessary for the Functioning of Organisms?

Cyclic AMP has shown a number of significant ways that are necessary for the functioning of different organisms and their life processes; this was supported by responses exhibited by different species when mediated by cAMP against the external environment. For instance, in an Archaeon, this encodes a functional AC within its genome and a *Halobacterium salinarum*, which illustrated some measurable cAMP oscillations (Baumann et al., 2007). Generally, most

organisms have ACs that play significant roles in their various signalling processes such as those in *Thermus thermophilus*, where cAMP activity was measured (Shinkai *et al.*, 2007) and in *Bacillus anthracis*, where an AC acts as a toxin within the host cells (Tang and Guo, 2009). However, there are those species that have no cAMP binding proteins and/or cAMP in their systems. These include the *Mycoplasma pneumoniae* (which has a diminished *genome*) (Yus *et al.*, 2009) and *Bacillus subtilis* (Chauvaux *et al.*, 1998). In some microorganisms such as the *E. coli cya*A strain, the genomic component is deliberately mutated to lack the cAMP system and such organisms still function properly even though some partial defects usually occur during their developmental and growth phases (Brickman *et al.*, 1973).

In higher plants, there is ample evidence that cellular signalling is mediated by cAMP (Gehring, 2010). Their various biological processes are shown to be mediated by cAMP and such include the activation of protein kinases in the rice leaf (Komatsu and Hirano, 1993) and also, the promotion of cell division in tobacco BY-2 cells (Ehsan *et al.*, 1998). In addition, recent studies have experimentally confirmed ACs in higher plants where cAMP acts as a signalling molecule in various transduction pathways. Such plants include the *Zea mays*, the *Arabidopsis thaliana,* the *Nicotiana benthamiana* and the *Hippeastrum hybridum* (Moutinho *et al.*, 2001; Hussein, 2012; Ruzvidzo *et al.*, 2013; Ito *et al.*, 2014; Swiezawska *et al.*, 2014; Al-Younis *et al.*, 2015).

1.2.6 Cellular Responses of Different Systems to Various Signals

Living organisms respond differently to various stimuli that affect them, resulting in cascades of cellular signalling responses. Different factors can affect organisms in a drastic manner which results in an imbalance in their cellular functioning. External factors affect organisms differently, and these factors are perceived by the cell through the plasma membrane and can affect targets as either primary or second messengers. There are various second messengers

that include the Ca^{2+} ion, lipid-based compounds, kinases and cyclic nucleotides; which play key roles in cellular responses (Martinez-Atienza *et al.*, 2007). Many other signalling pathways exist such as the tyrosine kinase (TK)-coupled receptor and G-protein-coupled receptor (GPCR) systems - two systems considered the major pathways of the plasma membrane receptors. It has been reported that these pathways act in a bi-directional cross-talking manner to regulate physiological processes, whereas in some cases, their effects work well together while in others, they work against each other (García-Sáinz *et al.*, 2010).

The TK signals regulate a number of cellular signalling processes such as activation of the Ras and phosphatidylinositide 3-kinase (PI 3-kinase) pathways (van der Geer *et al.*, 1994), cell development and insulin regulation to cancer (Li and Hristova, 2006). The GPCR receptor system influences the activation and regulation of other receptors such as the TK system (Luttrell *et al.*, 1999) and some post-translational modifications such as phosphorylation via the activating/deactivating kinases. Tyrosine plays an important role in the phosphorylation system of plants as compared to other residues such as the serine and threonine. It has been indicated that Tyr-phosphorylation is involved in controlling most of the developmental aspects and adaptation to environmental responses in higher plants. The *AtDsPTP1* from an Arabidopsis cDNA has been shown to play a vital role in the expression of stamens and pollens (Gupta *et al.*, 1998; Schmid *et al.*, 2005).

Signal transduction is a significant process that occurs when an extracellular signalling molecule activates specific receptors within the cell or on its surface to trigger a response, thereby resulting in a modification of the protein structure and having effects on its cellular activity, stability and localization. Protein phosphorylation occurs on serine, tyrosine, histidine and threonine residues, catalyzed by protein kinases (PKs) that transfer the phosphate group

from ATP or GTP to the modified residues (Hanks and Hunter, 1995). This process in animals is highly essential for the regulation of growth and differentiation. Through the use of molecular technologies, it has been noted that the highly remarkable role of tyrosine phosphorylation regulates similar processes in plants as is in animals. In higher plants such as *Arabidopsis thaliana*, most protein kinases (PKs), which are >800 (Arabidopsis Genome Initiative [AGI], 2000) and protein phosphatases (PPs), which are >150, have been identified (Kerk *et al.*, 2008).

1.2.6.1 Cellular Responses Due to Kinases

Among eukaryotes, cellular signalling cascades are also mediated by the action of two main groups of kinases; the receptor-like kinases (RLKs) found in plants and the receptor tyrosine kinases (RTKs) found in animals. Apparently, both the RLKs and RTKs have a sequence homology and similar architectural structures (Walker, 1994), and with such related appearances, both types might use similar mechanisms in performing common biological functions (Zhang, 1998). Even though these proteins do share some similarities, there are some distinct variances between the two families. Firstly, in that all plant RLKs identified so far have the serine/threonine kinase activity (Ulrich and Schlessinger, 1990), and secondly, in that they have evolved independently of the animal RTKs and receptor Serine/Threonine kinases (RSKs) (Johnson and Ingram, 2005). Existing insights indicate that the downstream interacting proteins of the plant RLKs differ from those of animal receptor kinases. In contrast, evolutionary studies have shown a close relationship between the plant RLKs and the *Drosophila melanogaster* Pelle (Belvin and Anderson, 1996) and also with the mammalian interleukin receptor-associated kinases (IRAKs) (Cao *et al.*, 1996; Flannery and Bowie, 2010).

Plant RLK family is a very large group, divided into 45 sub-groups, which differ in their domain organization and the sequence identity present on their extracellular domains (Shiu and Bleecker, 2001a). With such striking differences, there is a strong possibility that they might play a role in perception of a wide range of stimuli exposed to them. Plant systems have three major sub-groups of the RLKs, which are uniquely characterized based on the presence or absence of a receptor and/or kinase domain (Walker, 1994; Braun, 1996; Torii, 2008). Among all of those sub-groups, the leucine-rich repeat (LRR) RLK appears to be the largest one, which contains several tandem repeats of the 24 amino acids with conserved leucine residues in the extracellular domain (Zhang, 1998; Torii, 2008). The second sub-group being the S-domain RLK, which is the first class of RLKs to be fully detailed in plants, with a unique trait of containing a group of ten cysteine residues proximal to the transmembrane region, which is assumed to play an important role in folding of the extracellular domain (Shiu and Bleecker, 2001a). The last sub-group is the lectin receptor kinases RLK, which plays a key role in interactions with oligosaccharides or cell wall fragments (Buchanan *et al.*, 2000).

The diversity of the RLK family proteins was shown by various studies, which implicated them in a diverse range of cellular processes. These processes included the control of plant growth by CLAVATA1 (CLV1) (Clark *et al.*, 1993), the regulation of organ elongation by ERECTA (Torii *et al.*, 1996), cell signalling by the brassinosteroid insensitive 1 (BRI1) (Li and Chory, 1997; Wang *et al.*, 2001) and the control of self-incompatibility by the SCR/SP11 of the S-locus receptor kinase (SRK) from *Brassica spp* (Stein *et al.*, 1996; Kachroo *et al.*, 2001; Takayama *et al.*, 2001). Other RLKs play essential roles in microbe interactions and pathogen defense systems such as the one exhibited by rice (*Oryza sativa*) Xa21 (Song *et al.*, 1995) and Arabidopsis FLS2 in flagellin perception (Gomez-Gomez and Boller, 2000).

Signal transduction pathways and kinase activation are generally triggered by adhesion of the ligand/external stimuli to the plasma membrane that will then result in a heterodimeric receptor complex (Tichtinsky *et al.,* 2003; Torii, 2000). Thus the RLK dimerized receptor complex effects auto- or trans-phosphorylation in order to activate the complex (Trotochaud *et al.*, 1999; Trotochaud *et al.*, 2000). Such signal transduction pathways and kinase activation processes were evidenced by a kinase-associated protein phosphatase (KAPP), which had shown a unique trait through its *in vitro* interaction with only the phosphorylated RLK5 (*Arabidopsis* RLK), since the RLK5 is a serine/threonine. Therefore, an interaction between the kinase interaction (KI) domain and the RLK5 depends on specific phosphorylated residues (phosphoserine and/or phosphothreonine), and in order for the auto-phosphorylation process to occur and also for the phosphorylated kinases to carry out their normal activated signalling complexing (Horn and Walker, 1994; Stone *et al.,* 1994). Hence, this phosphorylation-dependent binding manner suggests that extracellular signals can be transduced and decoded in the cell, then resulting in the production of an intracellular response against a particular signal.

Generally, the active modulations of intracellular transducing proteins can be modified by an activation or inhibition of the effector proteins through a phosphorylation/de-phosphorylation process on specific amino acid residues; serine, threonine, histidine or tyrosine. Protein modifications occur through specialised processes that include phosphorylation and de-phosphorylation, whereby for phosphorylation, the activated kinases will transfer a γ phosphate moiety, normally from ATP, to a hydroxyl group of another protein substrate, and for de-phosphorylation, a phosphatase enzyme catalyzes the removal of phosphate moieties from proteins through hydrolysis. In addition, such modifications may result in either the activation or inhibition of the protein/enzyme activity (Schenk and Snaar-Jagalska, 1999). Protein kinases are very specific to the classes onto which they phosphorylate and such a specificity is

based on particular amino acid residues, where for example, if a kinase only phosphorylates specific serine and threonine residues within a protein, then that particular kinase is classified as a serine/threonine kinase, while when it phosphorylates the tyrosine residue, then it is termed a tyrosine kinase. In some instances, it can be found that a certain class of protein kinases can utilize both the serine/threonine and tyrosine residues and exhibit a dual specificity kinase activity for both types of amino acid residues. There are also some rare classes of kinases, found in plants, which possess a histidine phosphotransferase activity and these are known as histidine kinases (Nongpiur *et al.*, 2012). Other histidine kinases were found to exist in bacteria (Xie *et al.*, 2010; Ferris *et al.*, 2012).

The posttranslational regulatory modification of most proteins involves essential processes like phosphorylation/de-phosphorylation, of which for most proteins, this has shown their roles in performing various regulatory signalling mechanisms in response to external stimuli, and such processes including, subcellular localization, protein-protein interactions and a rapid turnover of the proteins involved. In general, the regulatory modification process achieves several outcomes such as decreasing or increasing the biological activity of the substrate protein; stabilising it or destroying its functional activities; or facilitating the dissociation of protein-protein complexes (Cohen, 2002). Research studies have also shown that the RLKs and proteins involved in transport systems are a greater target for regulatory modification, especially phosphorylation. Evidence was revealed by an *in vivo* study which showed various phosphorylation sites on Arabidopsis proteins extracted from the nuclear and cytosolic regions (van Bentem *et al.*, 2006), and thus really emphasising the prominent function of phosphorylation as a regulatory mechanism in eukaryotes, especially plants.

Phosphorylation has also been shown to have an essential role in the regulation of most cellular and stress related responses that are linked to cAMP-dependent responses. In a study on an *Arabidopsis thaliana* AKT2 protein, potassium (K^+) voltage-gated channels have been shown to be expressed in the mesophyll cells and phloem tissues (Lacombe *et al.*, 2000; Pilot *et al.*, 2003) and such similar responses also being regulated by the cAMP-dependent protein kinase (PKA). Furthermore, calcium channels have shown to be regulated by protein phosphorylation. Studies performed on *Arabidopsis* and *Vicia faba* guard cells have demonstrated that the release of intracellular Ca^{2+} for gating by abscisic acid and nitric oxide, requires protein phosphorylation, in order for subsequent cell signalling pathways to occur (Köhler and Blatt, 2002; Sokolovski *et al.*, 2005). In addition, cyclic nucleotides (cAMP or cGMP) have shown significant roles in plant signalling systems that might have a direct influence on cellular systems either via the cyclic nucleotide-gated ion channels or indirectly, through protein kinases. In particular and among higher plants, cAMP has essential functions that are critical for cellular responses and signal transduction pathways, and also for regulatory mechanisms. The main focus of this work was to study a novel molecule suspected to harbour the adenylate cyclase and kinase domains in the form of a pentatricopeptide repeat protein (AtPPRAC/K) from *Arabidopsis thaliana.*

1.2.7 Identification of the Pentatricopeptide Repeat (PPR) Protein

Of particular interest to this work was the pentatricopeptide protein whose gene (At1g62590) has been bioinformatically identified by Gehring (2010) from the Arabidopsis genome using a search motif consisting of functionally assigned amino acids in the catalytic centre of annotated and/or experimentally tested nucleotide cyclases (Table 1.1).

Table 1.1: 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 genes, followed by the nucleotide sequences suspected to be their adenylate cyclase catalytic sites, and the names to which each gene was bioinformatically inferred (annotations).

*The gene for the PPR protein that was functionally characterized in this research.

The identification of adenylate cyclase candidates in higher plants was done through a search query of the Arabidopsis genome using a 14-mer motif derived from the other annotated and/or experimentally confirmed adenylate cyclases from various lower and higher eukaryotic species. Previously, the identification of the first plant guanylate cyclases (other closely-related nucleotide cyclases to adenylate cyclases) was accomplished through a blast search of the Arabidopsis genome with a 14 conserved amino acid motif from functionally assigned amino acids of the annotated catalytic centre of eukaryote GCs (Figure 1.2A) (Ludidi and Gehring, 2003). Therefore, the first AC candidate was identified through a modification of this GC search motif whereby specific variations were made on amino acid residues that show substrate specificity for GTP binding to suit the AC motif for ATP binding and as is indicated in position 3 of Figure 1.2A & B below (Gehring, 2010). In that case, nine AC candidates were then identified of which the At1g62590 gene annotated as a pentatricopeptide protein was one among them (Table 1.1) (Gehring, 2010).

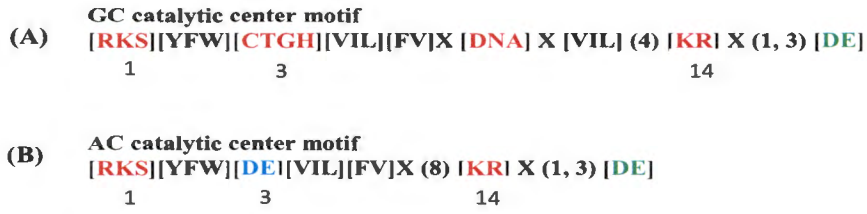

Figure 1.2: Structural features of the nucleotide cyclases catalytic motifs and identification of the first plant GC and AC candidates. (A) The guanylate cyclase (GC) and **(B)** adenylate cyclase experimentally confirmed catalytic centre motif present in plants. At position 1 (red), hydrogen bonds with the guanine of GTP in GCs while in ACs, it bonds with the adenosine residue of ATP. The amino acid in position 3 is responsible for substrate specificity where [CTGH] red residues from a GC motif has been modified to [DE] blue residues in an AC motif which is specific for ATP binding. In addition (**A and B**), the (red) residues in position 14 stabilises the transition (GTP/cGMP) or (ATP/cAMP), while the last residue in both GC and AC motifs (green), represents the metal cofactor (Mg^{2+}/Mn^{2+}) binding site on the C-terminal (Adapted from Gehring, 2010).

This identified pentatricopeptide protein has been bioinformatically shown to contain two domains; the adenylate cyclase and kinase domains (Gehring, 2010). The PPR family was first identified by two different research groups through a nucleotide sequencing of the *Arabidopsis thaliana* genome, in which a portion of its whole genome (1%) was occupied by genes of this orphan family, a finding that was found to be very unique to plants due to the absence of such similar sequences outside the plant kingdom (Aubourg *et al.*, 2000; Small and Peeters, 2000). When compared to ACs, kinases are enzymes capable of phosphorylating other protein candidates (including themselves) through the various modifications on serine, threonine, histidine or tyrosine residues (Hanks *et al.*, 1988), and these enzymes play an important role in plant cell signalling to respond to external environmental stimuli such as light, temperature, pathogen invasion, growth regulation factors and nutrition deprivation, all of which are essentially mediated by cAMP.

1.2.8 Structure of Pentatricopeptide Repeat (PPR) Proteins

Pentatricopeptide proteins have been members of the α-solenoid superfamily of helical repeat proteins (Figure 1.3) (Hammani *et al.*, 2014). This superfamily of protein is typically proposed

to contain a pentatricopeptide repeat (PPR) motif, which is known to be a 35 (pentatrico) degenerate amino acid system often arranged in tandem arrays of 2-27 repeats per peptide (Small and Peeters, 2000). The PPR family is divided into two sub-families, the P and PLS, with members of the P sub-family abundantly distributed among eukaryotes while the PLS subfamily are strictly restricted to plants (Lurin *et al.*, 2004). These two sub-families have their unique structural conformations (motifs) which relate them to how they function. The P sub-family contains only the canonical P motif which play roles in a range of RNA organelle processing activities (Barkan and Small, 2014). While, the PLS sub-family is noted to contain the PLS triplets (L, long variants of P; S, short variants of P) arranged in an array pattern with additional C-terminal domains, E/ E+ and DYW domains (Figure 1.3) (Lurin *et al.*, 2004; Shikanai and Fujii, 2013).

The PPR proteins have since been shown to closely resemble tetratricopeptides (TPRs) in structure, where they instead consist of 34 degenerate amino acid systems (Blatch and Lassle, 1999). These PPR and TPR motifs can be easily distinguished since the PPR are mostly abundant in eukaryotes and specifically in flowering plants such as *Arabidopsis thaliana* (about 441 genes) and rice (more than 655 genes) (Lurin *et al.*, 2004), while the TPRs are generally found in both prokaryotes and other eukaryotes such as yeast (*Sacharomyces cerevisiae)* and Drosophila (*Drosophila melanogaster*) (Desloire *et al.*, 2003).

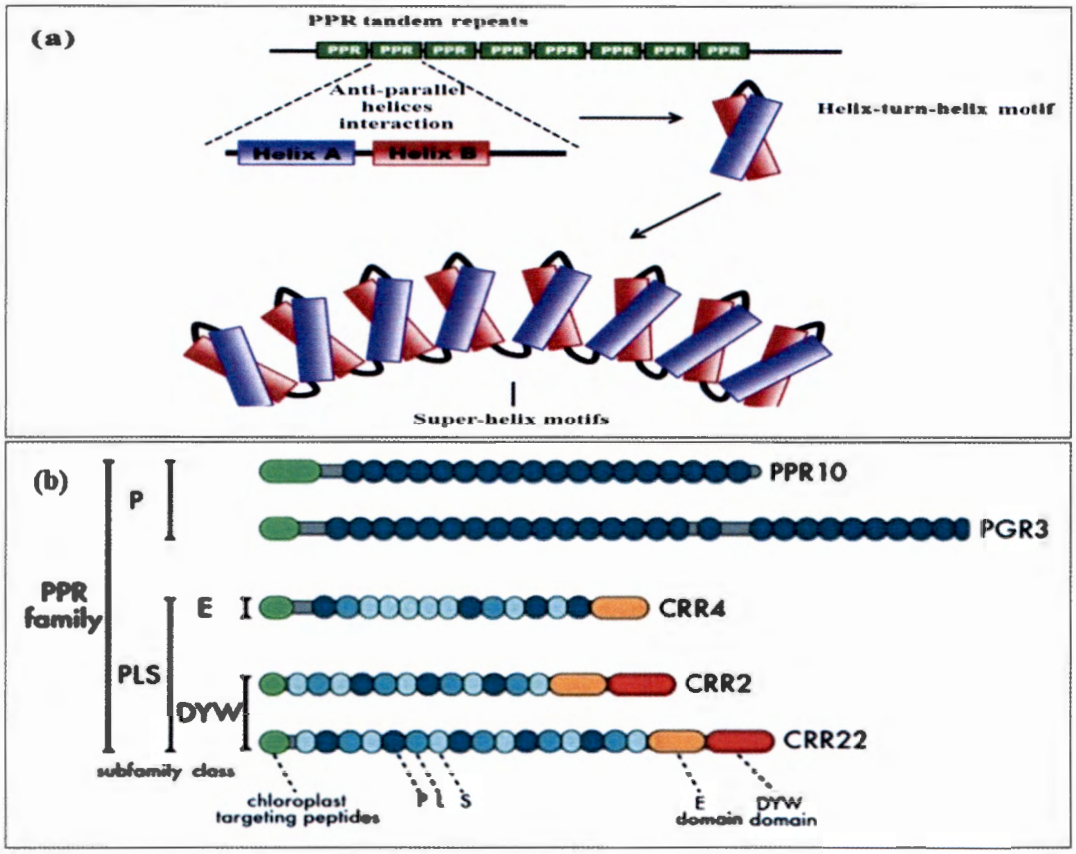

Figure 1.3: Structural features of the pentatricopeptide repeat protein. **(a)** PPR protein formed as a result of a tandem repeat array of PPR motifs which form two α -helices that interact together to form an alpha helix-turn-helix motif. A chain of such motifs organized together result in a super-helix structure (Hammani *et al.*, 2014). **(b)** The structural representation of pentatricopeptide repeat (PPR) protein domain organisation. The PPR protein family with its sub-families P & PLS (L, long variants of P; S, short variants of P) motifs and their sub-classes E/E+ and DYW domains. The PLS sub-family is arranged in tandem array repeats of PLS (P-dark blue, L-light blue, S-light green) with the E/E+ and DYW domains attached to the C-terminus while the P sub-family motifs are attached at the beginning of the N-terminus (Adapted from Shikanai and Fujii, 2013).

