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Recombinant expression and functional characterization of a putative pentatrico-peptide protein from *Arabidopsis thaliana*

by

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DECLARATION

I **Tshegofatso Bridget Dikobe** declare that the thesis entitled "Recombinant expression and functional characterization of a putative pentatricopeptide protein from *Arabidopsis thaliana*"submitted to the Department of Biological Sciences at University of North West for Master of Science in Plant Biotechnology has never been submitted at this university or in any other institution elsewhere. This is my own work and all the sources used or quoted have been indicated and acknowledged.

Student: **Dikobe BT**

BTDikobe ..... **Date:** 19/09/2013

Supervisor: **Dr. O. Ruzvidzo**

..... **Date:** 19/09/2013

DEDICATION

I dedicate this work to my family, Keitumetse, Kagiso, Adam and in memory of my mother and father Boitumelo and Tholo Dikobe who always served as my inspiration. I also dedicate this research to the Biotechnology Research Group.

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I thank **God** for His protection, guidance and strength to complete this research work.

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DEFINITIONS 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.

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

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

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

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

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 transmitting external cellular signals within the cell for the development of appropriate cellular responses through regulated gene expression and metabolic events.

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

LIST OF ABBREVIATIONS

AC: Adenylate cyclase

ANOVA: A one-way analysis of variance

AtCNGC: *Arabidopsis thaliana* cyclic nucleotide-gated channel

ATP: 3′, 5′-Adenosine 5′-triphosphate

bp: Base pairs

BLAST: Basic local alignment searching tool

cAMP: Cyclic 3′, 5′-adenosine monophosphate

cGMP: Cyclic 3′, 5′-guanosine monophosphate

cDNA: Copy DNA

CMS: Cytoplasmic male sterility

$CNGC_S$: Cyclic nucleotide-gated ion channels

EDTA: Ethylene diamine tetra-acetic acid

EGTA: Ethylene glycol tetra acetic acid

EIA: Enzyme immunoassay

GC: Guanylate cyclase

GTE: Glucose-Tris-Cl-EDTA

GTP: 3′, 5′-guanosine 5′-triphosphate

HR: Hypersensitive response

IBMX: 3-Isobutyl-1-methyl xanthine

IPTG: Isopropyl-β-D-thiogalactopyranoside

LB: Luria broth

MS: Murashige and Skoog

MWCO: Molecular weight cut off

Ni-NTA: Nickel-nitrilotriacetic acid

OD: Optical density

PBS: Phosphate buffered saline

PPR: Pentatricopeptide repeat

PMSF: Phenylmethylsulfonyl fluoride

rpm: Revolutions per minute

RT-PCR: Reverse transcriptase polymerase chain reaction

Rf: Restorer of fertility

SDS-PAGE: Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SNK: Student Newman Kuehls

STAND: Signal transduction ATPases with numerous domains

TAIR: The Arabidopsis Information Resource

TPR: Tetratricopeptide repeat

TEMED: N, N, N, N'- Tetra- methyl-ethylenediamine

TFB1: Transformation buffer 1

TIR-NBS-LRR: Toll interleukin receptor nucleotide-binding site leucine rich repeat protein

YT: Yeast-tryptone

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ABSTRACT

Food security appears to be heavily dependent on the development of crop plants with increased resistance to both biotic and abiotic stresses such as pathogen infections and droughts respectively. Plant biotechnology has focused strongly on protein molecules that systemically affect homeostasis in plants and, one such possible candidate molecule is the pentatricopeptide protein, whose gene (PPR, At1g62590) has recently been bioinformatically identified from the Arabidopsis genome and it harbours an adenylate cyclase (AC) catalytic motif. Adenylate cyclases (ACs) are enzymes that are capable of converting adenine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP) whose purpose is to function as a second messenger molecule in various physiological and biochemical cell signalling systems. The PPR protein family has previously been experimentally shown to have roles in RNA processing and the restoration of cytoplasmic male sterility. However, to date, there has been no study to characterise if the PPR protein encoded by At1g62590 has also AC activity. Therefore the aim of this study was to confirm if the PPR protein encoded by At1g62590 has any AC activity and if so, to further explore if it has any physiological roles in plant cell signalling systems. In order to attempt this aspect, the putative AC containing PPR gene was cloned in a prokaryotic expression vector (pCR®T7 TOPO®-NT) and expressed in *E. coli* BL21 (DE3) pLysS cells. In order to demonstrate the biological functionality of the PPR's adenylate cyclase catalytic centre, the recombinant protein was tested for its ability to generate cAMP endogenously, in *vitro* and *in vivo*. Results from these three assays all indicated that the recombinant PPR-AC does possess adenylate cyclase activity.

CHAPTER ONE

1.0. General Introduction and Literature Review

1.1. Introduction

Plants play an essential role in the lives of most organisms including humans and animals by providing services such as habitats and food. However, since climate changes will continue to occur and extreme stresses are likely to increase, we can expect increasing difficulties in growing crops in many parts of the world including South Africa (White *et al.*, 2004; Vinocur and Altman, 2005). Food security is therefore, heavily dependent on the development of crop plants with increased resistance to both biotic and abiotic stresses such as pathogen infections and droughts respectively. The urgent need to use rational approaches to develop crop plants with increased stress tolerance and yield has led to an impressive body of work in the areas of plant genetics, plant physiology, plant biochemistry and plant molecular biology, and a realization that only an integrated and systems-based approach can possibly deliver effective biotechnological solutions (Stuhmer *et al.*, 1989).