1.2.9 Organelle Localization and Functions of the PPR Proteins

Specifically, PPR proteins have been shown to be mostly organelle-localized; for instance, 80% of the Arabidopsis PPRs were predicted to target either the chloroplast or mitochondrion (Lurin *et al.*, 2004). As they target these organelles, they play a role in post-transcriptional processes such as RNA processing, RNA splicing, RNA stability, RNA editing and RNA translation (Delannoy *et al.*, 2007; Schmitz-Linneweber and Small, 2008; Pfalz *et al.*, 2009). While the TPRs interact mainly with other proteins, PPRs interact specifically with either the DNA-

binding and/or RNA-binding proteins (Ikeda and Gray, 1999; Lahmy *et al.*, 2000). When interacting with RNA, the PPRs facilitate several RNA processing roles such as RNA editing (Kotera *et al.*, 2005), transcript processing (Nakamura *et al.*, 2004) and translation initiation (Schmitz-Linneweber *et al.*, 2005). Actually, it has been predicted that the PPR proteins bind to RNA due to their structural morphology (concave surface) which facilitates the binding of extended hydrophilic and acidic ligands (Small and Peeters, 2000; Lurin *et al.*, 2004). This RNA-binding aspect of these proteins has been experimentally demonstrated both *in vitro* and *in vivo* using the RIP chip approach (Schmitz-Linneweber *et al.*, 2005).

Furthermore, a maize protein CRP1 has been shown to possess 13 PPR motifs, which localize mainly in the chloroplast stroma and facilitate the processing and translation of the *petD* and *petB* mRNAs (Fisk *et al.*, 1999). Additionally, a P67 protein from Arabidopsis and radish with two PPR motifs also reveals a similar functioning as is the one above (Lahmy *et al.*, 2000). Again, another maize protein PPR2 localized in the chloroplast stroma also functions in chloroplast biogenesis by regulating translation (Williams and Barkan, 2003). Most mutations in proteins are as a consequence of organelle defects in either the mitochondria or chloroplast that will ultimately affect protein functioning (Fisk *et al.*, 1999). The absence of PPR2 revealed that its mutant (*ppr2*) will prevent the accumulation of ribosomes in plastids and the assembling of translational apparatus, and thus resulting in non-functional chloroplast (Williams and Barkan, 2003). A rice protein OsPPR1 was shown to play a role in early biogenesis of plastids and was also found to target the chloroplast (Gothandam *et al.*, 2005). Two proteins (CCR4 and CCR2) have been shown to be similar in structure since they are both localized in the chloroplast of Arabidopsis. They perform different roles with the CCR4 involved in RNA editing of the *ndhD* gene (Shikanai, 2006), while the latter is responsible for RNA cleaving/splicing between the *rps7* and *ndhB* genes (Hashimoto *et al.*, 2003).

Pentatricopeptide genes have been noted to be unique among all other eukaryotes since they are composed of introns (Lurin *et al.*, 2004), and this will be important for determining their level and pattern of gene expression. Even though these PPR genes are very short, they encode introns which results in very large proteins of more than 650 amino acids (Lurin *et al.*, 2004), and thus an absence of these introns will affect the levels and patterns of expression. RNA editing serves as an essential process in plant organelles by regulating the expression of genes. Pentatricopeptides has also been noted to influence the process of RNA editing, where a cysteine (C) will be replaced by a uracil (U) in plant mitochondria (Covello and Gray, 1989; Gualberto *et al.*, 1989; Hiesel *et al.*, 1989). For instance, an Arabidopsis mutant *crr4 (chlororespiratory reduction)* has been found to regulate the efficiency of *ndhD* translation, yet it was specifically defective in the RNA editing of *ndhD-1* because this process is developmentally-regulated (Hirose and Sugiura, 1997).

Another group of plant specific PPR genes are the restorer of fertility (Rf) genes which are mainly targeted to the mitochondria. These genes are responsible for the restoration of cytoplasmic male sterility (CMS) which is maternally inherited and results in the inability of a plant to produce functional pollens (Pring *et al.*, 1995). These genes act to suppress the male sterility linked with CMS, a function related to the expression of mitochondrially-encoded sterility-associated genes. Restorer of fertility genes have been identified in petunia (Bentolila *et al.*, 2002), rice (Komori *et al.*, 2004; Fujimura, 2004), maize (Cui *et al.*, 1996) and radish (Koizuka *et al.*, 2003), and they belong to the P sub-family of PPR genes and all code for the PPR proteins (Brown *et al.*, 2003), except for the maize gene (Rf2), which encodes an aldehyde dehydrogenase protein (Iwabuchi *et al.*, 1993). The petunia Rf, the radish Rfk1 (Rfo), and the rice Rf-1 genes encode proteins consisting of 14, 16, and 18 tandem PPR repeats respectively. All fertility restorer genes encoding PPR-containing proteins reported so far have been found

to modify the expression of CMS-associated genes (Kadowaki *et al.*, 1990; Iwabuchi *et al.*, 1993; Koizuka *et al.*, 2003; Akagi *et al.*, 2004; Kotera *et al.*, 2005;). Unlike the PPR restorer, the maize gene Rf2 does not affect a build-up of the CMS-associated protein URF13 (Dewey *et al.*, 1987) but instead, it compensates for the metabolic scarcity of this protein (Liu *et al.*, 2002).

Pentatricopeptide genes have also been found to play key roles in plant embryogenesis (Cushing *et al.*, 2005; Ding *et al.*, 2006) and development (Oguchi *et al.*, 2004; Prasad *et al.*, 2005). Since PPR proteins are sequence specific, they bind to RNA and act as *trans*-acting factors, thus recruiting general factors to facilitate organellar gene expression by processing and stabilizing mRNA (Barkan *et al.*, 1994; Fisk *et al.*, 1999) and finally, translation (Schmitz-Linneweber *et al.*, 2005). Mutational evidence revealed that a gene containing PPR domain has a trans-acting factor (Kotera *et al.*, 2005), and this factor play an essential role in RNA editing (Lurin *et al.*, 2004). A trans-acting factor was initially identified in tobacco plastids using an *in vivo* approach (Chaudhuri *et al.*, 1995), where it was noted to decrease the editing efficiency of the *psbL* gene (Hirose and Sugiura, 2001).

Another PPR protein, CRR4 has been found to play a role in RNA editing of the *ndhD* gene in chloroplasts of Arabidopsis. The CCR4 protein has been identified to be a *trans*-acting factor since it interacts with a signature sequence which is nearby the ndhD-1 editing site and facilitates the recruitment of an editing enzyme such as the cytidine deaminase (C-deaminase) via a C-terminal E+ domain (Okuda *et al.*, 2006; Shikanai, 2006). Pentatricopeptide proteins also play major roles in gene expression, which is mainly organelle-based, either in mitochondrion or chloroplast (Taanman, 1999). They regulate mitochondrial RNA metabolism in fungi (Coffin *et al.*, 1997), yeast (Manthey and McEwen, 1995; Manthey *et al.*, 1998) and

humans (Hou *et al.*, 1994; Liu and McKeehan, 2002; Mili and Pinol-Roma, 2003) but except in plants where little evidence has revealed that it might be targeted to the mitochondrion. This is also despite the fact that these proteins have a high presence in plants when compared to the other organisms (Lurin *et al.*, 2004; Andres *et al.*, 2007).

To this day, there is no experimental or biochemical evidence for the existence of a multi-domain multifunctional protein with a dual catalytic function composed of the kinase and AC activities and the involvement of such domains in specific regulatory networks in plants as compared to the combined activities of a kinase and a GC that have since been experimentally demonstrated in *Arabidopsis thaliana* (Kwezi *et al.*, 2007; Meier *et al.*, 2010; Kwezi *et al.*, 2011). Therefore, given the unique twin-domain structural architecture of the AtPPR protein, harbouring both the kinase and AC domains (AtPPR-AC/K), this study describes the functional characterization of this putative protein so as to establish if both of its domains are catalytically functional. If so, to further establish whether there is any cross-talking scenario between the two domains and in relation to important cellular processes like growth, development and response to the various environmental stress factors.

CHAPTER TWO

Molecular Cloning, Partial Expression and Determination of the Endogenous Adenylate Cyclase Activity of the Recombinant AtPPR-AC/K Protein

Abstract

Bioinformatic studies have discovered the existence of two special groups of enzymes termed guanylate cyclases (GCs) and adenylate cyclases (ACs), both involved in plant signal transduction systems. GCs generate the second messenger 3′,5′-cyclic guanosine monophosphate (cGMP) from guanosine 5′-triphosphate (GTP) while ACs generate the second messenger 3′,5′-cyclic adenosine monophosphate (cAMP) from adenosine 5′-triphosphate (ATP). In the meantime, the existence and functions of GCs and cGMP have been widely studied and well-documented in plants, not so much is currently known about ACs and cAMP in higher plants. Recently, a study based on a rational search strategy of the Arabidopsis genome using a search motif based on conserved and functionally assigned amino acid residues in the catalytic centre of annotated and/or experimentally tested GCs has identified nine putative AC molecules in the *Arabidopsis thaliana,* among which a pentatricopeptide repeat protein (AtPPR), coded for by the At1g62590 gene, was present. This protein is in context, novel, as it harbours two putative catalytic domains, annotated as the AC and kinase domains (a twin-domain protein). Apparently and even though the AtPPR protein has previously been implicated in organellar gene expression processes that may be mediated by cAMP- and/or kinase-dependent signalling pathways, none of its domains has ever been shown to possess any functional activity. Therefore in order to investigate the possible functional activity of the AtPPR protein, its AC-kinase containing fragment domain (AtPPR-AC/K) was in this study, cloned and recombinantly expressed in chemically competent *E. cloni* EXPRESS BL21 (DE3)

pLysS cells. The AtPPR-AC/K activity was then practically demonstrated, by enzyme immunoassay, to have an ability to induce the endogenous generation of cAMP in these host prokaryotic expression systems.

2.1 Introduction

There is really a necessity for transcriptomic and proteomic approaches to be used to study the relationships between structures and functions of genes/proteins of living systems especially plants. These approaches will also further assist, more specifically, on how such an understanding would play a role in molecular modifications of crops for the establishment of sustainable agriculture practices. In addition, an application of such approaches would also play a significant role in the development of effective therapeutic drugs, biomaterials and genetic manipulations for the biotechnology/biochemistry industries (Wang *et al.*, 2016). Recombinant DNA technology has become handy in trying to solve these challenges, where large volumes of proteins are needed for biochemical characterization, industrial production and commercialization. As a result, researchers had to come up with ways to produce/express billion copies of recombinant proteins and their purification without requiring tons of biological fluids, animal and/or plant tissues (Rosano and Ceccarelli, 2014). Ideally, there are a few points that one needs to look at when attempting to recover a highly expressed protein; (i) a suitable heterologous expression host system that needs to be selected carefully, (ii) an expression plasmid to use, and (iii) a suitable host cell for such massive protein expressions.

Essentially, there are several host expression cell systems used amongst microorganisms such as the bacteria, yeast, filamentous fungi, and the unicellular algae - their choice being mainly based on the desired protein targeted (Demain and Vaishnav, 2009; Adrio and Demain, 2010). In this study, we chose *E. coli* as our heterologous expression host system for expression of the

adenylate cyclase and kinase domains of a pentatricopeptide protein from *Arabidopsis thaliana* (AtPPR-AC/K) - the reason being that it is simple, with a quick growth rate and a relatively low-cost usage. This system is also very compatible with most expression vector forms even though its most associated limitation in this case, is the inability to perform post-translational modifications, which are essentially required for the functional activities of most recombinant proteins under consideration (Yin *et al.*, 2007). Fortunately, there are a number of *E. coli* strains that are commonly used as hosts, including the BL21 (DE3) type and some of its derivatives. All these strains have unique genetic characteristics that prevent the degradation of a foreign protein because they are all deficient in the Lon protease enzyme (Gottesman, 1996) and another outer membrane protease *OmpT*, responsible for the extracellular degradation of foreign proteins (Grodberg and Dunn, 1988).

In order for any fusion heterologous protein construct to be obtained, it needs to be expressed in a suitable expression host cell such as *E. coli* and usually attached to an affinity tag. These affinity tags are essential for increasing the chances of attaining maximal protein solubility and purity. There are a number of small peptide tags used for such purposes, which include the poly-Arg-, the FLAG-, the poly-His-, the c-Myc-, S-, and the StrepII-tags (Terpe, 2003) that can be added at the N- or C- terminus of the desired protein. In this study a small tag, the hexa-histidine (6xHis tag) harboured on the pCRT7/NT-TOPO expression vector (Figure 2.1) was selected and used for the expression and affinity purification of the targeted recombinant AtPPR-AC/K. The tag was selected specifically for its size that has an added advantage of being less likely to interfere with both protein structure and function even though it may not enhance protein solubility (Niiranen *et al.*, 2007).

As for the choice of a suitable expression plasmid vector, one should have the following functional features; a promoter, a replicon, selection markers, multiple cloning sites as well as a fusion protein affinity tag and at times, the fusion protein removal strategy (Rosano and Ceccarelli, 2014). In most cases, a regulatable promoter such as the *lac* T7 would be most ideal, which when induced by a high concentration of the isopropyl-1-thio-D-galactopyranoside (IPTG) or L-arabinose will produce very high levels of the desired heterologous protein candidate. Fortunately, all of these expression features are available on the pCRT7/NT-TOPO (Figure 2.1) used in this study. The study details the heterologous expression of the AtPPR-AC/K in the *E. cloni* EXPRESS BL21 (DE3) pLysS DUOs cell system and the determination of its possible endogenous AC catalytic activity by enzyme-immunoassay system.

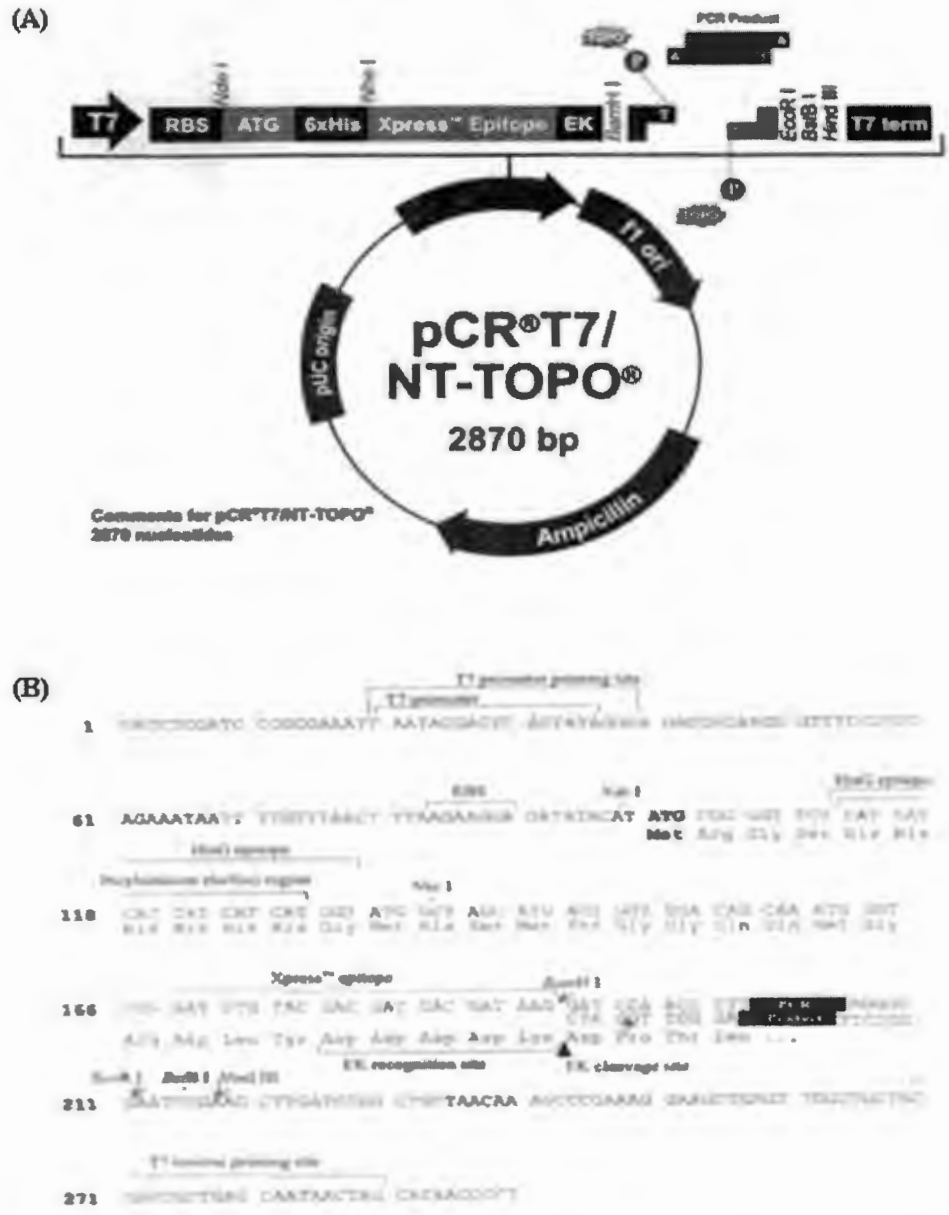

Figure 2.1: Structural features of a pCRT7/NT-TOPO expression vector. **(A)** A physical map illustration of the expression and purification features of the plasmid such as the T7 promoter for high level expression with the forward and reverse priming sites for both the restriction sites *Bam* HI and *Eco* RI within a multiple cloning site. In addition, there is also an ampicillin resistant gene that allows for screening of positive recombinants. For purification purposes, the vector expresses a recombinant 6-Histidine fusion protein. **(B)** A full sequence map of the vector showing the cloning site, where the AtPPR-AC/K insert was cloned as a PCR product (Figures adapted from www.lifetechnologies.com).

2.2 Materials and Methods

2.2.1 Generation and Propagation of *Arabidopsis thaliana* Plants

2.2.1.1 Seed Sterilization

About 10 mg of *Arabidopsis thaliana* ecotype Columbia seeds were placed into a sterile 1.5 mL Eppendorf tube, where 500 µL of 70% ethanol was added and vortexed for 30 seconds in order to sterilize the seeds surfaces. The seeds were left to settle down through gravity and the ethanol discarded. The seeds were then flooded with 500 µL sterilization buffer (0.1% SDS and 5% bleach (commercial) and vortexed for 30 seconds. The buffer was removed followed by a repeated wash of the seeds (5 times) with 2.5 mL of sterile distilled water, to remove traces of ethanol and any other remaining buffer components.

2.2.1.2 Seed Stratification

After sterilization, the seeds were stratified by submerging them into 500 µL of sterile distilled water followed by their introduction to a cold temperature of 4°C for 1-3 days. This process was undertaken to break seed dormancy and improve germination (uniform/synchronized germination) (Lack and Evans, 2001).

2.2.1.3 Seed Germination and Maintenance of Seedlings and Full Grown Plants

The sterilized and stratified seeds were placed on Murashige and Skoog medium [4.3 g Murashige and Skoog basal salts (Gibco®), 1% sucrose (w/v), 1X Gamborg's vitamins (v/v), 0.05% MES (w/v), 0.8% type M agar (w/v) (Sigma-Aldrich Corp., Missouri, USA), pH 5.7 with KOH] in petri dishes that were later sealed with parafilm and placed in a GC-300TL growth chamber (Lab Companion, Pretoria, RSA). The seeds were then allowed to germinate and grow over 14 days under long days (16-hour days) and short nights (8-hour nights) at a constant temperature of 25°C. After 14 days, the generated seedlings were transplanted with a

sterile blade into potting soil composed of 3 parts peat-based soil and 2 parts vermiculite, and then watered with double distilled water containing the Gaucho chemical (to systematically protect them from fungal attack). The transplanted seedlings were then allowed to grow for a further 2-4 weeks and under the same growth chamber conditions.