Due to these multiple factors such as drought, salinity, pests and diseases affecting plants this has led to the application of genetic modification as an important component to resolve these challenges (Jauhar, 2006, Edgerton, 2009, Anderson, 2010; Fedoroff *et al.*, 2010, Tester and Langridge, 2010). Since proteins do systemically affect homeostasis in plants and therefore make desirable candidates for biotechnology, one such molecule is the pentatricopeptide protein, whose gene (At1g62590) has recently been bioinformatically identified from *Arabidopsis thaliana*, and thus it needs to be extensively studied before it could be used for the improvement of crop yield. Pentatricopeptide repeat (PPR) has been bioinformatically identified as one of the possible plant adenylate cyclases which was previously proposed to

be involved in the signalling of molecules that have been implicated in regulation of important processes that includes pathogen response and gene transcription. PPR have been shown to be one of the largest protein families in the *Arabidopsis thaliana* genome comprising of about 466 genes (Aubourg *et al.*, 2000; Small and Peeters, 2000). Adenylate cyclases (ACs) are enzymes that are capable of converting adenine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). There has been no study to date to characterise if the PPR protein encoded by the At1g62590 gene is also a possible AC. The aim is to experimentally test if the PPR protein in question has AC activity, and if so, to further explore in future if it also has physiological roles in plant signalling, particularly in plant adaptation and tolerance to biotic and abiotic environmental stress factors.

1.2. Literature Review

Cellular signalling with cAMP in higher plants

Adenylate cyclases (ACs) are enzymes that catalyse the conversion of adenine-5′-triphosphate (ATP) to cyclic 3′, 5′-adenosine monophosphate (cAMP). In animals and lower eukaryotes, cAMP has been firmly established as an important signalling molecule and acting as a second messenger in several cellular signal transduction pathways (Donaldson *et al.*, 2004). However, in higher plants recently, the only annotated and experimentally confirmed AC is a *Zea mays* pollen protein (Moutinho *et al.*, 2001) capable of generating cAMP, which in turn is a second messenger with a role in polarized pollen tube growth. Otherwise not much is known in higher plants about ACs or their product cAMP as compared to animals and lower eukaryotes although the presence of cAMP in higher plants and its biological role in cell signalling have been extensively documented (Gehring, 2010).

By the mid-1970s, the molecule 3'-5'-cyclic adenosine monophosphate (cAMP) had been firmly established as an important signalling molecule and a second messenger in both animals and lower eukaryotes (Robison *et al.*, 1968, Goodman *et al.*, 1970; Gerisch *et al.*, 1975; Wiegant, 1978). cAMP generated from the hydrolysis of ATP can affect many different downstream signalling processes including the activity of kinases (Robison *et al.*, 1968). Given the growing realization of the importance of ACs and cAMP, that led to plant scientist's interest in learning if this signalling system was universal and therefore operating in plants too. The major reasons why AC and/or cAMP information was not readily available in plants as was in animals and lower eukaryotes were firstly, that the levels of cAMP detected in plants appeared to be very low (< 20 pmol/g fresh weight) (Ashton and Polya, 1978) as 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). However, the fact that signalling in plants at lower molecular levels is feasible is not uncommon because incidentally, low levels of another cyclic nucleotide, cGMP (< 0.4 pmol/g fresh weight) (Meier *et al.*, 2009), were also reported in plants where the molecule has a physiological role in specific responses to avirulent pathogens and defense mechanisms. In addition, the availability of more advanced analytical tools has dramatically improved the assaying systems in plants and the inference of solid conclusions.

An Arabidopsis orthologue of this protein (At3g14460) is annotated as 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). 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 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) is enabled by cAMP.

Identification of a pentatricopeptide repeat (PPR) protein

Of particular interest to this research is a pentatricopeptide protein whose gene (At1g62590) has recently been bioinformatically identified by Gehring (2010) from the *Arabidopsis* genome using a search motif consisting of functionally assigned amino acids in the catalytic centres of annotated and/or experimentally tested nucleotide cyclases (Table 1).

Table 1: The nine bioinformatically identified *Arabidopsis. thaliana* proteins containing the AC catalytic centre.

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

Bioinformatically identified *A. thaliana* proteins with AC catalytic domains: ATG represents the assigned *A. thaliana* gene bank numbers for the nine genes, followed by 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 to be functionally characterized in this project. AC catalytic centre: [RK][YFW][DE][VIL][FV]X(8)[KR]X(1,3)[DE] (Adapted from [Gehring, 2010]).

Structure of a pentatricopeptide repeat (PPR) protein

This protein is proposed to contain a pentatricopeptide repeat (PPR) motif, which is a 35 (pentatrico) degenerate amino acid system often arranged in tandem arrays of 2-27 repeats per peptide (Small and Peeters, 2000). It has further been established that the PPR family is divided into two sub-families, the P and PLS subfamilies, with members of the P sub-family abundantly distributed in eukaryotes while the PLS subfamily are strictly restricted to plants (Lurin *et al.*, 2004). PPRs 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, flowering plants such as the *A. thaliana* (about 441 genes) and rice (more than 655 genes) (Lurin *et al.*, 2004) while TPR are generally found in both prokaryotes and other eukaryotes such as the yeast (*Saccharomyces cerevisiae)* and Drosophila (Desloire *et al.*, 2003).

Function of the PPR proteins

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 mitochondria (Lurin *et al.*, 2004). TPRs interact mainly with other proteins whereas PPRs interact specifically with either the DNA-binding 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 the RNA editing (Kotera *et al.*, 2005), transcript processing (Nakamura *et al.*, 2004) and translation initiation (Schmitz-Linneweber *et al.*, 2005). It has been predicted that 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 of these proteins has been experimentally demonstrated both *in vitro* and *in vivo* using the RIP chip approach (Schmitz-Linneweber *et al.*, 2005). Further, a maize protein CRP1 has been shown to have 13 PPR motifs which localize mainly in the chloroplast stroma and facilitate the processing and translation of *petD* and *petB* mRNAs (Fisk *et al.*, 1999). Additionally, a P67 protein from Arabidopsis and radish with two PPR motifs also reveals similar functioning as 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).

The absence of PPR2 revealed that its mutant (*ppr2*) will prevent the accumulation of ribosomes in plastids and the assembling of translational apparatus 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 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 but they perform different roles with CCR4 involved in RNA editing of the *ndhD* gene (Shikanai, 2006) and the later being responsible for RNA cleaving/splicing between *rps7* and *ndhB* genes (Hashimoto *et al.*, 2003). PPR 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 would have coded for large proteins of more than 650 amino acids (Lurin *et al.*, 2004), and thus 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. PPRs have also been noted to influence the process of RNA editing, where cysteine (C) will be replaced by 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 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 pollen (Pring *et al.*, 1995). They act to suppress male sterility linked with CMS, a function related to the expression of mitochondrially-encoded sterility-associated genes. Rf 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 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, maize gene (Rf2) does not affect the 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).

PPR genes have also been found to play key roles in plant embryogenesis (Cushing *et al.*, 2005, Ding *et al.*, 2006) and developmental process (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). Evidence has revealed that a gene that contained a PPR protein has shown by genetic approach, to be a trans-acting factor (Kotera *et al.*, 2005), and this factor plays an essential role in RNA editing (Lurin *et al.*, 2004). A trans-acting factor was firstly 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* (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. CCR4 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 cytidine deaminase (C-deaminase) via C-terminal E+ domain (Okuda *et al.*, 2006; Shikanai, 2006). PPR proteins also play major roles in gene expression which is mainly organelle-based either in mitochondria 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 in humans (Hou *et al.*, 1994; Liu and McKeehan, 2002; Mili and Pinol-Roma, 2003), except in plants where little evidence has revealed that it might be targeted to mitochondria even though these proteins have a high presence in plants compared to other organisms (Lurin *et al.*, 2004; Andres *et al.*, 2007).

1.3.1. Problem statement

Despite the fact that mutational analysis approaches have demonstrated PPR to have roles in processes such as RNA processing and the restoration of cytoplasmic male sterility, no study in plants to date has attempted to characterize this protein as a possible plant AC. Considering that cyclic nucleotides have important and diverse roles in plant signalling (Newton and Smith, 2004), it is unlikely that a single AC can account for all the known cAMP-dependent processes in higher plants. In line with this hypothesis is the fact that a number of Arabidopsis genes with different AC catalytic domains have recently been bioinformatically identified (Gehring, 2010). Included among these genes that contained the AC domain was one (At1g62590) coding for a PPR protein. In order to understand the functional relevance of this PPR protein it is necessary that AC activity of this protein be experimentally tested and verified.

1.3.2. Research aim

The overall research question of this project is to establish experimentally the presence of any ACs in plants besides the currently described *Zea mays* pollen protein (Moutinho *et al.*, 2001) and if so, to determine the enzymatic activities of such molecules *in vitro*. This research question shall therefore be partially addressed by experimentally exploring the functional roles of the recently identified and annotated PPR gene in the Arabidopsis genome (Gehring, 2010).

1.3.4. Objectives

The following specific objectives were set:

1. To isolate and clone the annotated Arabidopsis PPR gene into stable and viable heterologous prokaryotic expression systems.
2. To optimize expression and purification strategies of the recombinant PPR protein.
3. To determine the biological/enzymatic activity of the recombinant PPR protein.
4. To further characterize the enzymatic activities of this PPR protein *in vitro*.

1.3.5. Significance of the research project

The following significant impact will be expected after the completion of this project:

1. The advance with increase functional characterisation of the annotated PPR protein will advance the knowledge on plant genes responsible for environmental stress responses and adaptation.