2.2.2 Isolation and Recombinant Cloning of the Targeted AtPPR-AC/K Gene Fragment

2.2.2.1 Designing and Acquisition of Sequence-specific Primers

The nucleotide sequence of the At1g62590 gene was retrieved from The Arabidopsis Information Resource (TAIR) (http://www.arabidopsis.org). The retrieved full genomic DNA sequence was then used to blast the "National Centre for Biotechnology Information" (NCBI) and searching for a homologous non-truncated copy DNA (cDNA) match; with which a translation into protein using the EXPASY Translate and EXPASY Protein Parameter Tools (http://au.expasy.org/cgi-bin/protparam) were undertaken and in order to determine the physical and chemical parameters of the resultant protein. The retrieved full length genomic DNA and its associated cDNA had an average nucleotide variance of 107 signifying that the At1g62590 gene has introns and thus primarily undergoing posttranscriptional modifications. Based on the cDNA sequence (TAIR protein locus At1g62590, GenPept accession number, NP_176447), two sequence-specific primers for the targeted AC/kinase containing segment of the At1g62590 were manually designed and both primers incorporating specific restriction sites (Figure 2.2) to enable directional cloning. The manually designed primers were then sent to Inqaba Biotechnical Industries (Pretoria, RSA) for both chemical synthesis and subsequent supply.

At1g62590-AC/K Forward

C**GG GAT CC**G AGC CAA ATC GAG AAG ATG AGG ATC TCG ATT

At1g62590-AC/K Reverse

C**GA ATT C**AG CAT TTC CAC CAT TTG ATC AAC CAA AGC TAC

Figure 2.2: Amino acid sequences of the truncated *Arabidopsis thaliana* pentatricopeptide repeat protein (AtPPR-AC/K) that was cloned and functionally characterized in this study. Above is the AtPPR-AC/K protein sequence showing the annotated kinase domain (underlined and highlighted in grey) and the AC catalytic centre (underlined and highlighted in red). The forward and reverse priming sites of this twin catalytic domain protein are highlighted in yellow and purple, respectively. The green highlighted amino acid residues indicate the protein phosphorylation sites. Below are the manually designed sequence specific primers for the AtPPR-AC/K whereby the forward primer is carrying a *Bam* HI site (bold and underlined) and the reverse primer carrying an *Eco* RI site (bold and underlined).

2.2.2.2 Isolation of the Total RNA from *Arabidopsis thaliana*

Total mRNA for isolation of the targeted AtPPR-AC/K gene fragment was extracted from the generated 6-week old Arabidopsis plants (Section 2.2.1.3), whereby about 0.1 g of plant leaf material was harvested followed by its processing with a Gene Jet Plant RNA Purification Mini Kit and as guided by the manufacturer's instructions (Catalog # K0801; Thermo Scientific Inc., Massachusetts, USA). Briefly, the weighed leaf material was quickly placed in liquid nitrogen and ground thoroughly with a cold pestle and mortar until it had formed a fine tissue powder. The fine tissue material was decanted into an RNase-free microcentrifuge tube and the liquid nitrogen was allowed to evaporate. A total volume of 450 µL of Buffer RLT was added to the tissue powder and the mixture vortexed vigorously. The lysate was transferred to a

QIAshredder spin column placed in a 2 mL collection tube and the collection centrifuged at 18000 *g* at 25°C. The supernatant was transferred carefully into a new microcentrifuge tube then a half volume of ethanol was added into it and the mixture then gently mixed by pipetting. The sample was transferred into an RNeasy spin column placed in a 2 mL collection tube and then centrifuged for 15 seconds at 12 750 *g* before the flow-through was discarded.

A total volume of 700 µL of Buffer RW1 was added into the spin column and centrifuged for 15 seconds at 8000 *g* so as to wash the spin column membrane. The flow-through was discarded. A total of 500 µL of Buffer RPE were added to the spin column and centrifuged for 15 seconds and 2 minutes, respectively, at 8000 *g* with the flow through discarded. The RNeasy spin column was placed into a sterile 1.5 mL tube and then 50 µL of RNase free water was added directly onto the spin column membrane and centrifuged for 1 minute at 8000 *g* so as to elute the RNA. Concentration of the total RNA was eventually determined with a NanoDrop ND 2000 spectrophotometer (Thermo Fisher Scientific Inc., Massachusetts, USA).

2.2.2.3 Amplification of the Targeted AtPPR-AC/K Gene Fragment by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

The targeted AtPPR-AC/K gene fragment was synthesized from the extracted total RNA (section 2.2.2.2), whereby the RNA was first used as a template to generate copy DNA (cDNA). Together with the acquired sequence-specific primers, the generated cDNA was then used to amplify the targeted AtPPR-AC/K fragment in a reverse transcriptase-polymerase chain reaction (RT-PCR) system using a Verso™ 1-Step RT-PCR Reddy Mix kit and in accordance with the manufacturer's instructions (Fermentas International Inc., Burlington, Canada). Reaction components for the RT-PCR system are shown in Table 2.1 below.

Table 2.1: Reaction components for the 1-step RT-PCR amplification of the targeted AtPPR-AC/K gene fragment.

Component	Volume (µL)	Final Concentration
Verso Enzyme Mix	1.0	
1-Step PCR Reddy Mix (2X)	25.0	1X
Forward Primer (10 µM)	1.0	200 nM
Reverse Primer(10 µM)	1.0	200 nM
RT Enhancer	2.5	
Water (PCR Grade)	14.5	
Template (RNA)	5.0	1 ng
Total Volume	50	

Following reconstitution, the RT-PCR process was then carried out on a C1000 Touch™ Thermal cycler (Bio-Rad Laboratories Inc., California, USA) and according to the Verso™ 1-Step RT-PCR Reddy Mix™ system (Fermentas International Inc., Burlington, Canada). The used thermocycling conditions are shown in Table 2.2 below.

Table 2.2: The 1-Step RT-PCR thermal cycling program for amplification of the targeted AtPPR-AC/K gene fragment.

Step	Temperature (°C)	Time (min)	Cycles
cDNA Synthesis	50	15.0	1
Verso Inactivation	95	15.0	1
Denaturing	95	0.3	45
Annealing	60	0.5	
Extension	72	1.0	
Final Extension	72	5.0	1

The amplified DNA fragment was then resolved through electrophoresis on a 1.0% agarose gel stained with 0.5 µg/mL ethidium bromide, immersed in 1X TBE buffer at 80 volts and 250 mA current for 50 minutes. The fragment was resolved alongside a 100 bp (0.1 µg/µL) DNA molecular weight marker (Catalog # SM1143; Fermentas International Inc., Burlington, Canada) and visualized under UV light on a 2000 Trans-illuminator (Bio-Rad Laboratories Inc., California, USA). Images were then captured with a Chemi Doc™ Imaging system, (Bio-Rad Laboratories Inc., California, USA) using the Bio-Image Lab™ software.

2.2.2.4 Cleaning of the Amplified AtPPR-AC/K Gene Fragment

The amplified AtPPR-AC/K gene fragment was cleaned from its RT-PCR reaction mixture using a DNA Clean & Concentrator™-5 kit and according to the manufacturer's instructions (Catalog # D4003; Zymo Research Inc., California, USA). Briefly, about 100 µL of the PCR product were transferred into a 1.5 mL Eppendorf tube followed by an addition of 500 µL of the DNA Binding buffer. The mixture was briefly vortexed before being carefully loaded into a Zymo spin column placed in a 2 mL Eppendorf tube. The collection was then centrifuged for 30 seconds at 10 000 *g* before the filtrate was discarded. About 200 µL of Wash buffer were added to the column and centrifuged twice for 30 seconds at 10 000 *g*. The washed Zymo

spin column was then placed into a new 1.5 mL Eppendorf tube and 30 µL of pre-warmed (65°C) sterile distilled water was added directly onto the column, allowed to incubate at room temperature for 2 minutes before being spun at 10 000 *g* for 1 minute to elute the cleaned PCR product.

2.2.2.5 Double Digestion of the AtPPR-AC/K Gene Fragment and the pCRT7/NT-TOPO Expression Vector

The cleaned AtPPR-AC/K gene fragment together with a commercially acquired pCRT/NT-TOPO plasmid vector (Invitrogen Corp., New York, USA) (Figure 2.1) were double-digested using a digestion kit and in accordance with the manufacturer's instructions (Catalog # EL0011; Fermentas International Inc., Burlington, Canada). Briefly, a 50 µl reaction mixture containing 10 units *Bam* HI, 10 units *Eco* RI, 2X Tango buffer, and 10 ng/µl plasmid or 20 ng/µl insert DNA was prepared followed by its incubation at 37°C on an AccuBlock Digital dry bath (Labnet International Inc., New Jersey, USA) for 4 hours. The restriction enzymes were then inactivated by heating on the AccuBlock Digital heating block (Labnet International Inc.,) at 80°C for 20 minutes. The double-digested DNA fragments were then both cleaned off their reaction components with a DNA Clean & Concentrator^{TM}-5 kit (Catalog # D4003; Zymo Research Inc., California, USA) as already been outlined in section 2.2.2.4 above.

2.2.2.6 Ligation of the AtPPR-AC/K Gene Fragment into the pCRT7/NT-TOPO Expression Vector

The double digested AtPPR-AC/K gene fragment was directionally cloned into the cloning site of the equally double digested pCRT/NT-TOPO expression vector using a ligation kit and in accordance with the manufacturer's instructions (Catalog # EL0014; Fermentas International Inc., Burlington, Canada) to produce a pCRT7/NT-TOPO:AtPPR-AC/K fusion expression

construct with an N-terminus His purification tag. Ideally, all fragments generated during a digestion process with any type II restriction enzyme like *Eco* RI and *Bam* HI, always form sticky ends (palindromic sequences), which will permit complementary alignments between the digested fragments (insert and vector), thus directional cloning. Briefly, a 20 µL ligation reaction mixture containing 20.0 ng/µL of the pCRT7/NT-TOPO plasmid vector, 60.0 ng/µL of the AtPPR-AC/K gene fragment, 1 unit T4 DNA ligase enzyme and 1X T4 DNA ligase buffer was prepared followed by its incubation at 22°C on a C1000 Touch Thermal cycler (Bio-Rad Laboratories., California, USA) for 60 minutes. The ligation mixture was further incubated overnight at 4°C before being kept at -20°C for further use. Finally, the nucleotide sequence of the resultant pCRT7/NT-TOPO-AtPPR-AC/K fusion expression construct was confirmed through chemical sequencing at Inqaba Biotechnical Industries (Pretoria, South Africa).

2.2.2.7 Transformation of the Chemically Competent *E. cloni* EXPRESS BL21 (DE3) pLysS Cells with the pCRT7/NT-TOPO-AtPPR-AC/K Fusion Expression Construct

Some commercially acquired chemically competent *E. cloni* EXPRESS BL21 (DE3) pLysS cells were transformed with the generated pCRT7/NT-TOPO-AtPPR-AC/K fusion expression construct and as guided by the manufacturer's instructions (Lucigen Corporation, Middleton., USA). Briefly, about 40 µL of the chilled cells were transferred into a sterile ice-cold Eppendorf tube followed by the addition of a 1 µL aliquot of the ligation mix. The mixture was gently mixed by stirring with a pipette tip and incubated on ice for 30 minutes. The mixture was heat-shocked on an AccuBlock Digital heating block (Labnet International Inc.,) at 42°C for 45 seconds and immediately placed on ice for 2 minutes. Subsequently, about 960 µL of a room temperature Recovery Expression medium (Lucigen Corp., Middleton, USA) was added to the mixture followed by incubation in a SI-600 Benchtop incubator (Lab Companion,

Johannesburg, RSA) shaking at 200 rpm at 37°C for 1 hour. After an hour, the transformation mix was then plated at 50 µL, 100 µL, 200 µL aliquots onto LB plates [1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, 0.5% (w/v) glucose and 1% (w/v) agar; pH 7.0] supplemented with 100 µg/mL ampicillin and 34 µg/mL chloramphenicol and then left to grow overnight at 37°C.

2.2.2.8 Colony Polymerase Chain Reaction

In order to ascertain that the transformed cells were carrying the desired generated pCRT7/NT-TOPO-AtPPR-AC/K fusion expression construct, a confirmatory PCR was conducted using a Standard MyTaq™ Mix kit and according to the manufacturer's protocol (Bioline, London, UK). Briefly, a reaction mix was prepared as is presented in Table 2.3 below and ensuring that in this case, an intact bacterial colony was acting as a template.

Table 2.3: Reaction components of a colony PCR to confirm presence of the pCRT7/NT-TOPO-AtPPR-AC/K fusion expression construct in the transformed *E. cloni* EXPRESS BL21 (DE3) pLysS cells.

Component	Volume (µL)	Final Concentration
Template DNA (colony)		
Forward Primer (10 µM)	1.0	200 nM
Reverse Primer (10 µM)	1.0	200 nM
MyTaq Red Mix, (2X)	25.0	1X
Water (PCR Grade)	23.0	
Total Volume	50.0	

Following the reconstitution, PCR was then conducted on a C1000 Touch™ Thermal cycler (Bio-Rad Laboratories Inc.,) and in accordance with the Standard MyTaq™ Mix protocol (Bioline, London, UK). The used thermocycling conditions are shown in Table 2.4 below.

Table 2.4: Thermocycling conditions for a step-by-step colony PCR amplification of the AtPPR-AC/K gene fragment.

Step	Temperature (°C)	Time (min)	Cycles
Initial Denaturation	95	1.00	1
Denaturation	95	0.25	25-35
Annealing	60	0.25	
Extension	72	0.17	

The amplified fragment was resolved, visualized and captured as is already been outlined in the last paragraph of section 2.2.2.3 above. A re-amplification of the AtPPR-AC/K gene fragment would then confirm the presence of the desired pCRT7/NT-TOPO-AtPPR-AC/K fusion expression construct in the transformed *E. cloni* EXPRESS BL21 (DE3) pLysS cells.

2.2.3 Partial Expression of the Recombinant AtPPR-AC/K Protein

A single colony of the freshly streaked plates in section 2.2.2.7 above was picked and used to inoculate 5 mL of a double strength yeast-tryptone (2YT) media [16 g tryptone powder, 10 g yeast extract, 5 g NaCl and 4 g glucose per L (pH 7.0)] containing 100 µg/mL ampicillin and 34 µg/mL chloramphenicol before the culture was incubated overnight at 37°C and shaking at 200 rpm. The next day, about 200 µL of the overnight culture were used to inoculate a fresh 10 mL 2YT media supplemented with the same antibiotics as was the overnight culture. The culture was then incubated in a SI-600 Benchtop Shaking incubator (Lab Companion) at 200 rpm at 37°C and up until the OD_{600} had reached 0.5, as measured by a Hekios spectrophotometer (Merck, Modderfontein, South Africa). Immediately, the culture was split into two equal volumes with one culture being induced to express the anticipated AtPPR-AC/K recombinant through the addition of isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma-Aldrich Corp, Missouri) to a final concentration of 1 mM while the other culture was left un-

induced (control). The two split cultures were then shaken in an incubator at 200 rpm at 37°C for a further 3-hour period. After the 3 hours of incubation, some 1 mL of cell aliquot were harvested at 8 000 *g* for 10 minutes followed by their analysis through sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.2.4 Determination of the Endogenous Adenylate Cyclase Activity of the Recombinant AtPPR-AC/K Protein

2.2.4.1 Protein Expression

An expression culture of the recombinant AtPPR-AC/K protein was prepared as already been outlined above in section 2.2.3 except for the fact that an un-induced culture from the same section was in this case used as an initial inoculum instead of a cell colony and that the volume of the second day culture was 20 mL instead of 10 mL. At an OD_{600} of 0.5, the culture was immediately placed on ice and split into four equal portions of 5 mL each. Protein expression was induced by the addition of 1 mM IPTG into three cultures while one culture was left un-induced (control). From two of the three induced cultures, one culture was supplemented with 100 µM forskolin (Sigma-Aldrich Corp., Missouri, USA) (the common AC inducer) while the other culture was supplemented with 100 µM 2′, 5′-dideoxyadenosine (Sigma-Aldrich Corp., Missouri, USA) (a potent AC inhibitor). All the four sets of cultures were then allowed to grow for a further 3-hour period in a shaking incubator. Cells were finally harvested by centrifugation at 9 200 g for 10 minutes and the pellets stored at -20°C for further analysis.

2.2.4.2 Activity Assaying

Cells from section 2.2.4.1 were lysed in 1 mL Lysis Buffer 1 (Amersham Healthcare, New Jersey, USA) supplemented with 2 mM IBMX (Sigma-Aldrich Corp., Missouri, USA) to inhibit phosphodiesterases. The samples were then shaken on an AGITADOR orbital shaker

(Comecta, Johannesburg, RSA) at 100 rpm at 37°C for 30 minutes and in order to intensify the cell lysis process. The samples were centrifuged at 16 300 *g* for 5 minutes and the lysate transferred into a fresh Eppendorf tube where 200 µL of the Lysis Buffer 2 (Amersham Healthcare, New Jersey, USA) was added and mixed. About 220 µL of the mixture was then transferred into a fresh Eppendorf where 11 µL of the acetylating reagent (Sigma-Aldrich Corp., Missouri, USA) was added and the mixture pulsed. The endogenous cAMP contents of each of the four cell lysates were then measured by a cAMP enzyme immunoassay kit (Catalog # CA201, Sigma-Aldrich Corp., Missouri, USA) following the acetylation version of its protocol and as was described by the manufacturer's manual. All assaying measurements were taken using a Microplate Reader (Labtech, International Ltd., East Sussex, UK) at 405 nm.

2.2.4.3 Statistical Analysis

All immunoassay measurements recorded in section 2.2.4.2 above were undertaken in three replicates whereby all corresponding responses of each process were subjected to an analysis of variance (ANOVA) Super-Anova, Stats graphics Version 7, 1993 (Stats graphics Corporation, USA). In addition, and wherever ANOVA was revealing some significant variations between treatments, the affected means ($n = 3$) were separated by a post hoc Student Newman Kuehls (SNK), multiple range test ($p \leq 0.05$).

2.3. Results

2.3.1 Isolation of the AtPPR-AC/K Gene Fragment from *Arabidopsis thaliana*

In order to test if the annotated dual domain of the At1g62590 gene had any functional activity, its truncated version (AtPPR-AC/K) was isolated from 6-week old *Arabidopsis thaliana* plants using a specialized 1-step RT-PCR system (ThermoScientific Inc., Massachusetts, USA). As shown in Figure 2.3 below, the expected 567 bp gene fragment was amplified, thereby

demonstrating a successful isolation of the desired AtPPR-AC gene fragment from the Arabidopsis plants.

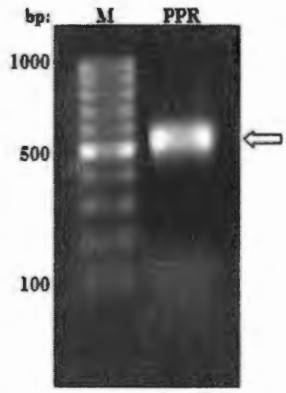

Figure 2.3: Isolation of the AtPPR-AC/K gene fragment from *Arabidopsis thaliana*. A 1% agarose gel showing a resolution of the AtPPR-AC/K gene fragment isolated from the 6-week old *A. thaliana* total mRNA via RT-PCR. M represents the 100 bp DNA ladder (Catalog # SM1143; Fermentas Int., Burlington, Canada); PPR represents the amplified AtPPR-AC/K gene fragment while the arrow is marking the resolved 567 bp AtPPR-AC/k band.

2.3.2 Colony PCR of the Cloned AtPPR-AC/K Gene Fragment

After isolation, the AtPPR-AC/K gene fragment was cloned into the pCRT7/NT-TOPO expression vector to yield a pCRT7/NT-TOPO-AtPPR-AC/K fusion expression construct. This construct was then used to transform some chemically competent *E. cloni* EXPRESS BL21 (DE3) pLysS cells for subsequent protein expression. However, before protein expression was pursued, a colony PCR of the transformed cells was undertaken so as to ascertain that these cells were indeed carrying the correct and desired pCRT7/NT-TOPO-AtPPR-AC/K fusion expression construct. As is shown in Figure 2.4 below, the 567 bp AtPPR-AC/K gene fragment was once again amplified, thus demonstrating its presence in the transformed cells.

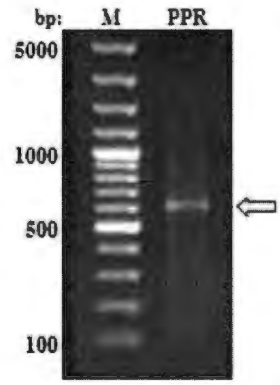

Figure 2.4: Colony PCR of the AtPPR-AC/K gene fragment. A 1% agarose gel resolution of the AtPPR-AC/K gene fragment following a colony PCR of the chemically competent *E. cloni* EXPRESS BL21 (DE3) pLysS cells transformed with the pCRT7/NT-TOPO-AtPPR-AC/K fusion expression construct. M represents the 100 bp DNA ladder plus (Catalog # SM1143; Fermentas Int.,); PPR represents the re-amplified AtPPR-AC/K gene fragment while the arrow is marking the resolved and expected 567 bp AtPPR-AC/K band.