CHAPTER TWO

2.0. Isolation and Molecular Cloning of the PPR Gene

2.1. Plant Generations and Growth Conditions

2.1.1 Seed Sterilisation

A. thaliana ecotype Columbia seeds were transferred into a sterile 1.5 mL Eppendorf tube where 500 µL of 70% ethanol was added and vortexed for 30 seconds. Seeds were left to settle through gravity and the ethanol was discarded. The seeds were then repeatedly washed 5 times with sterile distilled water. The seeds were then submerged into a 500 µL sterilization buffer (0. 1% SDS and 5% bleach (commercial) and vortexed for 30 seconds. The buffer was removed and the seeds were washed 5 times with 1 mL of sterile distilled water, and 500 µL of sterile distilled water were finally added onto seeds.

2.1.2 Seed Vernalisation

After sterilisation, the seeds were vernalized by introducing them to a cold temperature of 4°C for 1-3 days. This process was used to eliminate seed dormancy and improve germination rate (Lack and Evans, 2001).

2.1.3 Seed Germination

Sterilised seeds were seeded onto Murashige and Skoog medium [4.3g Murashige & Skoog basal salts (Gibco®), 1% Sucrose m/v, 1 X Gamborg's vitamins v/v, 0.05% MES m/v, 0.8% type M agar m/v (Sigma-Aldrich Corp., Missouri), pH 5.7 with KOH] in petri dishes that were later sealed with parafilm and incubated in a Labcon growth chamber (Type #: LTGC20, Labex, Labdesign Engineering, RSA). Seeds were allowed to germinate for 14 days under long 12 hour days and long 12 hour nights at a constant 25°C. After the 14 days,

the seedlings were transplanted using sterile blades to potting soil composed of 3 parts peat-based soil to 2 parts vermiculite and then watered with distilled water containing Gaucho to systematically protect them from fungal attack. The seedlings were then allowed to grow for a further 2-4 weeks or until further use (seed harvest). All seedlings and/or plants were grown under greenhouse conditions with long 16 hour days (light), at 10 000 LUX of light intensity and 8 hour nights (dark).

2.2. Designing and Acquisition of Sequence-specific Primers

Two sequence-specific primers were manually designed based on the PPR gene sequence shown in Figure 2.1 below and ensuring that they were both carrying restriction sites to enable their directional cloning (Forward primer with *Bam* HI site: 5′CG**GGATCC**GATGGGTGGCAGTGGTG 3′ and Reverse primer with *Eco* RI site: 5′GCG**GAATTC**TAGGCCGTGACAGTATCC 3′) into the pCR®T7TOPO®-NT expression vector (Invitrogen, Carlsbad, USA) shown figure 2.2. The designed primer sequences were then sent to the Department of Molecular and Cell Biology, University of Cape Town, South Africa for chemical synthesis and subsequent supply.

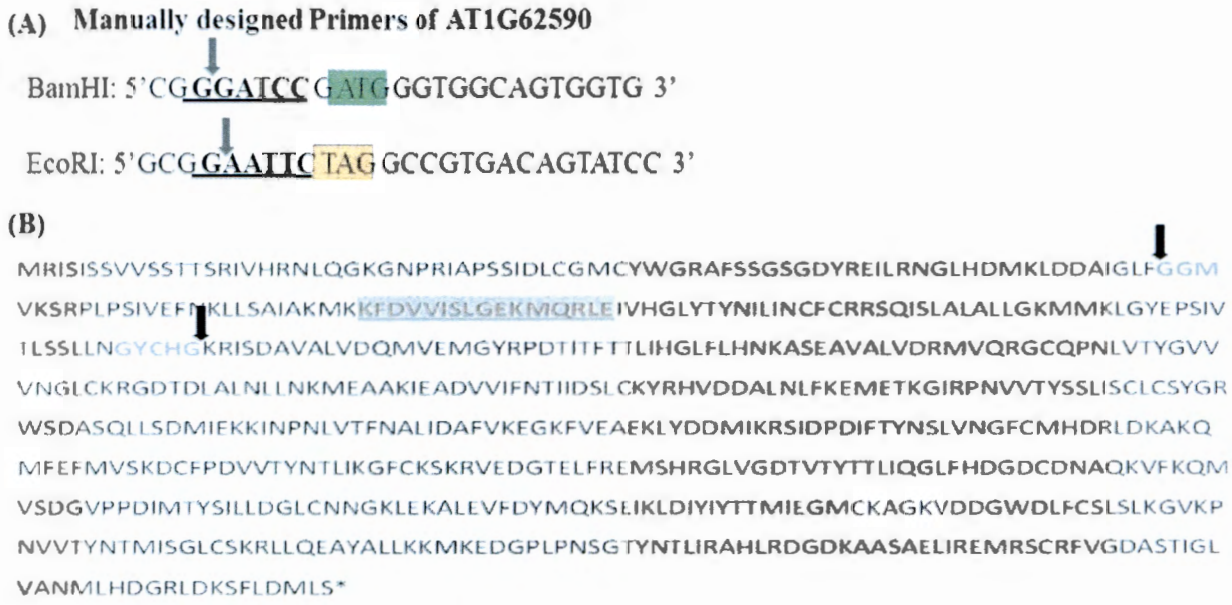

Figure: 2.1. Sequence Information for Primer Design. **(A)**Forward and Reverse Primer sequences designed to amplify AC catalytic centre of At1g62590 carrying restriction enzymes (*Bam* HI and *Eco* RI) indicating how these enzymes start to cut/digest as shown by arrows, start (green)and stop (red) codons (highlighted). **(B)** The amino acid sequence annotated as pentatricopeptide protein. Arrows marks the start and end of forward and reverse primer target region, with bold and underlined sequence being the AC catalytic centre.

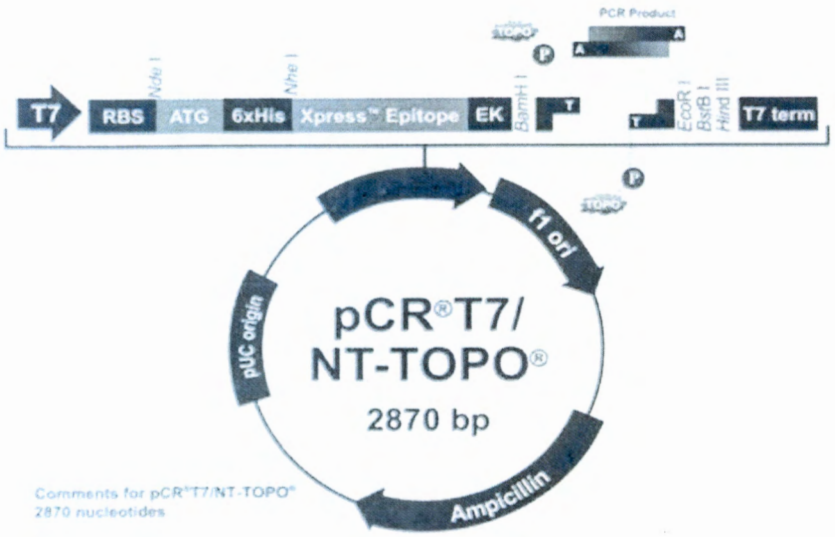

Figure 2.2: Structural features of a vector map of pCR®T7 TOPO®: The illustration shows expression and purification features of the plasmid such as the T7 promoter for high level expression with forward and reverse priming sites for both restriction sites *Bam* HI and *Eco* RI within a multiple cloning site. There is also a point of origin to facilitate replication of the plasmid in bacteria cells such as *E. coli*. In addition, there is an ampicillin resistant gene that allows for screening of positive recombinants. For purification purposes, the vector expresses a recombinant 6-Histidine fusion protein that can be affinity purified on positively-charged chromatographic columns (Adapted from www.lifetechnologies.com).

2.3. RNA extraction from *A. thaliana*

About 0.1 g of plant leaf material was harvested from 2 - 4 week old Arabidopsis plants, followed by isolation of RNA, where the weighed leaf material was placed in liquid nitrogen and ground thoroughly with a pestle and mortar until it formed a fine tissue powder. The tissue material was decanted into an RNase-free ® microcentrifuge tube and the liquid nitrogen was allowed to evaporate. A volume of 450 µL of Buffer RLT was added into the tissue powder and vortexed vigorously. The lysate was transferred to a QIAshredder spin column placed in a 2 mL collection tube and were centrifuged at 18 000 x *g* at 25°C. The supernatant was transferred carefully into a new microcentrifuge then 0.5 volumes of ethanol were added into the cleared lysate and were gently mixed by pipetting. The sample was transferred into an RNeasy spin column placed in a 2 mL collection tube and were

centrifuged for 15 seconds at 12750 x *g* then the flow through discarded. A volume of 700 µL of Buffer RW1 was added into to the spin column and centrifuged for 15 seconds at 8000 x *g* so as to wash the spin column membrane and then 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 x *g* with the flow through discarded. The RNeasy spin column was placed into a 1.5 mL tube and then 50 µL of RNase free water was added directly to the spin column membrane and centrifuged for 1 minute at 8000 x *g* so as to elute the RNA. Concentration of the total RNA was quantified using a nanodrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Massachusetts).

2.4 Amplification of the AC catalytic region of At1g62590 gene from *A. thaliana* RNA via RT-PCR

Copy DNA (cDNA) for the targeted PPR gene was synthesized from the total RNA using a Verso™ 1-Step RT-PCR Reddy mix™ Kit (Thermo Fisher Scientific Inc., Massachusetts) and the two designed sequence-specific forward and reverse primers, following the already established steps with minor modifications. The 50 µL polymerase chain reaction (PCR) mix containing 1 µL of Verso Enzyme mix, 25 µL of 2X 1-Step PCR Ready mix,, 1 µL of 10 µM Forward and Reverse primers, 2.5 µL of RT Enhancer, 5 µL of RNA template, and RNase free distilled water was prepared. Reaction amplification was then performed using a PCR Peltier Thermal Cycler (model-PTC-220 DYAD™ DNA ENGINE, MJ Research USA) under the following conditions: cDNA synthesis at 50°C for 15 minutes, Verso inactivation at 95°C for 2 minutes, followed by 45 cycles of Denaturation step at 95°C for 20 seconds, Annealing at 65°C for 30 seconds, Extension at 72°C for 1 minute, with a Final Extension step at 72°C for 5 minutes and the produced PCR products were then kept at 4°C.