2.3.3 Partial Expression of the Recombinant AtPPR-AC/K protein

Following the successful cloning of the AtPPR-AC/K gene fragment into the pCRT7/NT-TOPO expression vector and transformation of the chemically competent *E. cloni* EXPRESS BL21 (DE3) pLysS cells, with the pCRT7/NT-TOPO:AtPPR-AC/K fusion expression construct the recombinant AtPPR-AC protein was then partially expressed before its probable AC activity was tested. As is shown in Figure 2.5 below, the expected 27.290 kDa recombinant protein with an N-terminus His-tag was resolved, demonstrating a successful expression of the targeted and desired AtPPR-AC/K recombinant.

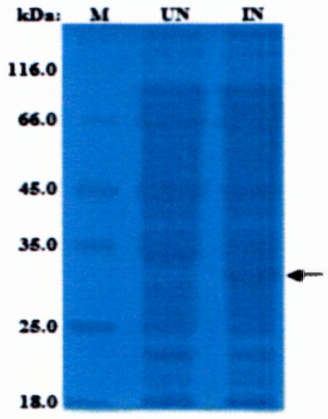

Figure 2.5: Partial expression of the recombinant AtPPR-AC/K protein. A 12% SDS PAGE analysis of un-induced (UN) and induced (IN) *E. cloni* EXPRESS BL21 (DE3) pLysS cell cultures (Lucigen Corp., Middleton., USA) harbouring the pCRT7/NT-TOPO-AtPPR-AC/K fusion expression construct. M represents the unstained molecular weight marker (Catalog # SM0431; Fermentas Int., Burlington, Canada) while the arrow is marking the partially expressed recombinant AtPPR-AC/K protein.

2.3.4 Determination of the Endogenous AC Activity of the Recombinant AtPPR-AC/K

After successfully expressing the desired AtPPR-AC/K in the transformed *E. cloni* EXPRESS BL21 (DE3) pLysS cells, its ability to induce the conversion of ATP into cAMP within these prokaryotic cells (endogenous) was then assessed. The assessment was carried out under various growth conditions, which included in the presence or absence of activators or inhibitors. The produced inherent cellular cAMP contents were then determined and quantified by a cAMP-linked enzyme immunoassay system (Catalog # CA201; Sigma-Aldrich, Missouri, USA) and as is shown in Figure 2.6 below.

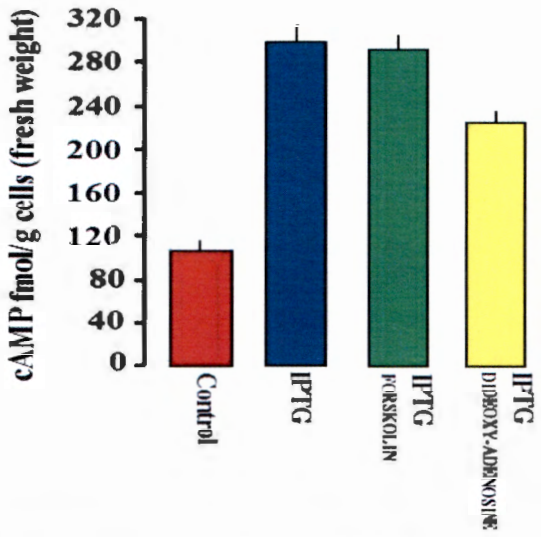

Figure 2.6: Determination of the endogenous AC activity of the recombinant AtPPR-AC/K protein. Cyclic AMP levels generated by the un-induced (Control) and induced (IPTG) *E. cloni* EXPRESS BL21 (DE3) pLysS cells harbouring the AtPPR-AC/K gene fragment, and also by the induced cells in the presence of the AC activator forskolin or the AC inhibitor dideoxy-adenosine. Such cAMP levels were determined and quantified with a cAMP Enzyme immunoassayi system (catalog # CA201; Sigma-Aldrich Inc., Missouri, USA), where error bars represent the standard errors (SEM) of the means of three independent and representative assays (n = 3).

2.4 Discussion

In most organisms, the majority of cellular processes are regulated by cAMP, which is a very significant molecule to many aspects of growth and development. However, and despite a wide recognition of this second messenger molecule in most organisms, there is still a wide gap regarding its functionality in plants that needs to be filled. Previously, a search query was undertaken in plants using a unique motif that was derived from functionally assigned residues in the catalytic centres of most guanylate cyclase (GC) molecules found across different species and such a search then resulted in the identification of multiple GC candidates (Ludidi and Gehring, 2003; Wong and Gehring, 2013). In line with that, a similar approach was also used for the identification and retrieval of nine adenylate cyclase (AC) candidate molecules from *A. thaliana* (Table I.1) one of which was the gene of interest for this study, At1g62590, annotated as a pentatricopeptide repeat (*PPR*) gene and coding for a putative pentatricopeptide (PPR) protein (Gehring, 2010). Specifically, the identification of these AC candidate molecules was

based on a specific site-directed mutagenesis, where a GC motif was converted into an AC motif through a modification of specific residues at the third position of the 14 amino acid search term from [CTGH] to [DE] and in order to suit specificity for ATP binding rather than the GTP, and also with the C-terminal metal cofactor binding part as is shown in Figure 1.2 (Tucker *et al.*, 1998; Roelofs *et al.*, 2001). Through a close sequence identity inspection, it was noticed that this At1g62590 gene harbours a stretching dual-catalytic domain structure consisting of an adenylate cyclase and kinase domains. An *in silico* analysis has predicted that a full length PPR protein sequence is made up of 1363 amino acids, with a molecular mass of approximately 156 kDa. In line with the basis of this somewhat intriguing plant molecule, this chapter then entailed to clone a truncated version of this gene harbouring the AC and kinase domains (AtPPR-AC/K gene fragment) into a pCRT7/NT-TOPO prokaryotic expression vector followed by partial expression and determination of its possible endogenous AC activity.

Ideally, the At1g62590 full length genome has intronic sequences; and therefore, in order to clone the intracellular AC and kinase domain fragment, its expressible cDNA sequence was targeted. The retrieved cDNA sequence was then used as a template to amplify the targeted 567 bp truncated region of the At1g62590 gene harbouring the AC and kinase domains (Figure 2.3). In order for us to clone the amplified AtPPR-AC/K gene fragment into a compatible and viable expression vector system, pCRT7/NT-TOPO vector was chosen (Figure 2.1) amongst other expression systems due to its unique characteristics of adding a very small and short purification fragment tag (6x His tag) to the N-terminus end of the resultant recombinant fusion protein, and thus owing a very minimal interference to both the expression and purification aspects of the recombinant product. A link between the amplified gene fragment and its selected expression vector yielded a pCRT7/NT-TOPO:AtPPR-AC/K fusion construct whose in-frame orientation was subsequently verified through sequencing at Inqaba Biotechnical

Industries, Pretoria (results not shown). The pCRT7/NT-TOPO:AtPPR-AC/K fusion construct was then used to transform competent *E. cloni* EXPRESS BL21 (DE3) pLysS expression cells and such a transformation was further verified by colony PCR (Figure 2.4). The transformed cells were then used to partially express the targeted AtPPR-AC/K recombinant - a process that yielded the expected fusion-tagged protein product of approximately 27.290 kDa shown in Figure 2.5.

After expression, the AC coding region of the produced AtPPR-AC/K recombinant protein was then tested for its ability to generate cAMP from ATP within the transformed *E. cloni* EXPRESS BL21 (DE3) pLysS cells (endogenously) and under various growth conditions. The conditions included growth in the presence or absence of 1 mM IPTG as well as growth in 1 mM IPTG in the presence of the AC activator 100 mM forskolin (Ehsan *et al.*, 1998) or the AC inhibitor 100 mM dideoxyadenosine (Volotovski *et al.*, 1998). As is shown in Figure 2.6, cells that were induced with 1 mM IPTG had their cAMP content increased to about 3.2 times that of un-induced cells. In addition, cells that were grown in the presence of dideoxyadenosine had their cAMP content reduced by a factor of 1.6 while forskolin had no apparent effect. These findings correspond very well with findings of a related previous study where the PSiP coding region of a pollen-specific putative AC from *Agapanthus umbellatus* (Liliaceae) was cloned into bacterial cells and treatment of the cloned cells with 1 mM IPTG resulting in a net cAMP increase of about 3.0 fold (Moutinho *et al.*, 2001). More so, the potent AC inhibitor, dideoxyadenosine (Volotovski *et al.*, 1998) has also been shown to reduce the levels of cAMP (accompanied with a temporary arrest in growth) in growing pollen tubes of *Zea mays* by a factor of 1.1 (Moutinho *et al.*, 2001). The lack of a response to forskolin by the AtPPR-AC/K proposes this recombinant as a probable soluble AC (sAC) because only sACs are insensitive

to forskolin while all transmembrane ACs (tmACs) are responsive (Seamon *et al.*, 1981; Forte *et al.*, 1983; Laurenza *et al.*, 1989).

Therefore, outcomes of this chapter revealed that the cloned and expressed recombinant AtPPR-AC/K protein may be a *bona fide* soluble adenylate cyclase (sAC) capable of converting ATP to cAMP or any other signalling plant molecule capable of stimulating other resident adenylate cyclases (*E. coli* ACs in this case) to produce cAMP. In addition, the findings also show that the AC fragment component of the twin-domain recombinant AtPPR-AC/K is a functional and catalytically active domain. Furthermore, this AC domain is also capable of showing functional activity despite its co-existence with yet another domain (kinase domain in this case) that might share the same substrate (ATP) with itself.

2.5 Conclusion

Findings from this chapter indicate that the expressed recombinant AtPPR-AC/K protein may either be a *bona fide* functional higher plant AC or simply another signalling plant molecule capable of stimulating other resident ACs (*E. coli* ACs in this case) to generate cAMP.

2.6 Recommendation

From the findings obtained in this chapter, it is highly recommended that further *in vivo* and *in vitro* assays be undertaken in order to clearly establish whether or not this AtPPR-AC/K protein is indeed a functional higher plant AC capable of generating cAMP.

CHAPTER THREE

Determination of the *In Vivo* Adenylate Cyclase Activity of the Recombinant AtPPR-AC/K Protein

Abstract

Naturally, cellular signal transduction is a typical system by which both eukaryotes and prokaryotes communicate and respond to the various environmental stimuli. In plants, this system is mainly modulated by the various cAMP-dependent signaling pathways enhanced by adenylate cyclases (ACs). Both the ACs and their enzymatic product, cAMP, are known to relay external signals (environment) to the internal effectors (cytoplasm) so that cells can effectively respond to such signals, resulting in appropriate physiological processes. Apparently, even though the ACs and cAMP are both involved in the regulation and mediation of most signal transduction pathways necessary for the adaptation and survival of most organisms, their existence and/or functional roles in plants have been very elusive or rather a subject of huge controversy. To date, only five annotated higher plant AC molecules have been experimentally confirmed; one each in the *Zea mays*, *Nicotiana benthamiana* and *Hippeastrum hybridum* plants, and two in *Arabidopsis thaliana*. Hence, in an effort to identify yet another additional AC molecule in higher plants and following the recent annotation of a pentatricopeptide protein from *Arabidopsis thaliana* (AtPPR) as a probable AC molecule, we hereby cloned the AC/kinase-containing fragment domain of this putative protein (AtPPR-AC/K) into a non-lactose fermenting mutant strain of *Escherichia coli* (the *cya*A SP850 cells) and unequivocally confirmed its ability to generate cAMP from ATP. Actually, the generated cAMP complemented the mutant strain to behave as if it was now a wild type capable of fermenting lactose.

3.1 Introduction

Adenylate cyclases (ACs) are enzymes capable of generating cyclic adenosine 3′,5′-monophosphate (cAMP) from adenosine 5′-triphosphate (ATP) and this cAMP is naturally degraded by phosphodiesterases (PDEs). Cyclic AMP is largely known as a universal second messenger that is ubiquitous and widely distributed among prokaryotes and eukaryotes. It is a signalling molecule responsible for relaying external signals to the internal effectors so that cells can respond appropriately and result in various physiological processes (McDonough and Rodriguez, 2012). In the prokaryotic bacterium *E. coli*, cAMP was found to specifically bind to a cAMP receptor protein (CRP) leading to a conformational change, which results in a cAMP-CRP complex that modulates the initiation of a variety of catabolite-sensitive operons (Ullmann and Danchin, 1983). The activated operons in turn, then regulate transcription of a diverse of physiological processes such as carbon catabolism, nitrogen assimilation, iron uptake, biofilm formation, antibiotic resistance, chemotaxis and virulence (Bai *et al.*, 2011; Coggan and Wolfgang, 2012; McDonough and Rodriguez, 2012).

Cyclic AMP has also been shown to play an essential role in the catabolite repression of a wide variety of aerobic and facultative anaerobic bacteria (Pastan and Adhya, 1976; Botsford and Harman, 1992). It is also involved in responses and regulations of most signal transduction pathways that are significant and essential for the adaptation and survival of various organisms (Bretschneider *et al.*, 1999; Bahn *et al.*, 2007; Biswas *et al.*, 2011) as evidenced in cyanobacteria, where its enhanced levels respond to environmental signals. In *Anabaena cylindrica*, cAMP was shown to transduce light signalling and cellular transduction systems (Ohmori *et al.*, 1988). In *E. coli*, it is involved in the positive regulation of the *lac* operon (Ullmann and Danchin 1983),

Apparently, and despite the fact that most bacteria require cAMP as an essential molecule for both signalling and survival, *E. coli* naturally has only a single AC that is coded for by a *cya*A gene (Côté *et al.*, 2010). This one and only enzyme was first isolated and purified in 1983 by Yang and Epstein and is very critical for the fermentation of lactose, where it produces the most wanted cAMP that activates the *lac* operon (Cotta *et al.*, 1998). In 1992, Gross *et al.* used site-directed (deletion) mutagenesis and genetic recombination to knock out this gene, resulting in the production of a mutant strain known as the SP850 (Shah and Peterkofsky, 1991). This strain lacks the AC activity and therefore, cannot metabolize carbon sources such as lactose, maltose, galactose, arabinose and glycerol (Ullmann and Danchin, 1983; Moutinho *et al.*, 2001). When grown on MacConkey agar, there is a very distinct phenotypic difference between this mutant and its wild type, whereby it appears white or yellowish while the wild type appears deep red or purplish (Cotta *et al.*, 1998).

Technically, the *cya*A SP850 mutant provides a very viable biochemical platform for testing the probable AC activity of foreign recombinant proteins via a complementation phenomenon of the mutation (Cotta *et al.*, 1998). It has already been demonstrated that when a *cya*A mutant is transformed with a heterologous system, which encodes a putative AC gene, the AC gene will rescue the *cya*A mutation (complementation) (Cotta *et al.*, 1998), allowing the mutant strain to now ferment lactose and thereby producing deep red or purplish colonies on MacConkey agar (Perlman and Pastan, 1969). Therefore, the specific goal for this chapter was to experimentally assess the probable AC catalytic activity of the annotated *Arabidopsis thaliana* pentatricopeptide repeat protein (AtPPR-AC/K) encoded by the At1g62590 gene through its possible complementation of the *cya*A mutation in the *E. coli* SP850 mutant strain.

3.2 Materials and Methods

3.2.1 Isolation and Purification of the pCRT7/NT-TOPO:AtPPR-AC/K Fusion Expression Construct

A 5 mL overnight culture of the *E. cloni* EXPRESS BL21 (DE3) pLysS cells harbouring the pCRT7/NT-TOPO:AtPPR-AC/K fusion expression construct was prepared as already outlined in sections 2.2.3 and 2.2.4. The next morning, cells were harvested through centrifugation in a Z300k Benchtop centrifuge (Hermile Corp., Berlin, Germany) at 8 000 *g* for 5 minutes. The pCRT7/NT-TOPO:AtPPR-AC/K fusion expression construct was then isolated from the harvested cells using a GeneJET Plasmid Miniprep kit and in accordance with the manufacturer's instructions (Catalog # K0502; Thermo Fisher Scientific Inc., Massachusetts, USA). Briefly, the harvested cell pellets were resuspended in 250 µL of a Resuspension solution by pipetting up and down, and the cell resuspension transferred into a microcentrifuge tube. A total volume of 250 µL of a Lysis solution was added to the cell resuspension and the mixture mixed thoroughly through inversion for 4-6 times or until the solution had become viscous. To this mixture, a total volume of 350 µL of a Neutralization solution was added and mixed thoroughly through gentle inversion for 4-6 times. The solution was then centrifuged for 5 minutes at 8 000 *g* and to pellet the chromosomal DNA and other cell debris. The supernatant was carefully transferred into a supplied Gene Jet spin column fitted into a collection tube, and the collection tube centrifuged for 1 minute at 8 000 *g*. The flow-through was discarded and the spin column placed into the same collection tube.

The column was washed twice with 500 µL of the Wash solution apiece and through centrifugation at 8 000 *g* for 30-60 seconds while the flow-through was discarded and the same collection tube re-used. The spin column was re-centrifuged at 8 000 *g* for an additional minute and in order to remove the excess and residual Wash solution. The spin column was transferred

into a fresh microcentrifuge tube, where 50 µL of the Elution buffer was added to the centre of the column membrane and in order to elute the plasmid DNA. The set up was left to incubate for 2 minutes at room temperature before being centrifuged at 8 000 *g* for 2 minutes. The spin column was discarded while the eluted plasmid DNA was kept at -20°C for further downstream uses.

3.2.2 Preparation of Chemically Competence *E. coli cya*A SP850 Mutant Cells

An overnight culture of the *E. coli cya*A SP850 mutant cells [lam-, el4-, relA1, spoT1, cyaA1400 (::kan), thi-1] obtained from the Coli Genetic Stock Centre (Yale University, New Haven, Connecticut, USA) was prepared in Luria-Bertani (LB) media [1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl and 0.5% (w/v) glucose; pH 7.0] supplemented with 15 µg/mL kanamycin, and as has already been outlined in section 2.2.3 of Chapter 2. The next morning, a sub-culture of the overnight culture was then prepared in a similar fresh LB media and as has already been described in section 2.2.3 of Chapter 2. At an OD_{600} of 0.5, the cell culture was cooled on ice for 5 minutes, then transferred into a pre-chilled centrifugation tube before being centrifuged at 4 000 *g* for 5 minutes at 4°C,. The supernatant was discarded while the cell pellet was kept on ice. The cell pellet was resuspended in 30 mL of an ice-cold Transformation buffer 1 (TFB1) [30 mM KAc, 50 mM $MnCl_2$, 100 mM RbCl, 10 mM $CaCl_2$, 15% glycerol, pH: 5.8] before being incubated on ice for 90 minutes. The resuspension culture was centrifuged at 4 000 *g* for 5 minutes at 4°C and the supernatant was discarded while the cell pellet was kept on ice. The cell pellet was resuspended in 4 mL of an ice-cold Transformation buffer 2 (TFB2) [10 mM MOPS, 75 mM $CaCl_2$, 10 mM RbCl, 15%. glycerol, pH: 6.8] before aliquots of 100 µL were prepared in 1.5 mL Eppendorf tubes.

3.2.3 Transformation of the Chemically Competent *E. coli cya*A SP850 Cells with the pCRT7/NT-TOPO-AtPPR-AC/K Fusion Expression Construct

About 10 µL of the ice-cold pCRT7/NT-TOPO-AtPPR-AC/K fusion expression construct were added to 100 µL of the ice-cold chemically competent *E. coli cya*A SP850 cells and the mixture mixed gently through stirring with a pipette tip before being incubated on ice for 20 minutes. The mixture was heat-shocked at 42°C on an AccuBlock Digital heating block (Labnet International Inc.,) for 90 seconds before being immediately placed on ice for 5 minutes. About 500 µL of the pre-warmed SOC medium (Lucigen Corp., Middleton, USA) was added to the mixture followed by incubation in a SI-600 Benchtop Shaking incubator (Lab Companion) at 200 rpm at 37°C for 1 hour. After an hour, the transformation mix was plate spread at 50 µL, 100 µL, 200 µL aliquots onto LB plates supplemented with 100 µg/mL ampicillin and 15 µg/mL kanamycin before the plates were allowed to grow overnight at 37°C.

3.2.4 Testing for Complementation of the *cya*A mutation in SP850 *E. coli* Cells by the AtPPR-AC/K Recombinant Protein

A MacConkey agar plate [2% (w/v) peptone, 1% (w/v) lactose, 0.5% (w/v) bile salts, 1% (w/v) NaCl, 0.0075% (w/v) neutral red and 1% (w/v) agar; pH 7.1) supplemented with 15 µg/mL kanamycin and 0.1 mM IPTG (Sigma-Aldrich Corp.,) was prepared and then sub-divided into 4 equal quadrants using a permanent marker. The first quadrant was left un-cultured (no *cya*A cells), the second quadrant was streaked with the non-transformed *E. coli* SP850 *cya*A mutant cells, the third quadrant was streaked with the *cya*A mutant cells transformed with the empty pCRT7/NT-TOPO vector, while the last quadrant was streaked with the *cya*A mutant cells transformed with the pCRT7/NT-TOPO:AtPPR-AC/K fusion expression construct. The plate was then inverted and incubated at 37°C for 40 hours. After the incubation, all quadrants were then visually inspected for various phenotypic characteristics shown by means of coloured or

non-coloured colonies. A complementation of the *cya*A mutation would result in deep red or purplish colonies while a non-complementation of the mutation would result in whitish or light yellow colonies.