The amplified DNA was resolved through electrophoresis on a 0.8% agarose gel stained with ethidium bromide (0.5 µg/mL) at 100 volts for 60 minutes. All samples were resolved along a 100 bp (0.1 µg/µL) DNA molecular weight marker (Catalog# SM1143-Fermentas International Inc., Burlington, Canada) and visualized under UV light (420nm) (Bio-Rad Laboratories., USA) as previously described by (Sambrook *et al.*, 1989). Gene Genius Bio Imaging system (syngrene, Synoptics; UK) was used to capture the gel image using a Gene Snap (version 6.00.022) software.

2.5. Bacterial Culture Enrichment

A pCRT7/NT-TOPO plasmid was supplied by the Department of Biotechnology, University of the Western Cape, South Africa, in *E. coli* BL21 (DE3) plysS bacterial host cells. The culture was enriched by inoculating a single colony into 10 mL of Luria Bertani broth that was supplemented with 100 µg/mL ampicillin (v/v) and 34 µg/mL chloramphenicol (v/v). The bacterial culture was then grown in an incubator overnight at 37°C with vigorous shaking at 220 rpm so as to allow enough aeration for 12-16 hours.

2.6. Isolation of Plasmid DNA from Bacterial Culture Using Alkaline Lysis Method

After 16 hours of incubation, cells were harvested through centrifugation in a Hermile Z300k centrifuge at 8000 x *g* for 5 minutes. The cell pellets were re-suspended in ice-cold 200 µl GTE (50 mM Glucose, 25 mM Tris-Cl, pH 8.0, 10 mM EDTA). A total volume of 400 µl of lysis buffer (1M NaOH and 10% SDS) was added and mixed by a gentle inversion of 4-6 times followed by incubation on ice for a further 5 minutes. To this mixture, 300 µl of 3M KAc (potassium Acetate) pH 5.5 was added and mixed by gentle inversion and incubated on ice for 5 minutes. This was then centrifuged for 5 minutes at 8000 x *g*. The supernatant was transferred to a clean 1.5 mL Eppendorf tube where 0.1 volume of KAc (40 µL) and 0.7

volume isopropanol (240 µL) were added and incubated at -20°C for an hour. After an hour of incubation, the reaction mixture was centrifuged at 8000 x *g* for 5 minutes and the supernatant discarded as waste. The pellets were washed twice with 500 µL ice-cold 70% ethanol by centrifuging at 8000 x *g* for 2 minutes and the supernatant discarded as waste in between washes. The plasmid DNA pellet was air dried at room temperature and subsequently dissolved into 50 µL of TE (10 mM Tris-Cl and 1 mM EDTA (Ethylene Diamine Tetra-Acetic Acid Disodium Salt) buffer. The plasmid DNA was resolved on 0.8% agarose gel to determine its quality and integrity.

2.7. Isolation and Purification of Plasmid DNA from Agarose

DNA extraction was done using the Isolate PCR and Gel Kit, following manufacturer's instructions (Catalog#52029, Bioline, USA), where 300 mg of agarose gel slice was excised with a sterile blade and transferred into an Eppendorf tube. To the agarose gel slice 650 µL of gel solubilizer was added and then incubated for 10 minutes at 50°C in a waterbath until all the agarose had completely dissolved. To the dissolved solution, 50 µL of binding optimizer was added and mixed by pipetting, and then 750 µL of the sample was transferred into a spin column placed in a 2 mL collection tube and centrifuged at 13 300 x *g* for 1 minute. The filtrate was discarded and the collection tube re-used in subsequent steps. A volume of 700 µL of wash buffer A was added to the spin column and centrifuged at 13 300 x *g* for 1 minute. After the wash steps, the column was centrifuged at 13 300 x *g* for an additional 2 minutes to remove excess ethanol present. The spin column was placed into a 1.5 mL elution tube, where 50 µL of the elution buffer was added directly onto the column membrane and then incubated at room temperature for 2 minutes, after which it was centrifuged at 13 300 x *g* so as to elute the plasmid. Plasmid DNA quality was then analyzed on a 1% agarose gel

and viewed under UV light of a transilluminator (Bio-Rad Laboratories., USA) and then stored at -20°C.

2.8. Purification of the Amplified PCR product

The PCR product was cleaned using a DNA clean & concentrator ^{TM}-5 kit (Catalog # D4003, Zymo Research). 100 µL of PCR product was added into an Eppendorf tube with 500 µL of DNA binding buffer and the mixture was briefly vortexed. The mixture was loaded into a Zymo-spin column placed in a 2 mL Eppendorf tube and then centrifuged at 10 000 x *g* for 30 seconds. The filtrate was discarded. 200 µL of wash buffer was added to the column and centrifuged 10 000 x *g* for 30 seconds twice. The Zymo-spin column was placed into a new 1.5 mL Eppendorf tube and 30 µL of pre-warmed sterile distilled water were then added directly onto the column and spun for 1 minute to elute the PCR product. The product was then kept at -20°C.