3.3 Results

After determining the AtPPR-AC/K's ability to induce the generation of cAMP within the transformed *E. cloni* EXPRESS BL21 (DE3) pLysS cells, its ability to exclusively generate cAMP *in vivo* was then tested. The test was undertaken in *E. coli cya*A SP850 mutant host cells (Coli Genetic Stock Center, Yale University, Connecticut, USA), which naturally lack AC activity and therefore, cannot ferment lactose but when rescued by an exogenous AC candidate (like the AtPPR-AC/K in this case), can then ferment the sugar resulting in distinctively deep red or purplish colonies on the differential and selective MacConkey agar. In this test, the mutant cells were transformed with the pCRT7/NT-TOPO vector harbouring the AtPPR-AC/K recombinant (pCRT7/NT-TOPO:AtPPR-AC/K) followed by an analysis of their phenotypic appearances on MacConkey agar as shown in Figure 3.1 below.

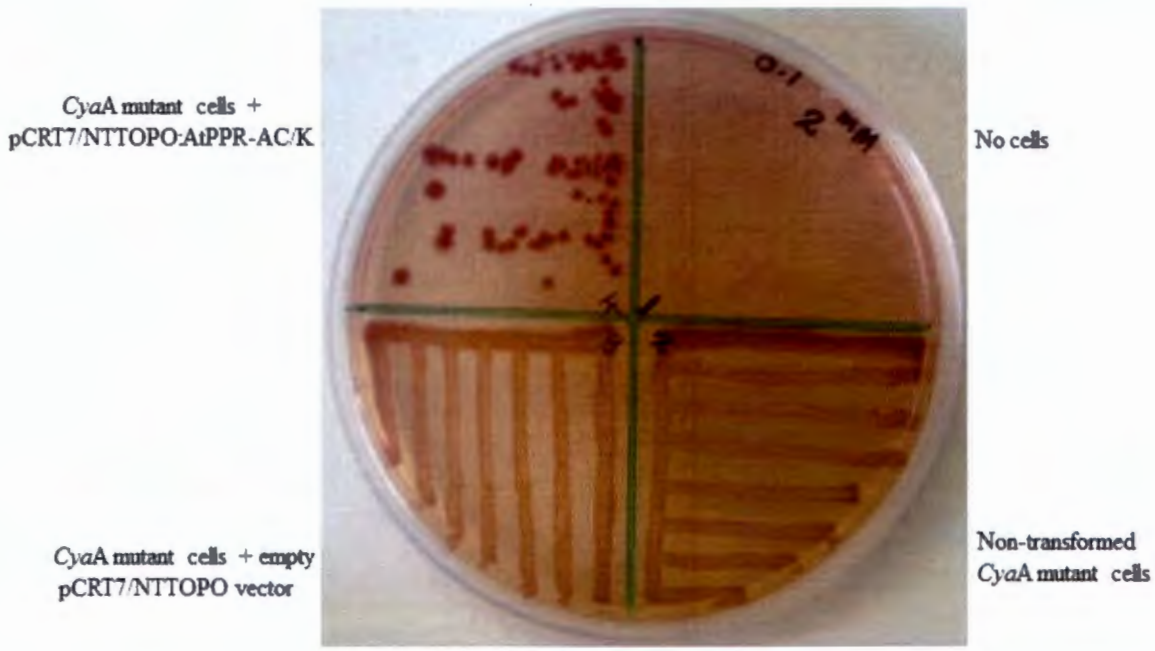

Figure 3.1: Determination of the *in vivo* AC activity of the recombinant AtPPR-AC/K protein. Different *E. coli cya*A cells were plated onto MacConkey agar supplemented with 0.1 mM IPTG and 15 µg/mL kanamycin. The plate was divided with a permanent marker into four equal quadrants. In quadrant 1, no cells were plated; in quadrant 2, non-transformed cells were plated, in quadrant 3, cells transformed with the empty pCRT7/NT-TOPO vector were plated while in quadrant 4, cells transformed with the pCRT7/NT-TOPO:AtPPR-AC/K fusion expression construct were plated. The plate was then incubated at 37°C for 40 hours followed by an analysis of the phenotypic features of the plated cells. Cells in segments 2 and 3 are both non-lactose fermenters and therefore produced white or yellowish colonies while those in segment 4 have now picked a lactose fermentation trait and thus becoming deep purple in colour. The appearance of cells in segment 4 clearly demonstrates the AtPPR-AC/K's ability to generate cAMP and thus rescuing the involved cells.

3.4 Discussion

Cyclic nucleotides (cAMP and cGMP) have been established as important signalling molecules involved in various biological and physiological processes exhibited by both prokaryotes and eukaryotes (Lemtiri-Chlieh *et al.*, 2011). To date, cAMP and the enzymes that produce it, adenylate cyclases (ACs), appear to be some of the most extensively researched signalling molecules in animals, lower eukaryotes and bacteria while in plants, both their presence and biological functions have somewhat remained elusive and controversial (Amrhein, 1977). Based on these aspects, it was therefore very imperative to conduct a further functional analysis

of the recombinant AtPPR-AC/K produced in Chapter 2 and determine whether it was a *bona fide* functional AC or not.

In such an analysis, a complementation testing of the *E. coli* mutant host, the SP850 *cya*A strain by the recombinant AtPPR-AC/K protein was undertaken. This type of *E. coli* strain is characterized by a lack of the endogenous AC activity as a result of a mutation of its AC gene (*cya*A) (Shah and Peterkofsky, 1991), and therefore it cannot catabolize polysaccharides such as lactose and maltose (Ullmann and Danchin, 1983; Moutinho *et al.*, 2001). In this regard, it therefore mean that this host strain is incapable of synthesizing cAMP, which is needed for activation of the *lac* operon necessary for sugar fermentation. As such, when grown on a differential and selective medium like MacConkey agar, this mutant strain displays a white or yellowish phenotype as opposed to the deep red or purplish phenotype exhibited by its wild type (Perlman and Pastan, 1969). However, if the mutant is augmented with a functional exogenous AC protein, it overcomes its mutation and regains its wild type sugar fermentation characteristic – a scenario that is taken advantage of by biochemists to test for the probable AC functionality of unknown and foreign proteins (Cotta *et al.*, 1998).

Therefore, in order to investigate the probable *bona fide* AC functionality of the recombinant AtPPR-AC/K protein, complementation testing was carried out whereby the pCRT7/NT-TOPO:AtPPR-AC/K fusion expression construct was used to transform chemically competent *E. coli* SP850 *cya*A mutant host cells followed by a visual inspection of the displayed colony phenotypes on MacConkey agar. As shown in Figure 3.1, the pCRT7/NT-TOPO:AtPPR-AC/K fusion expression construct successfully rescued the transformed *E. coli* SP850 *cya*A mutant host cells and thus signifying that the harboured recombinant AtPPR-AC/K protein was indeed a functional higher plant AC capable of generating cAMP from ATP. This outcome is very

similar to findings obtained from the various previous studies undertaken on the *E. coli cya*A SP850 mutant strain with different unknown foreign proteins. Such proteins include the D31d protein from the anaerobic bacterium *Prevotella ruminicola* and cyanobacterium *Anabaena cylindrica* (Cotta *et al.*, 1998); the PSiP protein from *Zea mays* (Moutinho *et al.*, 2001); the MR-1 protein from the anaerobic bacterium *Shewanella oneidensis* (Charania *et al.*, 2009); the AtPPR-AC protein from *Arabidopsis thaliana* (Ruzvidzo *et al.*, 2013); the HpAC1 protein from *Hippeastrum hybridum* (Swiezawska *et al.*, 2014); and the AtKUP7 protein from *Arabidopsis thaliana* (Al-Younis *et al.*, 2015). In this regard therefore, the recombinant AtPPR-AC/K protein becomes the seventh ever AC protein amongst other species to be confirmed this way while at the same time; it is the fifth ever higher plant AC to be confirmed in the same way.

In summary, since results obtained from the previous chapter (Chapter 2) could not conclusively establish whether the recombinant AtPPR-AC/K was a *bona fide* functional AC or not, this chapter now firmly affirms it as a *bona fide* functional AC capable of generating cAMP from ATP, and possibly with functional roles in higher plants that are mediated and/or regulated by cAMP.

3.5 Conclusion

Findings from this chapter provide adequate evidence that the expressed recombinant AtPPR-AC/K protein from *Arabidopsis thaliana* is indeed a *bona fide* functional AC molecule and thereby becoming the sixth ever such candidate to be identified and experimentally confirmed in higher plants while at the same time it also becomes the third ever such molecule to be identified in *Arabidopsis thaliana*.

3.6 Recommendation

Since findings from this chapter have now firmly established the AtPPR-AC/K recombinant protein as a *bona fide* and biologically functional AC, it is therefore recommended that more work be undertaken so as to determine its exact molecular properties and possibly, its exact physiological roles in higher plants.

CHAPTER FOUR

Affinity Purification of the Recombinant AtPPR-AC/K Protein and Determination of its *In Vitro* Adenylate Cyclase Activity

Abstract

The 3′,5′-cyclic adenosine monophosphate (cAMP) from adenosine 5′-triphosphate (ATP), like its analogue, the 3′,5′-cyclic guanosine monophosphate (cGMP) from guanosine 5′-triphosphate (GTP), are essential molecules for cell signalling and multifunctional physiological processes of nearly all kingdoms of life. For several years, the demonstration of potential activity for adenylate cyclases (ACs) (enzymes responsible for the generation of cAMP) in higher plants has always been conducted with the histochemical and/or biochemical methods. However, such conventional methods have since been seriously criticized on the basis of their inherent weakness of heavily relying on either intact cells and/or crude cellular extracts, which in turn always resulted in the final findings being more of rather presumptive deductions rather than firm or conclusive outcomes. It thus become very apparent that only the use of pure protein extracts would surely provide a more convincing and rather highly reliable functional evidence for this very important group of plant molecules. Thus following the recent annotation of a pentatricopeptide protein from *Arabidopsis thaliana* (AtPPR) as a probable AC molecule, we hereby detail the recombinant expression and affinity purification of its AC/kinase-containing fragment domain (AtPPR-AC/K), followed by a practical demonstration of its *in vitro* AC enzymatic activity. In addition, we also show that this truncated recombinant protein is indeed a soluble AC (sAC), whose functional activities are mediated by cAMP via a calmodulin-dependent signalling system.

4.1 Introduction

The optimal expression of recombinant proteins in various vectors and prokaryotic expression systems, including *E. coli*, is very crucial particularly if the targeted recombinant protein is meant for subsequent biochemical, biophysical and/or functional characterization. Generally, a small scale expression is required on early optimization of growth conditions needed for protein detection, and once such conditions have been optimized, then a large scale or over-expression of the target protein can be effected. Apparently, recombinant proteins expressed in *E. coli* at small scale levels normally result in production of soluble native forms whereas those expressed at large scale and in most cases, produce aggregates or inclusion bodies, which are a result of mis-folds and/or mis-localizations of the recombinant proteins in foreign hosts (Yesilirmak and Sayers, 2009). Such aggregated or mis-folded proteins are always insoluble and non-functional even though strategies such as expression in the periplasmic space, or with a tag, and/or utilization of different hosts may improve the situation (Hu and Reddy, 1997).

In order to regain biological or functional activity in an insoluble or aggregated protein molecule, this can be achieved through a solubilization/refolding process. Such a process was once demonstrated on the Arabidopsis thaumatin-like protein (ATLP3), which was purified from its inclusion bodies and then refolded to display a detectable activity against some pathogenic fungi (Hu and Reddy, 1997). Other methods normally used in resolving the problem of inactivity in insoluble proteins include dialysis, slow dilution, rapid dilution, pulse re-naturation as well as chromatography (Lilie *et al.*, 1998; Li *et al.*, 2003; Li *et al.*, 2004). In the solubilization and refolding method, the protein is first dissolved in a chaotropic agent such as 8 M urea or 6 M guanidine chloride followed by a slow and linear dilution of such an agent, to allow the proper formation of correct inter- and intra-molecular interactions (Li *et al.*, 2004). In this Chapter, we describe a step-by-step over-expression, affinity purification, chemical

solubilization and refolding of the recombinant AtPPR-AC/K protein followed by functional characterization of its inherent *in vitro* AC activity.

4.2 Materials and Methods

4.2.1 Over-expression of the Recombinant AtPPR-AC/K Protein

An expression culture of the *E. cloni* EXPRESS BL21 (DE3) pLysS cells harbouring the recombinant AtPPR-AC/K protein was prepared as already outlined in section 2.2.4 of Chapter 2, except that the final concentration of the inducing IPTG (Sigma-Aldric) was now at 3 mM instead of 1 mM. This elevated concentration of the inducer was used so as to attain the targeted high levels (over-expression) of the recombinant AtPPR-AC/K protein. After expression, the induced cells were harvested through centrifugation at 8 000 *g* for 10 minutes and their cell pellet kept at -20°C for further use.

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

After expression, the solubility and/or insolubility nature of the over-produced recombinant AtPPR-AC/K protein was determined to ascertain how much of its fraction could be readily used for the subsequent purification and assaying steps. Briefly, the pelleted induced cells from above (Section 4.2.1) were resuspended in 5 mL of Phosphate Buffered Saline (1X PBS) [140 mM NaCl, 3 mM KCl, 4 mM $Na_2HPO_4.2H_2O$), 1.5 mM KH_2PO_4] supplemented with 1 µg/mL of Lysozyme (Catalog # 1120; Sigma-Aldrich Inc., Missouri, USA) and 1 mM phenylmethylsulfonyl fluoride (PMSF) (Catalog # 62970; Sigma-Aldrich Inc., Missouri, USA) and incubated on ice for 30 minutes. After incubation, the cells were sonicated for 6 cycles: 30 seconds pulsing and 30 seconds chilling cycles on ice and in order to rupture and solubilize the cell contents. The cellular components (crude lysate) were then centrifuged at 10 000 *g* at

4°C for 10 minutes and their supernatant [cleared lysate (with the soluble protein fraction)] transferred into an ice-cold 1.5 mL Eppendorf tube. Both the supernatant and its associated cellular pellet (with the insoluble protein fraction) were then stored on ice. Portions of both fractions were then analyzed by SDS-PAGE and in order to determine the presence and relative proportions of the soluble or insoluble portions of the recombinant AtPPR-AC/K in the expressed protein.

4.2.3 Affinity Purification of the Recombinant AtPPR-AC/K Protein

After realizing that the targeted recombinant AtPPR-AC/K protein was wholly expressed as an insoluble product, the recombinant protein was then purified under non-native denaturing conditions using a HIS-Select nickel-nitrilotriacetic acid (Ni-NTA) affinity matrix and in accordance with the manufacturer's protocol (Catalog # P6611; Sigma-Aldrich Inc., Missouri, USA) as is briefly described below.

4.2.3.1 Preparation of the Cleared Lysate

The whole insoluble fraction (cell pellet) from section 4.2.2 above was resuspended through mixing for an hour in 10 mL of Urea 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]. After 1 hour, the crude lysate was then clarified into a cleared lysate through centrifugation at 10 000 *g* for 20 minutes and the cleared lysate kept for the subsequent downstream purification steps. Part of the cleared lysate (about 20 µL) was also saved for analysis by SDS-PAGE.

4.2.3.2 Binding of the Recombinant AtPPR-AC/K Protein onto the Ni-NTA HIS-Select Affinity Matrix

About 2.5 mL of the nickel-nitrilotriacetic acid (Ni-NTA) slurry matrix (Lot # MG159557; Thermo Scientific Inc., Rockford, USA) were washed twice with 10 mL of sterile distilled water on a rotary mixer for 5 minutes. The washed Ni-NTA beads were then equilibrated for 5 minutes in 10 mL of the Urea Lysis buffer. After equilibration, the Ni-NTA beads were then mixed through rotation on an adjustable Bench Revolver (Labnet International Inc., New Jersey, USA) at 30 rpm at room temperature with the AtPPR-AC/K cleared lysate generated from section 4.2.3.1 was used so that the AtPPR-AC/K protein could bind to the provided bead matrix using its histidine tagged segment. After binding, the mixture was then centrifuged at 10 000 *g* for 5 minutes before the supernatant (flow-through) was removed and part of it (about 20 µL) saved for analysis by SDS-PAGE.

4.2.3.3 Washing of the Bound Ni-NTA HIS-Select Affinity Matrix

After binding, the unbound proteins were all washed off the Ni-NTA bead matrix through repeated washes (three times) with 10 mL of a Urea 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) whereby on each wash, the beads were completely resuspended followed by a total removal of the wash buffer. Portions of each wash together with part of the washed bound beads (about 20 µL) were then all saved for analysis by SDS PAGE.

4.2.3.4 Refolding of the Denatured Purified Recombinant AtPPR-AC/K Protein

The Ni-NTA beads carrying the bound and purified AtPPR-AC/K recombinant protein was pre-packed into an empty 30 cm XK16 plastic Econo-Column (Bio-Rad Laboratories Inc., California, USA). The packed beads were then pre-equilibrated in the Refolding buffer A [8

M urea, 200 mM NaCl, 50 mM Tris-Cl; pH 8.0, 500 mM glucose, 0.05% (w/v) poly-ethyl glycol (PEG), 20 mM β-mercaptoethanol] and in preparation for refolding on a BioLogic F40 Duo-Flow Chromatography System and in accordance with the manufacturer's guidelines (Bio-Rad Laboratories., California, USA). The bound, denatured and purified recombinant AtPPR-AC/K protein was then refolded into its native and functional form using a controlled linearized gradient system, whereby the 8 M urea salt in the Refolding buffer A was gradually diluted to 0 M concentration using a Refolding buffer B [200 mM NaCl, 50 mM Tris-Cl; pH 8.0, 500 mM glucose, 0.05% (w/v) poly-ethyl glycol (PEG), 4 mM reduced glutathione, 0.4 mM oxidized glutathione, and 0.5 mM phenylmethanesulfonylfluoride (PMSF)]. The used linear gradient parameters are shown in Table 4.1 below.

Table 4.1: The linear gradient operation settings for refolding of the denatured purified recombinant AtPPR-AC/K protein, using a BioLogic Duo-Flow medium pressure chromatography system.

Variable	**Value**
Column volume	2.00 mL
Flow rate	0.5 mL/min
Column pressure limit	2.80 MPa
Average time for UV	5 min
System pump for automatic pressure and flow regulation	Normal
Starting concentration of refolding buffer B	0.00%
Targeted concentration of refolding buffer B	100%
Equilibrate column with	4.00 cv
Length of gradient	500 min
Cleaning of the refolded bound beads with washing buffer	5.00 mL
Protein elution with elution buffer	4.00 mL

4.2.3.5 Elution of the Refolded Purified Recombinant AtPPR-AC/K Protein

After refolding, the renatured histidine tagged recombinant AtPPR-AC/K protein was eluted off the Ni-NTA beads with 2 mL of a non-denaturing native Elution buffer [200 mM Nacl, 50 mM Tris-Cl, 0.5 mM PMSF, 250 mM imidazole; pH 8.0] that was directly connected to the Biologic Duoflow system for easier monitoring/controlling with a UV detector at 280 nm.

4.2.3.6 Concentration and Desalting of the Recombinant AtPPR-AC/K Protein

The eluted protein fraction obtained from section 4.2.3.5 was freed from its buffering salts and excess water by pouring it into the upper chamber of a Spin-X UF Concentrator device with a molecular weight cut off (MWCO) limit of 5000 (Product # 431482; Corning Life Sciences Corp., New York, USA). The loaded Spin device was then spun at 2 540 *g* for 4 hours at 4°C or until the final volume has reached 0.1 mL, using a swing-out bucket rotor Z300k centrifuge (Hermle Labortechnik, Wehingen, Germany). The de-salted and concentrated protein fraction was then collected in a 1.5 mL Epperndof tube and its concentration subsequently determined with a NanoDrop ND 2000 spectrophotometer (Thermo Scientific Inc., California, USA). A small portion of the eluted protein fraction (about 20 µL) was then set aside for analysis by SDS-PAGE while the rest was stored at -20°C for further downstream uses.

4.2.4 Functional Characterization of the Purified Recombinant AtPPR AC/K Protein

4.2.4.1 Sample Preparations and Enzyme Immunoassaying

The *in vitro* enzymatic activity of the purified AtPPR-AC/K recombinant protein was determined by assessing the ability of its AC catalytic domain to convert ATP to cAMP in a Tris-buffered system using the cAMP enzyme immunoassay kit (Catalog # CA201; Sigma-Aldrich Corp., Missouri, USA). To determine this activity, 10 µg of the purified recombinant AtPPR-AC/K were assessed in 200 µL reaction mixtures containing the various additives as is

outlined in Table 4.2 below. The assay process included a determination of the functional effects of various molecules and ions (ATP, GTP, Mg^{2+}, Mn^{2+}, Ca^{2+}, HCO_3^-, and F^-) on the enzymatic activity of the AtPPR-AC/K. Alongside this, a measurement of the residual cAMP levels was also done in a setting (control) that contained all incubation components except for the AtPPR-AC/K protein. In all cases, reactions were incubated at room temperature (24°C) for 20 minutes and then terminated by the addition of 1 mM EDTA as well as boiling for 5 minutes. Furthermore, samples were centrifuged for 5 minutes at 9 200 *g* before their lysates were assessed for cAMP content using the kit and following the acetylation version of its protocol. Measurements were taken at 405 nm in triplicate sets using the Microplate Reader (Labtech, International Limited East Sussex, UK) and results were subjected to the statistical analysis of variance (ANOVA).