2.9. Digestion of PCR Products and Plasmid DNA (pCRT7/NT-TOPO)

In each Eppendorf tube, 10 µL of 13.1 ng/µL plasmid DNA and 20 µL of 11.2 ng/µL insert (pCRT7/NT AtPPR-A) were digested with 10 units of *Bam* HI and *Eco* RI, in the presence of 10 µL of 2X Tango buffer (Fermentas International Inc., Burlington, Canada) at a total reaction volume of 50 µL made up with filtered-sterilized distilled water. The reaction mixture was then mixed gently and spun down for 2 seconds, and then incubated in a water bath at 37°C for 4 hours. The reaction was halted by heating at 80°C for 20 minutes and the sample stored at -37°C.

2.10. Ligation of the Amplified DNA into the pCRT7/NT-TOPO Expression Vector

The RT-PCR gene product (AtPPR-AC) was cloned into the cloning site of the pCRT/NT-TOPO expression vector system using a kit and following the manufacturer's instructions (catalog# EL0014, Fermentas International Inc., Burlington, Canada) to make a pCRT7/NT-TOPO-AtPPR-AC fusion expression construct with an N-terminus His purification tag. Fragments created during restriction enzyme digestion formed sticky ends and were ligated at 1:3 vector-to-insert molar ratio, where 1 µL of 126.9 ng/µL (NT-TOPO) vector was added to 2 µL of 80.9 ng/µL (AtPPR-AC) insert. A volume of 2 µL of 10X T4 DNA ligase buffer and 1 µL of 1 unit T4 DNA ligase were added to the Eppendorf tube with the insert and vector. The reaction mixture was filled up to 20 µL with filter sterilized water and incubated at 22°C for 60 minutes in a PCR (mini cycler CG1-96, Corbett Research, Australia), and then further incubated overnight at 4°C. The ligation mixture was then kept at -20°C.

2.11. Preparation of BL21 (DE3) Competent Cells

Escherichia coli BL21 (DE3) Star pLysS cells obtained from the Department of Biotechnology, University of the Western Cape, South Africa were prepared to become chemically competent following the manufacturer's instruction (Invitrogen, Carlsbad, USA). The supplied cells were removed from a storage vial using a sterile wire loop and streaked onto the Luria Bertani (LB) agar plates containing chloramphenicol to a final concentration of 34 µg/mL and were then incubated at 37°C overnight. After 24 hours, fresh 10 mL Luria Bertani (LB) broth containing 34 µg/mL chloramphenicol only was inoculated with a single colony, and then incubated at 37°C overnight in a shaking incubator at 220 rpm. On the subsequent day, 10 mL of fresh media was inoculated with 1 mL overnight culture and then shaken at 200 rpm, at 37°C and monitored until the OD_{600} had reached 0.5. At this OD, the

culture was cooled on ice for 5 minutes and transferred to a sterile round bottom centrifuge tube, and then centrifuged at 4000 x *g*, 4°C for 5 minutes. The supernatant was discarded and cells were kept on ice. The cells were re-suspended with 30 mL of ice-cold TFB1 (Transformation buffer 1) [30 mM KAc, 50 mM $MnCl_2$, 100 mM RbCl, 10 mM $CaCl_2$, 15% glycerol, pH: 5.8] and kept on ice for 90 minutes. Cells were then harvested by centrifugation at 4000 x *g* for 5 minutes at 4°C and kept on ice. 4 mL of ice-cold transformation buffer 2 (TFB2) [10 mM MOPS, 75 mM $CaCl_2$, 10 mM RbCl, 15%. glycerol, pH: 6.8] was used to resuspend the cells and aliquots of 100-200 µL were then prepared in Eppendorf tubes and snap frozen in liquid nitrogen and kept at -80°C.

2.12. Transformation of Competent BL21 (DE3) pLysS Cells with the pCRT7/NT-TOPO-AtPPR Fusion Expression Construct

About 10 µL of the ligation mix (pCRT7/NT-AtPPR) was transferred into a clean ice-cold Eppendorf tube and kept on ice. An aliquot of competent cells was thawed on ice and its 100 µL portion was then added to the ligation mix. The mixture was gently mixed by stirring with a pipette tip and kept on ice for 20 minutes. The mixture was heat-shocked in a water bath at 42°C for 90 seconds. The mixture was immediately placed on ice and 500 µL of SOC broth was added. The mixture was then shaken in an incubator at 220 rpm, at 37°C for 60-90 minutes. The transformation mix was finally plated at 50 µL, 100 µL, 200 µL aliquots onto LB plates with both 100 µg/mL ampicillin and 34 µg/mL chloramphenicol and then left to grow at 37°C overnight.