Table 4.2: Reaction components for the AC functional characterization of the AtPPR-AC/K recombinant.

Reaction Additives	**Tube 1**	**Tube 2**	**Tube 3**	**Tube 4**	**Tube 5**	**Tube 6**	**Tube 7**	**Tube 8**	**Tube 9**
Tris	50 Mm	50 mM	50 mM	50 mM	50 mM	50 mM	50 mM	50 mM	50 mM
IBMX	2 mM	2 mM	2 mM	2 mM	2 Mm	2 mM	2 mM	2 mM	2 mM
$MnCl_2$	5 mM	5 mM	-	5 mM	5 mM	5 mM	5 mM	5 mM	5 mM
Protein	-	10 µg	10 µg	10 µg	10 µg	10 µg	10 µg	10 µg	10 µg
ATP	1 mM	1 mM	1 mM	1 mM	1 mM	1 mM	-	1 mM	1 mM
$MgCl_2$	-	-	5 mM	-	-	-	-	-	-
$CaCl_2$	-	-	-	250 µM	-	-	-	-	-
GTP	-	-	-	-	-	1mM	1mM	-	-
$NaHCO_3$	-	-	-	-	50 mM	-	-	-	-
NaF + $AlCl_3$	-	-	-	-	-	-	-	-	10 mM + 30 µM

4.2.4.2 Statistical Analysis

In vitro results for AC enzymatic immunoassays were performed in triplicates in which data from all corresponding responses of each process were subjected to an analysis of variance (ANOVA) Super-Anova, Stats graphics Version 7, 1993 (Stats graphics Corporation, USA). In addition, wherever the ANOVA revealed some significant variations between treatments, the affected means (n = 3) were then separated by a post hoc Student Newman Kuehls (SNK), multiple range test ($p \leq 0.05$).

4.3 Results

After establishing that the recombinant AtPPR-AC/K, had both the inherent endogenous and *in vivo* AC activities (Chapters 2 and 3 respectively), we then sought to characterize further the AC activity of this protein in its purest form. The approach began with a determination of the solubility/insolubility nature of this protein, followed by its affinity purification and refolding, and then functional characterization.

4.3.1 Determination of the Solubility/Insolubility Nature of the Recombinant AtPPR-AC/K Protein

After expression and in preparation for purification, the recombinant AtPPR-AC/K protein was checked to see how much of it was soluble or insoluble. As is shown in Figure 4.1 below, the protein was being wholly expressed as an insoluble product probably as a result of the production of inclusion bodies due to the inherent mis-foldings and/or mis-localizations of heterologous proteins in foreign hosts (Yesilirmak and Sayers, 2009).

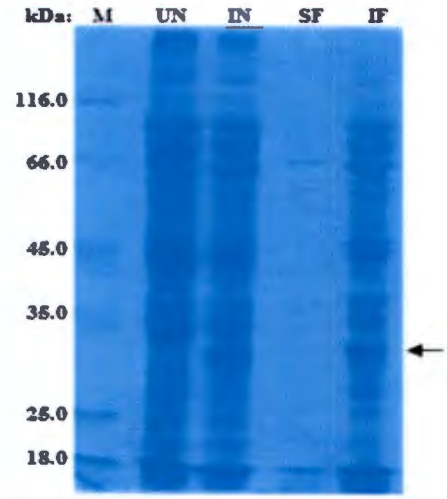

Figure 4.1: Determination of the solubility/insolubility nature of the expressed recombinant AtPPR-AC/K protein. A 12% SDS PAGE showing the relative proportions of the expressed recombinant protein as appearing in the soluble and insoluble fractions of the native extraction buffer. M represents the unstained molecular weight marker (Catalog # SM0431; Fermentas Int.,); UN represents the un-induced cell culture; IN represents the induced cell culture while SF and IF respectively represent the soluble and insoluble fractions of the extraction buffer. The arrow marks the expressed and resolved AtPPR-AC/K protein.

4.3.2 Purification of the Recombinant AtPPR-AC/K

After noting that the produced recombinant AtPPR-AC/K protein was being wholly expressed in an insoluble form, its purification process was then carried out under non-native denaturing conditions. Figure 4.2 below presents the purification regime of this insoluble recombinant protein, which was successfully undertaken.

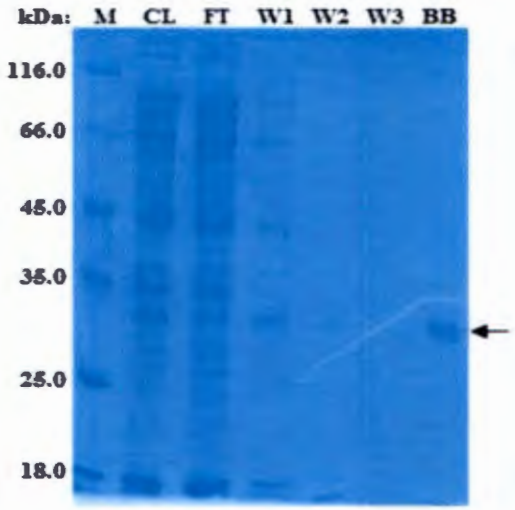

Figure 4.2: Affinity purification of the recombinant AtPPR-AC/K protein. An SDS-PAGE of the purification profile of the recombinant AtPPR-AC/K protein bound on the Ni-NTA agarose affinity matrix. M represents the unstained molecular weight marker (Catalog # SM0431; Fermentas Int.,); CL represents the cleared lysate obtained after clarification of the ruptured/lysed inclusion bodies in a urea lysis buffer, FT represents the flow-through which was obtained after passing the cleared lysate through the Ni-NTA agarose affinity matrix; W1, W2 and W3 represent the three successive washes that were undertaken on the bound Ni-NTA agarose affinity matrix; while BB represents the purified recombinant protein on the Ni-NTA agarose affinity matrix. The arrow marks the AtPPR-AC/K recombinant protein.

4.3.3 Refolding, Elution, Concentration and Desalting of the Purified Recombinant AtPPR-AC/K Protein

Since the recombinant AtPPR-AC/K protein purified in section 4.3.2 on the Ni-NTA agarose affinity matrix was still in its non-native denaturing form, its refolding into its native and biologically active form was therefore, very necessary. In this regard, the recombinant AtPPR-AC/K protein was then refolded into its native functional form through a linear dilution of the 8 M urea buffer to a 0 M concentration. The refolded protein was then eluted off the Ni-NTA agarose affinity matrix before being desalted and concentrated for the subsequent *in vitro* functional characterizations. Figure 4.3 below shows the successfully refolded, eluted, desalted and concentrated recombinant AtPPR-AC/K.

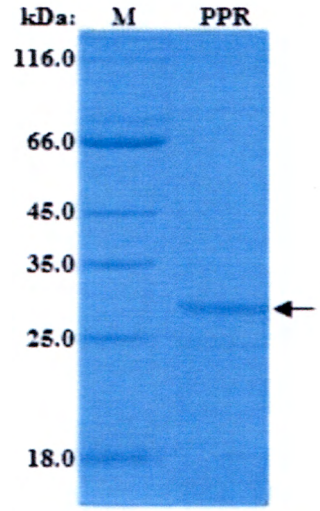

Figure 4.3: Refolding, elution, desalting and concentration of the purified recombinant AtPPR-AC/K protein. A 12% SDS-PAGE showing purified AtPPR-AC/K recombinant protein after refolding into a native and biologically active product on a Ni-NTA agarose affinity matrix. The refolded protein was then eluted into a native solution before being desalted and concentrated. M represents the unstained protein molecular weight marker (Catalog # SM0431; Fermentas Int.,) while PPR represents the refolded, eluted desalted and concentrated recombinant AtPPR-AC/K protein. The arrow marks the resultant and final protein product.

4.3.4 *In Vitro* Characterization of the AC Activity of the Recombinant AtPPR AC/K Protein

After obtaining the purified and refolded form of the recombinant AtPPR-AC/K protein, its AC enzymatic activity was then further characterized *in vitro* using the cAMP-linked enzyme immunoassay system (Catalog # CA201; Sigma-Aldrich Inc., Missouri, USA). Essentially, the AtPPR-AC/K's ability to generate cAMP from ATP as well as its different responses to the various molecules and chemical ions (ATP, GTP, Mg^{2+}, Mn^{2+}, Ca^{2+}, CO_3^{2-}, F^-) *in vitro* were assessed and determined as is shown in Figure 4.4 below.

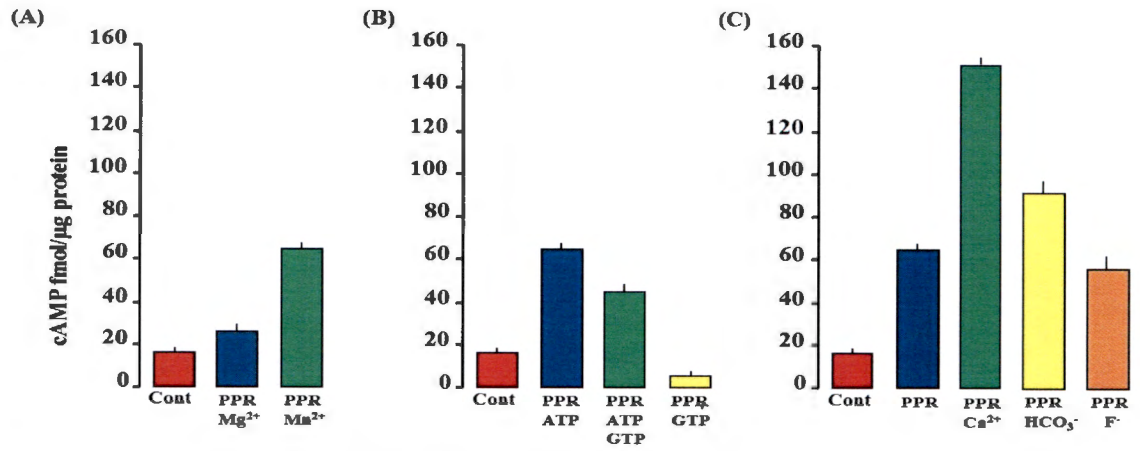

Figure 4.4: *In vitro* characterization of the AC Activity of the recombinant AtPPR-AC/K protein. A reaction mixture containing 10 µg of the purified recombinant AtPPR-AC/K protein, 50 mM Tris-HCl; pH: 8.0, 2 mM IBMX, 5 mM Mn^{2+}, 1 mM ATP and/or in the presence of other additives was incubated at room temperature for 20 minutes. The generated cAMP was then measured with a cAMP-specific enzyme immunoassaying kit (catalog # CA201; Sigma-Aldrich Inc.,) based on the acetylation version of its protocol. (A) Cyclic AMP levels generated with the purified recombinant AtPPR-AC/K protein in the presence of either Mn^{2+} or Mg^{2+} ions, (B) cAMP levels generated with the purified recombinant AtPPR-AC/K in the presence of either ATP and/or GTP, and (C) cAMP levels generated with the purified recombinant AtPPR-AC/K protein in the presence of Ca^{2+}, CO_3^{2-} or F^- ions. All error bars represent the standard errors (SEM) of the means of three independent and representative assays (n = 3).

4.4 Discussion

In order to properly determine the molecular properties of the expressed recombinant AtPPR-AC/K protein (Chapter 2), such experiments were undertaken with a purified version of this recombinant. However, before the actual purification process was undertaken, it was very crucial to first determine the solubility or insolubility nature of the expressed protein so that a suitable purification strategy could then be effected. As is shown in Figure 4.1, the produced recombinant AtPPR-AC/K protein was wholly expressed in an insoluble form that is always biologically non-functional (Yesilirmak and Sayers, 2009). This is probably because when *E. coli* is used as a host system to over-express foreign or heterologous proteins, in most cases, it yields such proteins in form of aggregates or inclusion bodies as a result of both mis-folds and mis-localizations (Yesilirmak and Sayers, 2009). To most prokaryotic hosts including *E. coli*, this is a survival strategy when faced with a high demand of expressing somewhat unnecessary

foreign proteins, which in most cases require some posttranslational modifications (Nishihara *et al.*, 1998). However, and in some instances, a co-expression of foreign proteins with chaperones comes in handy and rescue the situation (Nishihara *et al.*, 1998).

After establishing the insolubility nature of the expressed recombinant AtPPR-AC/K, a non-native denaturing affinity purification strategy was then tactically selected and utilized. The method involved utilization of the Ni-NTA affinity system, which has a specific and reversible binding to all poly-histidine tagged fusion recombinants like AtPPR-AC/K. This system is typically very rapid and simple while at the same time, allowing an easy removal of other contaminants, even under the strongly and harsh denaturing conditions required to initially solubilise the inclusion bodies (Crowe *et al.*, 1994). As is shown in Figure 4.2, the recombinant AtPPR-AC/K protein was successfully purified using this outlined approach.

Since the recombinant AtPPR-AC/K protein was purified under denaturing conditions, it was then subjected to a subsequent refolding system (Table 4.1) to convert it into a native and biologically active form (Singh and Panda, 2005). The refolding process was undertaken whilst the recombinant AtPPR-AC/K protein was still bound onto the Ni-NTA affinity matrix. This aspect is strongly encouraged because firstly, the matrix provides a suitable scaffold for the refolding protein (QIAexpressionist, 2003) and secondly, the bound His-tag of the fusion recombinant would have limited interferences with forming protein structure and thus its ultimate function (Ramos *et al.*, 2004). The refolded purified recombinant AtPPR-AC/K protein was then eluted off the Ni-NTA matrix (Figure 4.3) before being functionally characterized *in vitro*.

After purification, refolding and elution, the AC activity of the recombinant AtPPR-AC/K was then further characterized *in vitro* using the cAMP enzyme immunoassay system (Catalog # CA201, Sigma-Aldrich Corp., Missouri, USA). Previously, it has been shown that the divalent cations, Mg^{2+} and Mn^{2+} play essential roles in the regulation of activity of both ACs and GCs (Hurley, 1999; Seebeck *et al.*, 2008). With such an insight on our study, we then assessed the enzymatic activity of the recombinant AtPPR-AC/K with these two metal ions and realized that the protein preferred Mn^{2+} to Mg^{2+} as its co-factor of activity, where it exhibited a more than 2.1-fold increase with the former than the latter (Figure 4.4A). This particular trend has also been previously noticed in a sAC of the rat testis, where the AC activity was strongly dependent onto the Mn^{2+} metal ion but being highly insensitive to the Mg^{2+} ion (Braun, 1974; Braun, 1975). More so, it has also been noticed that the truncated recombinant AtPPR-AC/K contains a glutamate [E] amino acid within its catalytic centre that is essential for the specific binding of divalent metal ions (Kitada *et al.*, 1995).

Substrate specificity for the recombinant AtPPR-AC/K protein between ATP and its structural analogue GTP was also assessed by testing its ability to generate cAMP from either or both of the two molecules (Figure 4.4B). Between the molecules as possible substrates, the recombinant protein exhibited a more than 6-fold increase in activity with ATP than with GTP and when both substrates were simultaneously used at equimolar concentrations; the ATP-dependent protein activity was lowered by a factor of more than 1.6. By considering the nature of these outcomes, it is likely that there is competition between the two analogues for binding onto the catalytic site of the recombinant AtPPR-AC/K protein, yet ATP is the preferred substrate for catalysis. This competitive inhibitory scenario was previously and inversely seen in rat intestines, whereby the GTP-dependent activity of a particulate GC was inhibited by the presence of the ATP substrate (Parkinson *et al.*, 1994). Conceivably, in all class III ACs

including AtPPR-AC/K; the specificity for binding an adenine moiety involves the Lys and Asp residues (Sunahara *et al.*, 1998) both of which are present in the catalytic centre of AtPPR-AC/K, and thus the demonstrated high preference for ATP than GTP.

The activity of the recombinant AtPPR-AC/K protein was further assessed against the various commonly known AC modulators; calcium, hydrogen carbonate and sodium flouride. With calcium, the enzymatic activity of AtPPR-AC/K was enhanced by a factor of 2.4, with hydrogen carbonate, by a factor of 1.3 while with sodium flouride; there was no apparent effect (Figure 4.4C). From these outcomes, we firmly established that the AtPPR-AC/K protein was indeed a cytosolic soluble AC because of the two known forms of ACs, only sAC (Lomovatskaya *et al.*, 2008) are functionally activated by the calcium or bicarbonate ions (Okamura *et al.*, 1985; Garty and Salomon, 1987; Carricarte *et al.*, 1988; Visconti *et al.*, 1990; Chen *et al.*, 2000) but not by the fluoride ion, which on the other hand, only activates transmembrane ACs (tmACs) (Kamenetsky *et al.*, 2006) and not sACs (Rall and Sutherland, 1958). This, together with findings from other sections of this study, indicates that all sACs and not tmACs are dependent on the Mn^{2+} metal ion as a co-factor for activity (Figure 4.4A) (Braun, 1974; Braun, 1975) while on the other hand, all tmACs and not sACs are modulated by forskolin (Figure 2.6; Chapter 2) (Seamon *et al.*, 1981; Forte *et al.*, 1983; Laurenza *et al.*, 1989). Notably, all sACs are mediated by the second messenger, cAMP via control mechanisms that are regulated by the calcium-binding protein (calmodulin) as compared to the tmACs, which are mediated by the second messenger, cAMP via control mechanisms that are regulated by the GTP-binding proteins (G-proteins) (Kamenetsky *et al.*, 2006).

4.5 Conclusion

Findings from this chapter have unequivocally established the AtPPR-AC/K protein as a *bona fide* soluble AC (sAC) whose functional activities are regulated by cAMP via a calmodulin.

4.6 Recommendation

Now that the functional activity of the AC domain within the twin-domain AtPPR-AC/K protein has been firmly established, it is thus crucial that extra work is undertaken to further understand its probable cross-talking scenarios with the co-existing kinase domain within this protein and possibly, establishing the exact functional role of this twin-domain protein in plants.

CHAPTER FIVE

Determination of the *In Vitro* Kinase Activity of the Recombinant AtPPR-AC/K Protein

Abstract

In higher plants, cell communication and cell signalling systems are mainly mediated by two typical protein components, the adenylate cyclases (ACs) and kinases, both of which utilize adenosine 5′-triphosphate (ATP) as the sole substrate. While ACs hydrolyse ATP into the second messenger 3′,5′-cyclic adenosine monophosphate (cAMP), kinases use the ATP to phosphorylate other protein candidates in a process termed trans-phosphorylation or to phosphorylate themselves in a process termed auto-phosphorylation. Once the cAMP and phosphorylated molecules have been produced, the various downstream cellular signalling cascades can now then be cordially activated and appropriately driven. Plant kinases exist as a special group of protein molecules termed receptor-like kinases (RLKs), which mostly phosphorylate the serine and threonine amino acid residues, with a sequence homology and structural similarity to the other related group termed the receptor tyrosine kinases (RTKs) found in animals and mostly involved in the phosphorylation of the tyrosine residues. Within the RLKs group, most of the proteins bear an architectural structure that consists of an extracellular domain that binds to the different types of signals/ligands, followed by a single transmembrane-spanning domain, which functions in anchoring the protein onto the cell membrane and an intracellular region containing the Ser/Thr kinase-like domain that transduces the signals. In some cases, the kinase domain does encapsulate or co-exists with an intrinsic guanylate cyclase (GC) or AC domain, both of which are very crucial for a variety of plant cellular signalling processes. In this study, therefore, we detail the recombinant expression and affinity purification of the truncated version of an Arabidopsis

pentatricopeptide (AtPPR) protein annotated to contain both the kinase and AC domains (AtPPR-AC/K) followed by a practical demonstration of this protein's intrinsic *in vitro* trans-phosphorylation and auto-phosphorylation kinase activities.

5.1 Introduction

In nature and for any living organism to enable the regulatory mechanisms necessary for its cell communication and signalling systems, various types of signal molecules such as proteins, small peptides, amino acids, nucleotides, steroids and retinoids have to be released from its internal environment so that they are then used in these specified cellular systems (Alberts *et al.*, 2002). Irrespective of the type of an external signal, a target cell has to respond with only a specific receptor, which will bind the arriving signal molecule and then initiate an appropriate response in the target cell (Alberts *et al.*, 2002). Within this context, a specialized type of enzymes, termed kinases have been identified and shown to catalyze the phosphorylation of various substrates, which in turn, results in a modification of the conformational structures of such substrates altering their activation states and also forming switches in signalling cascades of the involved target cells (Hanks *et al.*, 1988). Another specialized type of enzymes within this same context are the adenylate cyclases (ACs), which catalyze the formation of 3′,5′-cyclic adenosine monophosphate (cAMP) from adenosine 5′-triphosphate (ATP), after which the generated cAMP would then play significant roles in the cell signalling and transduction pathways of the involved target cells (Cassel and Selinger, 1976; Donaldson *et al.*, 2004).

In both eukaryotes and prokaryotes, signal transduction pathways have been elucidated by a number of well characterized receptor protein kinases, during which a ligand-binding scenario will trigger the kinase activity of the involved receptor, and then a subsequent signal transduction system occurring via the various protein kinase cascades (Trewavas and Malho,

1997). Other remaining and related receptors will then activate their respective cytoplasmic or plasma membrane localized members of the GTPase superfamily, which will result in the synthesis or release of second messengers like cAMP or cGMP (Trewavas and Malho, 1997). In plant systems, a vast number of receptor-like kinases (RLKs) have been identified with more than 400 and 1000 RLK genes present in *Arabidopsis thaliana* and rice (*Oryza sativa*) respectively (Shiu and Bleecker, 2001b; Shiu *et al.*, 2004), and a few of them having been fully characterized (Heffani *et al.*, 2004). Among those RLK members found in *A. thaliana*, some of them have since been shown to possess some intrinsic *in vitro* guanylate cyclase (GC) and kinase activities; such members being the brassinosteroid receptor (BRI1; brassinosteroid insensitive 1) (Kwezi *et al.*, 2007), the wall-associated kinase-like 10 (Meier *et al.*, 2010), the elicitor peptide 1 receptor (Qi *et al.*, 2010) and the phytosulfokine receptor 1 (PSKR1) (Kwezi *et al.*, 2011). Apparently, and even after having illustrated such an intrinsic dual catalytic property of the RLKs, it is still unknown as to which mechanisms are involved in the regulation and modulation of such functions in these protein molecules.

Ideally, all plant RLKs genes code for proteins with an architectural structure that consists of an extracellular domain that binds the different types of signals/ligands, followed by a single transmembrane-spanning domain, which functions in anchoring the protein onto the cell membrane and an intracellular region containing the Ser/Thr kinase-like (or at times, a Tyr kinase-like domain), which transduces the signal (Walker, 1994). In some cases, the kinase domain does encapsulate or co-exists with an intrinsic GC domain (Braun *et al.*, 1997), both of which are known to play crucial roles in a variety of cellular processes like disease resistance, self-incompatibility, hormone perception and development (Flor, 1971; Stein *et al.*, 1996; Clark et al., 1997; Li and Chory, 1997). Apparently, while the existence of RLKs with a GC/kinase twin domain has been reported in plants (Kwezi *et al.*, 2007; Meier *et al.*, 2010;

Qi *et al.*, 2010; Kwezi *et al.*, 2011), to-date, no report on the existence of any RLK molecule with an AC/kinase twin domain has ever been released. In this regard and following the recent annotation of a pentatricopeptide repeat protein from *Arabidopsis thaliana* (AtPPR) as an RLK harbouring a probable AC/kinase twin domain (Gehring, 2010), we therefore detail the recombinant expression and affinity purification of its truncated AC/kinase-containing fragment domain (AtPPR-AC/K) followed by an *in vitro* functional characterization of its probable kinase activity. In addition, we also attempt to establish a probable cross-talking scenario between these two co-existing catalytic domains of the AtPPR-AC/K.

5.2 Materials and Methods

5.2.1 Determination of the Kinase Activity of the Recombinant AtPPR-AC/K Protein

The purified native protein produced in Chapter 4 was used in this chapter to assess the possible kinase activity of the recombinant AtPPR-AC/K protein. Both the trans-phosphorylation and auto-phosphorylation capacities of this recombinant were assessed *in vitro* together with the possible effects of cAMP on trans-phosphorylation.

5.2.1.1 The Trans-phosphorylation Activity

The trans-phosphorylation capacity of the purified recombinant AtPPR-AC/K protein was assessed *in vitro* by measuring its ability to direct the phosphorylation of special substrate peptides as is described by the Omnia™ Recombinant system (Catalog # KNZ1241/KNZ3101; Life Technologies, Carlsbad, USA). Briefly, a 50 µL reaction system containing 10 µg of the purified recombinant AtPPR-AC/K, 1X reaction buffer, 1 mM of ATP and/or GTP, 0.2 mM DTT, and 25 µM Ser/Thr or Tyr-peptide was prepared in a Black FluoroNunc Maxisorp 96-well plate (AEC Amersham, Little Chalfont, UK) and incubated at 30°C for 5 minutes. This was followed by an immediate measurement of the phosphorylation activity in form of

fluorescence signals at 485 nm (λ_{em} 485) after a reaction excitation at 360 nm (λ_{ex} 360) using a Fluoroskan Ascent FL fluorometer (AEC Amersham, Little Chalfont, UK). All activity readings were recorded at 30°C after every 1 minute for 30 minutes, in triplicate forms and as relative fluorescence units (RFUs).

5.2.1.1.1 Effects of cAMP on Trans-phosphorylation

Reaction set-up spiked with 0.0, 0.2, 0.4, 0.6, 0.8 and 1.0 µM cAMP were prepared and recorded as has already been described in section 5.2.1.1 above. These specific reactions were set out so as to assess the apparent effect of the AC domain onto the kinase domain within the recombinant AtPPR-AC/K protein and as the two co-existing domains share a common substrate (ATP).

5.2.1.1.2 The Trans-phosphorylation Reaction Kinetics

Individual reaction set-up with increasing concentrations of the Omnia™ recombinant Ser/Thr-peptide (1.56; 3.125; 6.25; 12.5 and 25.0 µM) and 1 mM ATP or 1 mM GTP as the sole substrate of phosphorylation were prepared and recorded as has already been described above (section 5.2.1.1). Initial velocity for each of the used peptide concentration was then determined followed by a sketching of the Hanes plot (Meier *et al.*, 2010; Kwezi *et al.*, 2011). The kinetic constants (K_m and V_{max}) for the recombinant AtPPR-AC/K with either ATP or GTP as the sole substrate of phosphorylation were then derived from this plot, where K_m was determined as the negative value of the x-intercept ($x = -K_m$, when $y = 0$) of a linear fit of the data while V_{max} was calculated from the y-intercept ($y = K_m/V_{max}$, when $x = 0$) of the same linear fit.

5.2.1.2 The Auto-phosphorylation Activity

The auto-phosphorylation capacity of the purified recombinant AtPPR-AC/K protein was assessed by first setting a de-phosphorylation reaction whereby 10 μg of the recombinant AtPPR-AC/K protein were mixed with 400 units of the lambda phosphatase enzyme in the presence of 100 mM Nacl, 50 mM HEPES, 2 mM DTT, 0.1 mM $MnCl_2$, 0.1 mM EGTA, 50% glycerol and 0.01% (v/v) Brij 35; pH 7.5 (Merck Group, Darmstadt, Germany). The reaction mix was then incubated for 2 hours at room temperature before either termination with 2X SDS loading buffer [125 mM Tris-HCl pH 6.8, 20% (v/v) glycerol, 0.01% (w/v) bromophenol blue, 4% (w/v) SDS and 200 mM DTT] or a re-phosphorylation treatment following the addition of a 4X PhosStop reagent (Roche Products, Basel, Switzerland). In the second step, a re-phosphorylation reaction was initiated by adding 1 mM ATP or 1 mM GTP to the de-phosphorylated samples in the presence of 20 mM Tris-HCl; pH 7.5, 15 mM $MgCl_2$ and 0.2 mM DTT and incubating for 30 minutes at room temperature. The reactions were then terminated with the addition of a 2X SDS loading buffer and boiling for 5 minutes at 95°C. After boiling, the samples were allowed to cool down and then centrifuged at 10 000 *g* for 1 minute.

The de-phosphorylation, re-phosphorylation as well as the untreated protein sample (control) were all resolved on a 12% SDS-PAGE gel followed by staining with a Coomassie solution (20% ethanol, 20% methanol, 20% acetic acid, and 0.5% Coomassie stain) for 60 minutes. The stained gel was de-stained for 60 minutes (or until the bands were clear enough to be visualized) in a de-staining solution (20% ethanol, 20% methanol, and 20% acetic acid). The de-stained gel was snap-photographed on a Chemi-Doc™ Imaging system (Bio-Rad Laboratories Inc.,) using the Bio-Image Lab™ software. The gel was then completely de-stained overnight in another de-staining solution (80% acetic acid, 20% ethanol, and 20% methanol). This de-

staining solution besides removing all the Coomassie stain from the gel, it also fixed the gel by ensuring a complete removal of the SDS buffer that could later on interfere with the phosphorylation staining signal.

The next morning, the de-stained gel was washed three times (10 minutes per wash) with distilled water before being stained with the Pro-Q Diamond phosphoprotein G solution (Life Technologies Inc., Carlsbad, USA) in the dark for 90 minutes. The stained gel was then de-stained three times (30 minutes per each step) with the de-staining solution [20% acetonitrile and 50 mM sodium acetate; pH 4.0] in the dark. The de-stained gel was finally washed twice (5 minutes per wash) with distilled water. The resultant phosphoprotein bands were then analyzed on a Chemi-Doc™ Imaging system (Bio-Rad Laboratories Inc.,) using Bio-Image Lab™ software at 488 nm blue laser and 520 nm band-pass (BP) and under a 40 emission filter. The specific pixel intensities were then finally quantified using Image-J software (http://rsbweb.nih.gov/ij/).

5.2.1.3 Statistical Analysis of the *In Vitro* Kinase Activity Assays

Data from all *in vitro* kinase activity assays were obtained from three replicates in which readings from all corresponding responses were subjected to an analysis of variance (ANOVA) Super-Anova, Stats graphics Version 7, 1993 (Stats graphics Corporation, USA). In addition, and wherever ANOVA revealed some significant variations between treatments, the affected means ($n = 3$) were then separated with a post-hoc Student Newman Kuehls (SNK), multiple range test ($p \leq 0.05$).

5.4 Results

5.4.1 The Trans-Phosphorylation Activity of the Recombinant AtPPR-AC/K Protein

When the purified recombinant AtPPR-AC/K was tested for its possible ability to trans-phosphorylate other molecules, it showed a very significant activity that was amenable with both the ser/thr and tyr residues even though its activity was relatively higher for the former than the latter (Figure 5.1A). In addition, the recombinant preferred the use of GTP to ATP as its sole source of the phosphate group even though both substrates could be ultimately utilized (Figure 5.1B).

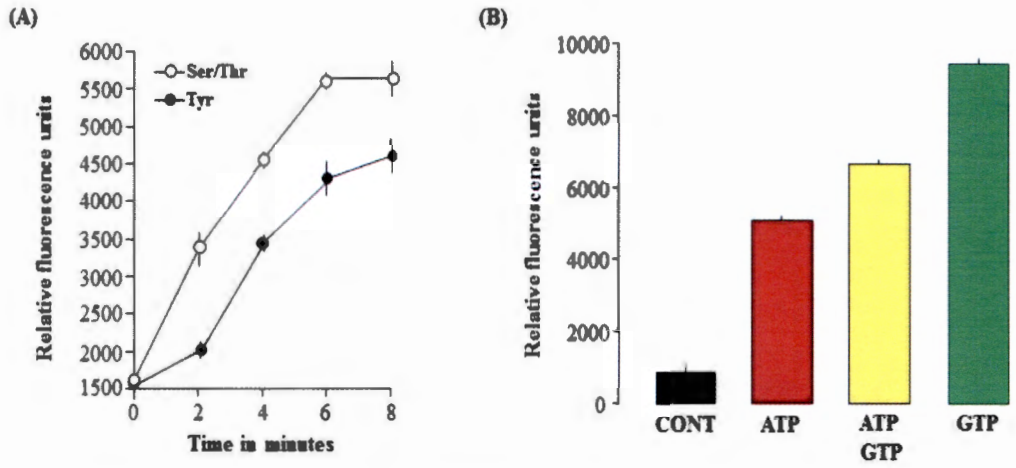

Figure 5.1: Demonstration of the trans-phosphorylation activity of the recombinant AtPPR-AC/K protein. (A) Time-dependent (calibration) curve produced by 10 µg of the recombinant AtPPR-AC/K protein in a reaction mixture containing 1X reaction buffer, 1 mM ATP, 0.2 mM DTT, and 25 µM Ser/Thr-peptide or 25 µM Tyr-peptide. **(B)** Effects of GTP on the kinase activity of the recombinant AtPPR-AC/K, where CONT is the control, ATP is when ATP is the sole substrate, ATP/GTP is when both ATP and GTP are substrates (co-substrates) and GTP is when GTP is the sole substrate. All experiments were performed in triplicate sets, and error bars represent the standard errors (SEM) of the means of three independent and representative assays.

5.4.1.1 Effects cAMP on Trans-phosphorylation

Since the recombinant AtPPR-AC/K protein has already been shown to be capable of generating cAMP from ATP (Chapters 2; 3 and 4), we then sought to test if such generated cAMP had any apparent effect (suppression or enhancement) on the kinase activity of this

recombinant and as part of a possible cross-talking scenario between the two co-existing domains in this protein. As is shown in Figure 5.2A below, cAMP had a massive suppressive effect on the kinase activity of the recombinant AtPPR-AC/K when ATP was used as the sole substrate but no apparent effect when GTP was used as the sole substrate. In Figure 5.2B, a calibration curve of the suppressive effects of the cAMP onto the kinase activity of the recombinant AtPPR-AC/K protein when ATP is the sole substrate, is also shown.

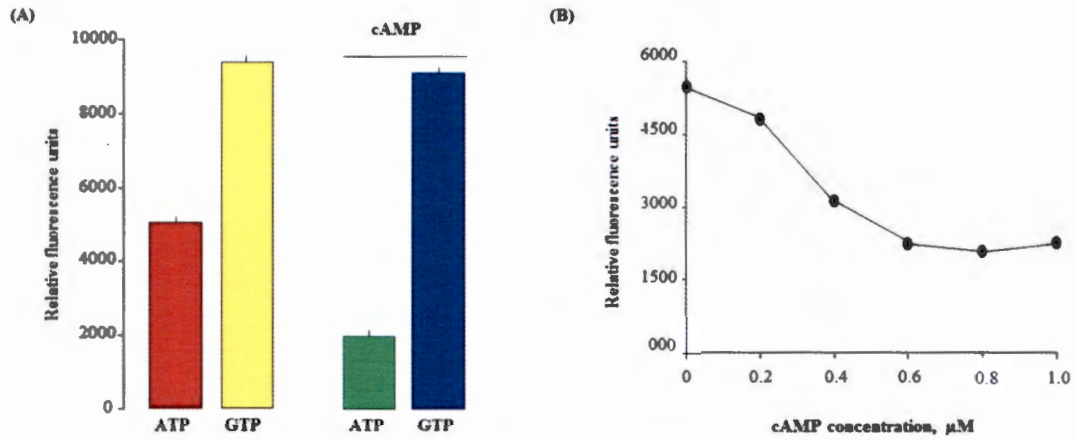

Figure 5.2: Demonstration of the effects of cAMP on the kinase activity of the recombinant AtPPR-AC/K protein. (**A**) Effects of 1.0 µM cAMP on the kinase activity of the recombinant AtPPR-AC/K in a reaction mixture containing 1X reaction buffer, 1 mM ATP or 1 mM GTP, 0.2 mM DTT, and 25 µM Ser/Thr-peptide. (**B**) A calibration curve of the cAMP (0-1.0 µM) when ATP is the sole substrate. Error bars represent the standard errors (SEM) of the means of three independent and representative assays.

5.4.1.2 The Trans-phosphorylation Reaction Kinetics

In order to determine the reaction kinetics of the recombinant AtPPR-AC/K protein, a titration reaction system of the Ser/Thr-peptide (0.0, 1.56, 3.125, 6.25, and 12.5 µM) using either ATP or GTP as the sole substrate of phosphorylation was undertaken followed by a sketching of the Hanes plot. From this plot, the relative reaction kinetics values for the recombinant AtPPR-AC/K protein, using either of the two sole substrates (ATP or GTP), were then derived and determined. As is shown in Figure 5.3 below, the recombinant AtPPR-AC/K had a relatively lower reaction kinetics rate when ATP was the sole substrate of phosphorylation ($K_m \approx 2.96$

μM and V_{max} ≈ 2276.92 nmoles/min/mg protein) (Figure 5.3A) than when GTP was the substrate (K_m ≈ 1.87 μM and V_{max} ≈ 4794.87 nmoles/min/mg protein) (Figure 5.3B).

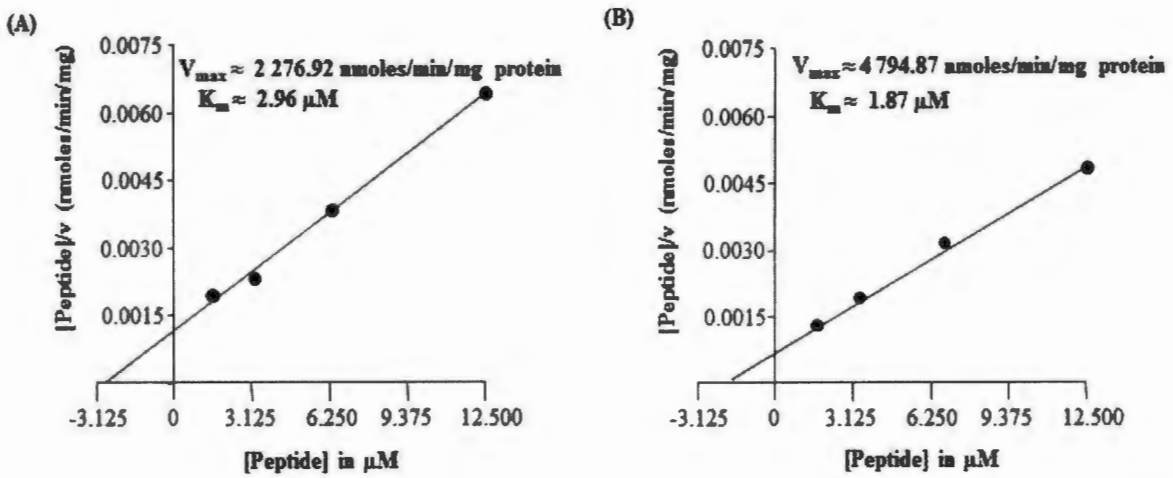

Figure 5.3: Determination of the reaction kinetics rates of the recombinant AtPPR-AC/K protein. Hanes plots for determining the reaction kinetics rates of the recombinant AtPPR-AC/K using either (**A**) ATP or (**B**) GTP as the sole substrate of phosphorylation. For both substrates, K_m was determined as the negative value of the x-intercept ($x = -K_m$, when $y = 0$) of the linear fit of the data while V_{max} was calculated from the y-intercept ($y = K_m/V_{max}$, when $x = 0$) of the same linear fit. All values obtained indicate the means of three independent and fully representative experiments ($n = 3$).

5.4.2 The Auto-phosphorylation Activity of the Recombinant AtPPR-AC/K Protein

After determining the trans-phosphorylation activity of the recombinant AtPPR-AC/K protein above (section 5.4.1), its probable auto-phosphorylation capacity was also assessed. Generally, auto-phosphorylation is a biochemical process by which proteins undergo during their posttranscriptional modifications and has critical roles in central cellular processes such as signalling and regulation (Hutti *et al.*, 2004). As is shown in Figure 5.4, the recombinant AtPPR-AC/K had a fully viable auto-phosphorylation activity that was highly pronounced when ATP was used as a sole substrate than GTP.

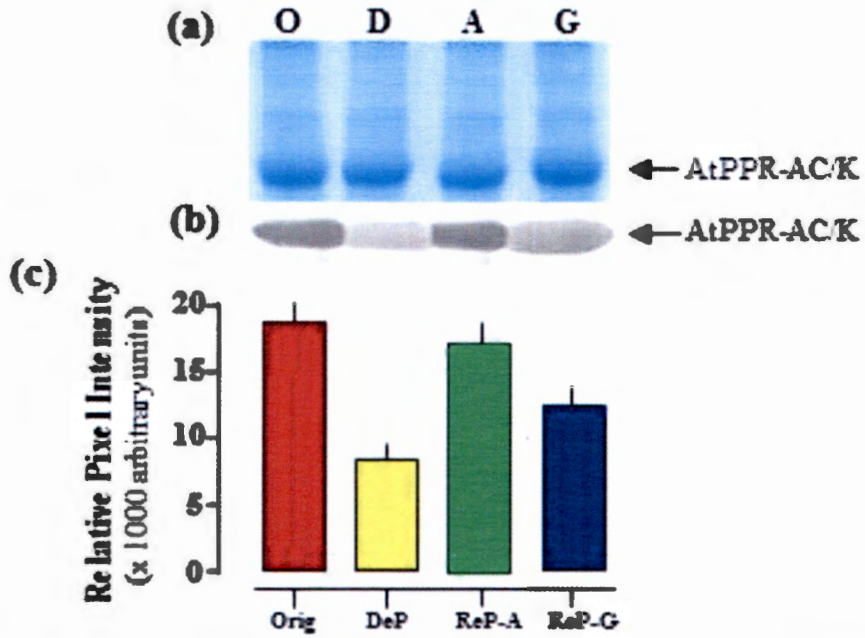

Figure 5.4: Demonstration of the auto-phosphorylation capacity of the recombinant AtPPR-AC/K protein. The purified (10 µg) recombinant AtPPR-AC/K (O/Orig or control) was de-phosphorylated for 2 hours using a lambda phosphatase (D/Dep) and then allowed to re-phosphorylate itself in the presence of either ATP (A/Rep-A) or GTP (G/Rep-G) followed by an SDS-PAGE resolution of such samples. (**a**) Resolved samples as stained with Coomassie brilliant blue, (**b**) resolved samples as stained with the ProQ-Diamond phosphoprotein stain and (**c**) phosphoprotein band pixel intensities as analyzed by the Image J software (ANOVA; $P < 0.05$; n = 3).

5.5 Discussion

The 3′,5′ cyclic adenosine monophosphate (cAMP) is a key second messenger molecule produced from ATP through the hydrolytic action of adenylate cyclases (ACs) that has been shown to play fundamental roles in a number of physiological cellular responses in higher plants (Thomas *et al.*, 2013). Plants, due to their sessile nature, tend to experience constant challenges from their periodic exposure to the various environmental stimuli which affect their productivity and developmental systems. In that case, many research groups have therefore, invested most of their time in attempting to discover and/or explain the various complex mechanisms by which plants can cope with the different biotic and abiotic stress factors.

The pentatricopeptide repeat protein (AtPPR) coded for by the At1g62590 gene was identified as a probable twin-domain molecule harbouring two annotated catalytic domains namely, the kinase and adenylate cyclase (AC) domains (Gehring, 2010). In addition, it was also found to be primarily localized with the plasma membrane and involved in various post-transcriptional modifications such as RNA splicing, RNA editing, RNA processing and RNA translation of the main cellular organelles like the chloroplast and mitochondria (Nakamura *et al.*, 2004; Kotera *et al.*, 2005; Schmitz-Linneweber *et al.*, 2005). This AtPPR protein belongs to a super group of plant proteins termed the receptor-like kinases (RLKs), which forms one of the largest gene families that regulate critical plant physiological processes such as growth, development and responses to the various environmental stress factors (Osakabe *et al.*, 2013). These RLKs have also been noted to be centrally involved in the mediation processes of perceiving extracellular stimuli and activating signalling responses in plants, which eventually leads to a modification of various cellular concentrations that activates protein phosphorylation pathways. Thus, such pathways do control the expression of stress-to-adaptive response genes in plants, with an ultimate goal of reducing the negative effects of stress factors or completely eliminating such causative environmental stress factors (Lee *et al.*, 2011; Xing *et al.*, 2011).

Principally, the regulation of cellular and physiological processes in plants is mainly driven by two specialized types of enzymes namely, the ACs which catalyze the conversion of ATP into cAMP (Cassel and Selinger, 1976; Codina *et al.*, 1983; Gilman, 1987) and kinases, which add a phosphate moiety (from ATP or GTP) to other molecules or to themselves through trans-phosphorylation and auto-phosphorylation respectively (Hanks and Hunter, 1995; Shiu and Bleecker, 2001b). This specialized process of protein phosphorylation has been reported to mainly occur on the serine (Ser), threonine (Thr), tyrosine (Tyr) and histidine (His) amino acids as key residues for the posttranslational modification of various proteins required for cell signal

transduction in eukaryotes (Nongpiur *et al.*, 2012). This process actually influences protein activity by inducing conformational changes on the involved proteins as a result of exposing, activating or hiding their catalytic sites (Hutti *et al.*, 2004).

One group of plant RLKs termed, receptor guanylate cyclase (GC), has been shown to possess a unique structural domain organization in which the receptor kinase is harboured within the putative GC catalytic centre or co-existing within the C-terminal end of the protein (Wong and Gehring, 2013). Such experimentally tested and successfully confirmed twin-domain receptor GCs include, the AtWAKL10 (Meier *et al.*, 2010), the AtBRI1 (Kwezi *et al.*, 2007), the AtPepR1 (Qi *et al.*, 2010) and the AtPSKR1 (Kwezi *et al.*, 2011) - all containing the overlapping functional kinase and GC catalytic domains. From this unique structural domain organization, it might suggest that this widespread and novel molecular structural design may be mediated by second messenger molecules like cAMP and cGMP in the regulation of complex networks involved in the various cell signalling pathways. Therefore, by considering the fact that the AtPPR protein from *Arabidopsis thaliana* has already been annotated as a probable twin domain molecule harbouring the kinase and AC domains (Gehring, 2010) and also that its truncated version (AtPPR-AC/K) has already been shown in this study to possess an intrinsic AC activity (Chapters 2-4), more work on determination of the possible kinase activity of the AtPPR-AC/K recombinant was hereby undertaken in this chapter. In addition, the work was also undertaken in light of attempting to elucidate a probable crossing-talking scenario between the two co-existing catalytic domains.

Using ATP as a sole substrate and testing the recombinant AtPPR-AC/K protein for its ability to phosphorylate the serine, threonine or tyrosine residues, the recombinant protein showed that it could phosphorylate all of the three amino acid residues even though its activity was

more pronounced when phosphorylating the serine or threonine residues than when phosphorylating tyrosine (Figure 5.1A). This observation directly concurs with those made from other previous studies where AtBRI1 (Bojar *et al.*, 2014; Oh *et al.*, 2009) and AtPSKR1 (Muleya *et al.*, 2016) could phosphorylate all of these three amino acid residues. It is also worthy to note that by phosphorylating the tyrosine residue, the AtPPR-AC/K (which is an RLK) had somewhat revealed a unique fingerprinting aspect of the receptor tyrosine kinases (RTKs) predominantly found in animals (Oh *et al.*, 2000; Oh *et al.*, 2009; Tang *et al.*, 2008; Jaillais *et al.*, 2011b; Oh *et al.*, 2011). These mammalian RTKs have been extensively shown to play key roles in most signalling cellular pathways including hormone response, cell differentiation, tissue development, and cancer responses (Blume-Jensen and Hunter, 2001). In plants, tyrosine phosphorylation has been shown to predominantly play important roles in plant cell signalling for tissue development and responses to external environmental stimuli such as light, temperature, pathogen invasion, growth regulatory factors and nutrition deprivation, all of which are essentially mediated by cAMP (Gupta *et al.*, 1998; Schmid *et al.*, 2005).

Furthermore, substrate specificity for the recombinant AtPPR-AC/K between ATP and its structural analogue GTP was also assessed by testing its ability to phosphorylate the serine/threonine residues (Figure 5.1B). Between the two molecules as possible substrates for the protein, the recombinant AtPPR-AC/K exhibited more than a 2-fold increase in activity with GTP than with ATP and when both substrates were simultaneously used at equimolar concentrations, the ATP-dependent kinase activity was only increased by a mere factor of 1.4. By considering the nature of these outcomes, it is really apparent that there is competition between the two analogues for binding onto the catalytic site of the recombinant AtPPR-AC/K protein yet GTP is the most preferred substrate for catalysis. This competitive inhibitory

scenario between the two structural analogues (ATP and GTP) with the same recombinant protein has already been witnessed in chapter 4 (Figure 4.4B), where in this case, ATP was the most preferred substrate for the AC domain than GTP, hence the scenario therefore not being an unusual outcome. In this regard, it can be confidently concluded that the AC domain of the recombinant AtPPR-AC/K prefers ATP as its sole substrate for activity while the kinase domain of the same protein prefers GTP as its sole substrate for the trans-phosphorylation activity.

However, because the kinase domain of the recombinant AtPPR-AC/K can still use ATP (a sole substrate for the AC domain) for its trans-phosphorylation activity, it was therefore still very necessary to assess how the product of the AC domain (cAMP) could influence the activity of the co-domain (kinase domain). As is shown in Figure 5.2A, cAMP had a significant inhibitory effect (more than 2.5 folds) onto the catalysis of ATP by the kinase domain, a scenario that was not observable when GTP was the sole substrate for the same kinase domain. Conceivably, such a suppressive effect of the cAMP onto the kinase domain was as effective as long as this compound was available in the system (Figure 5.2B). In this regard therefore, this outcome was then rationalized to explain a regulatory system whereby a direct competitive scenario between the two co-existing catalytic domains of the recombinant AtPPR-AC/K for the same substrate (ATP) is somehow, metabolically and catalytically minimized. Based on this same concept, we then proposed a functional model for the recombinant AtPPR-AC/K whereby when ATP is available, the AC domain will get more priority than the kinase domain, and the kinase domain then concentrating on GTP.

After establishing that the kinase domain of the recombinant AtPPR-AC/K did prefer GTP to ATP as its sole substrate for trans-phosphorylation, we then sought to calculate the reaction

kinetics of this recombinant protein for both of these two possible substrates. As is shown in Figure 5.3, GTP had a higher reaction kinetics ($K_m \approx 1.87$ μM and $V_{max} \approx 4794.87$ nmoles/min/mg protein) when the Ser/Thr-peptide was used (Figure 5.3B) than ATP ($K_m \approx 2.96$ μM and $V_{max} \approx 2276.92$ nmoles/min/mg protein) when the same Ser/Thr-peptide was used (Figure 5.3A), and thus further firmly confirming its outright preference as a sole substrate for trans-phosphorylation by the recombinant AtPPR-AC/K protein. With either substrate, the obtained reaction kinetics values for the recombinant AtPPR-AC/K protein relate very well with the other previously obtained values in related studies. For AtWAKL10, the K_m value was 2.7 μM while the V_{max} was 2269.74 nmoles/min/mg protein (Meier *et al.*, 2010), for the mouse PKA, the K_m value was 1.8 μM while the V_{max} was 3700 nmoles/min/mg protein (Shults and Imperiali, 2003; Shults *et al.*, 2006), for the AtPSKR1, the K_m value was 7.5 μM while the V_{max} was 1800 nmoles/min/mg protein (Kwezi *et al.*, 2011), for the related LRR RLK kinase domain of AtBRI1, the K_m value was 8.2 μM while the V_{max} was 1324 nmoles/min/mg protein (Oh *et al.*, 2000; Wang *et al.*, 2005). The kinetics values of the AtPSKR1 and AtBRI1 appear to be a bit lower than those of the AtPPR-AC/K because these two recombinants used the BRI12-peptide instead of the Ser/Thr-peptide as the trans-phosphorylation substrate (Oh *et al.*, 2000; Kwezi *et al.*, 2011).

After determining the trans-phosphorylation capacity of the recombinant AtPPR-AC/K protein its auto-phosphorylation capacity was also assessed. As is shown in Figure 5.4, the recombinant AtPPR-AC/K could viably auto-phosphorylates itself as much as it could previously trans-phosphorylate other separate substrates (Figures 5.1-5.3). However, and most interestingly, this kinase activity of the recombinant AtPPR-AC/K had a higher preference for ATP (~100%) than GTP (~67%) as the sole source of the pyrophosphate group, a scenario that was totally opposite to the one exhibited when the protein was undertaking trans-

phosphorylation. Based on this information, we then firmly concluded that besides possessing the AC activity (Chapters 2-4), the recombinant AtPPR-AC/K protein also possesses the kinase activity and specifically, the trans-phosphorylation and auto-phosphorylation activities, thereby becoming a bi-functional molecule.

Overally, we then proposed a functional and metabolically viable model for the recombinant AtPPR-AC/K as a bi-functional molecule. In this case and when the molecule is functioning, it probably follows the following sequential steps: (i) auto-phosphorylation of the kinase domain using ATP to activate the whole twin-domain molecule, (ii) conversion of the ATP into cAMP by the AC domain, (iii) initiation of the trans-phosphorylation activity by the auto-phosphorylated kinase domain using GTP and not ATP so that together with the generated cAMP, all the necessary downstream signalling cascades can now then be cordially driven and appropriately mediated.

5.6 Conclusion

Findings from this chapter firmly establish the recombinant AtPPR-AC/K protein as a functional kinase molecule with both the intrinsic trans-phosphorylation and auto-phosphorylation activities while at the same time, this protein becoming the first ever higher plant enzyme molecule to possess a dual functional property composed of an AC and a kinase.

5.7 Recommendation

Since the AtPPR protein was initially identified together with the other Arabidopsis proteins as possible AC/kinase enzymes yet now that the recombinant AtPPR-AC/K protein has been firmly established as a dual catalytic enzyme molecule, it is now therefore very necessary that the other outstanding molecules are also similarly characterized so as to establish a complete family of such specialized types of enzymes in the *Arabidopsis thaliana* system.

General Discussion, Conclusion and Future Outlook

Plants are the backbone of all life on earth; they play an essential role in the lives of most organisms including humans and animals, by providing services such as habitats, food and shelter. Even though they play such fundamental roles, plants tend to experience constant constraints throughout their life-cycles by being continuously exposed to various environmental stress stimuli such as pathogen infections, pest attacks, droughts, wounding and high salt contents. In all cases, these adverse environmental conditions always affect the plant's normal growth, productivity and developmental systems. As a result, plants therefore have to develop some coping mechanisms against such stressful conditions, mostly through cell signalling and molecular transduction systems (Tuteja, 2007; Ning *et al.*, 2010). Hence and in this regard, food security is therefore heavily dependent on the development and production of crop plants with increased resistance to both biotic and abiotic stress factors (Denby and Gehring, 2005). Thus, there is really an urgent need to use rational approaches to develop crop plants with increased stress tolerance, which nowadays has led plant geneticists, plant physiologists, plant biochemists and plant molecular biologists to develop new improved techniques with biotechnological solutions that would assist in the possible alleviation of hunger and poverty (Denby and Gehring, 2005).

Apparently, with the current increased rate of biotic and abiotic stress factors affecting crop development and yields, plant biotechnology has thus become handy in attempting to bring about rational approaches to counteract these continuous challenges. Such strategies, among others, include the over-expression of some specific and target genes to confer increased resistance and tolerance to both biotic and abiotic stress factors. However, and even though the plant biotechnological strategies may play a role in conferring resistance and tolerance to stress factors, there is also a possible disadvantage that may arise from such strategies, causing

some metabolic imbalances that would be ineffective and non-beneficial (Meier and Gehring, 2006). Therefore, and in order to resolve this challenge, a systemic approach will need to be put in place, where bioengineering may focus on regulatory genes or gene encoding molecules that activate signalling cascades in a stimulus-specific manner (Denby and Gehring, 2005). Application of these systemic approaches have resulted in the identification of specialized enzymes, the adenylate cyclases in higher plants (Gehring, 2010). Adenylate Cyclases, have been demonstrated to synthesize cAMP, which play a key role as a second messenger involved in cellular processes such as signalling and regulation (Hutti *et al.*, 2004). Ideally, proteins that systemically affect homeostasis in plants are a promising target candidate group for plant biotechnology, of which one such candidate molecule is the pentatricopeptide repeat protein from *Arabidopsis thaliana* (AtPPR) coded for by the At1g62590 gene (Gehring, 2010). This protein, together with the other related proteins, when queried bioinformatically with a search motif derived from conserved and functionally assigned amino acid residues in the catalytic centre of annotated and/or experimentally tested nucleotide cyclases, showed that it is actually a multi-domain, multi-functional protein, characterized by the possession of an overlapping adenylate cyclase (AC) and kinase architectural structure (Gehring, 2010). These two co-existing domains have been thought to possess essential functions in some important cell communication and cell signalling systems such as stress response and adaptation mechanisms that are naturally mediated by cAMP and/or protein phosphorylation (**Chapter 1**).

Based on this notion, a truncated version of this putative AtPPR protein harbouring the AC/kinase twin domain (AtPPR-AC/K) was in this study, prepared and its mode of action practically elucidated. Initially, when this recombinant protein was transiently expressed in competent *E. cloni* EXPRESS BL21 (DE3) pLysS cells, it induced a forskolin-insensitive (Forte *et al.*, 1983) generation of the endogenous cAMP in these prokaryotic host systems,

thereby proposing itself as either a *bona fide* soluble AC (sAC) capable of generating cAMP from ATP or is simply another functional signalling plant molecule capable of stimulating other resident sACs (*E. coli* sACs in this case) to produce cAMP (Yan *et al.*, 1998; Buck *et al.*, 1999) (**Chapter 2**). Secondly, when its cDNA was cloned into a non-lactose fermenting mutant strain of *E. coli* (*cya*A SP850 cells) (Ullmann and Danchin, 1983; Cotta *et al.*, 1998; Moutinho *et al.*, 2001; Charania *et al.*, 2009) via a pCRT7/NT-TOPO:AtPPR-AC/K fusion expression construct, followed by the growth of such cells on MacConkey agar supplemented with 0.1 mM IPTG, the recombinant AtPPR-AC/K protein succeeded to generate cAMP, which in turn then made the mutant cells to behave as if they were wild types and now ultimately fermenting lactose. This brilliant finding therefore, thus firmly established the recombinant AtPPR-AC/K protein as a biologically functional AC and thereby becoming the sixth ever such molecule to be identified in higher plants after the PSiP protein from *Zea mays* (Moutinho *et al.*, 2001); the AtPPR-AC protein from *Arabidopsis thaliana* (Ruzvidzo *et al.*, 2013); the NbAC from *Nicotiana benthamiana* (Ito *et al.*, 2014); the HpAC1 protein from *Hippeastrum hybridum* (Swiezawska *et al.*, 2014); and the AtKUP7 protein from *Arabidopsis thaliana* (Al-Younis *et al.*, 2015) (**Chapter 3**).

More so, when the recombinant AtPPR-AC/K protein was chemically purified followed by a rigorous *in vitro* functional characterization of its AC activity, it exhibited itself as a manganese-depended enzymatic protein (Okamura *et al.*, 1985), whose AC functional activity was positively enhanced by both the calcium and carbonate ions (Garty and Salomon, 1987; Carricarte *et al.*, 1988). This thus un-debatably, confirmed this protein as a *bona fide* sAC (Okamura *et al.*, 1985; Visconti *et al.*, 1990; Chen *et al.*, 2000), whose functional activities are mediated by cAMP via a calmodulin-dependent signalling system (Man-Ho *et al.*, 2012) (**Chapter 4**). Lastly, when the purified recombinant AtPPR-AC/K was further tested for its

possible kinase activity, it showed that it was indeed a functional kinase molecule, with both intrinsic trans-phosphorylation and auto-phosphorylation activities (Smith *et al.*, 1993). In this regard therefore, the recombinant AtPPR-AC/K became the first ever higher plant molecule to possess a dual functional property composed of an AC and a kinase, otherwise all the other previously identified bi-functional plant molecules like the AtBRI1, AtWAKL10, and AtPSKR1 had their architectural structures composed of a GC and a kinase (Kwezi *et al.*, 2007; Meier *et al.*, 2010; Kwezi *et al.*, 2011) (**Chapter 5**).

By summing up all findings of this whole study, it is conceivable to state that the pentatricopeptide protein from *Arabidopsis thaliana* (AtPPR) was unequivocally, established as a *bona fide* bi-functional plant molecule, possessing both the AC and kinase activities. In addition, this work also undoubtedly established that there is a cross-talking scenario between the two involved catalytic domains of this protein - an aspect that partly explains the ultimate regulatory system of this putative protein in higher plants. Finally, this study also firmly established the AtPPR protein as the sixth ever AC molecule to be identified and experimentally confirmed in higher plants (Moutinho *et al.*, 2001; Ruzvidzo *et al.*, 2013; Ito *et al.*, 2014; Swiezawska *et al.*, 2014; Al-Younis *et al.*, 2015), while at the same time, it becomes the first ever higher plant molecule to possess a dual functional property composed of an AC and a kinase (Kwezi *et al.*, 2007; Meier *et al.*, 2010; Kwezi *et al.*, 2011).

For possible future studies , it is generally understandable that this study has this far provided a reasonable platform for the possible further characterization of AtPPR-AC/K so as to clearly understand its exact functional roles in plants and, particularly, in critical cellular processes such as growth, development and responses to stressful environmental factors. This proposed further characterization may include studying the recombinant AtPPR-AC/K protein in either

detached or fixed living plant tissues or in full-grown plants so as to further augment our current data with the *in vivo*, *in situ* and/or *in planta* evidence. Other related but modern approaches such as mass spectrometry, structural biology, computational simulations, modelling and bioinformatics may also prove to be very useful. Lastly, all other plant protein molecules so far annotated to contain both the AC and kinase catalytic domains should also be similarly assessed as is being proposed for the AtPPR-AC/K. Such a focus will immensely contribute towards an establishment of part of the growing family of complex multiple-domain enzymes in plants, and thus indirectly, implying that a significant number of ACs and kinases in higher plants is still yet to be discovered.

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