2.13. Determination of the Cloning and Transformation Success

Cloning and transformation success was determined by performing some pilot expression screens, where 200 µL of overnight transformation culture was inoculated to a fresh 20 mL

double strength yeast-tryptone (2YT) media (16 g tryptone, 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 in a Labcon shaking incubator (Labex, RSA) at 200 rpm at 37°C. The culture was incubated for 2 hours at 37°C at 200 rpm up until the OD_{600} had reached 0.6 and as measured by a Hekios spectrophotometer (Merck, South Africa). Immediately, the culture was split into two falcon tubes with one culture being induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma-Aldrich Corp, Missouri) to a final concentration of 2 mM and the other remaining un-induced. The split cultures were then shaken in an incubator at 37°C for 3 hours. After the 3 hours, 500 µL samples were centrifuged at 8000 x *g* for 5 minutes and the pellets stored at -20°C before being analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Glycerol stocks of all positive cultures were then prepared and stored at -80°C.

2.14. Confirmation on Transformation and Recombination Success by PCR

From the positive clones that had shown the expression of the recombinant AtPPR-AC, the pCRT7/NT-AtPPR-AC expressed vector construct was extracted using Zyppy™ miniprep kit and following the manufacturer's instructions (Zymo-Research., USA), where 600 µL of the bacterial culture was added into a 1.5 mL Eppendorf tube and centrifuged for 30 seconds at 9200 x *g* and the pellet re-suspended in 600 µL of water. A volume of 100 µL of the 7X Lysis buffer was added to the suspension and mixed by inversion 4-6 times, and then 350 µL of cold Neutralization buffer was also added to the suspension and mixed thoroughly by inversion 2-3 times. The sample was centrifuged at 11 000 x *g* for 4 minutes, then 900 µL supernatant of was transferred into the Zymo-Spin™ column which was placed into a collection tube and centrifuged for 15 seconds at the same speed. The flow-through was discarded then 200 µL of Endo-wash buffer was added to the column and centrifuged at 11

000 x *g* for 30 seconds. About 400 µL of Zyppy™ wash buffer was added to the column and centrifuged for a minute at the same speed. The Zymo-Spin™ column was then transferred into a fresh 1.5 mL Eppendorf tube where 30 µL of the Zyppy Elution Buffer was added directly onto the column matrix and and allowed to stand for 1 minute at room temperature before being centrifuged at 11 000 x *g* for 30 seconds so as to elute the plasmid DNA. The eluted plasmid was then stored at -20°C.

For confirmatory PCR, the purified plasmid DNA was used as template for the two PPR sequence-specific primers using on the Taq DNA Polymerase (recombinant) Kit #EP0401 (Fermentas International Inc., Burlington, Canada) in a reaction mixture that contained 5 µL of 10X Taq buffer, 7.5 µL of dNTP mix, 0.5 µM Forward and Reverse primers, 4 µL of 2 mM $MgCl_2$, 2 µL of plasmid DNA template, and 11 µL of RNase free distilled water to make a final reaction volume of 50 µL. The samples were gently vortexed. Amplification of the template DNA was performed using a PCR Peltier Thermal Cycler (model-PTC-220 DYAD™ DNA ENGINE, MJ Research USA) under the following condition: Initial denaturation at 95°C for 3 minutes, Verso inactivation at 95°C for 2 minutes, followed by 40 cycles of Denaturation step at 95°C for 30 seconds, Annealing at 65°C for 30 seconds, Extension at 72°C for 1 minute, and a Final Extension at 72°C for 15 minutes. The amplified PCR product was then resolved on 1% agarose gel stained with ethidium bromide (0.5µg/mL) and visualized under UV light (420nm) (Bio-Rad Laboratories., USA) as previously described by (Sambrook *et al.*, 1989). The Gene Genius Bio Imaging system (syngrene, Synoptics; UK) visualised and captured image using the Gene Snap (version 6.00.022) software.

2.15. Recombinant Expression and Purification of the PPR Protein

2.15.1 Sample Preparation for stable Transformants and its Confirmation by SDS-PAGE

Positive stable transformants of clones were verified by SDS-PAGE by resolving the extracted total protein on a 12% polyacrylamide gel. The frozen cell pellets were thawed on ice and re-suspended with 1 mL of sterilized distilled water and vortexed for 30 seconds. Forty µL of both the induced (Experiment) and un-induced (control) samples were transferred into different 1.5 mL Eppendorf tubes and stored on ice, 10 µL of 5X loading buffer (6 mL of 100% glycerol, 1.25 mL of 0.5M Tris-HCL, 2 mL of 10% SDS, 0.25 mL of 0.5% Bromophenol blue, 0.5 mL of 100% β-mercaptoethanol) was added to each sample and were placed onto boiling water for 5 minutes then pulsed for 10 seconds in a microcentrifuge. Five microliter of the unstained protein marker (Catalog# 26632 Thermo Scientific., USA) and 20 µL from each sample was loaded into the gel, and electrophoresed at 200 volts for 60 minutes. After electrophoresis the gel was stained with Coomassie staining solution (100 % Ethanol, 100% Methanol, 100% Acetic acid,0.5% Coomassie) for 15 minutes , then destained (100% Ethanol,100% Methanol,100% Acetic acid) for 30 minutes shaking on an ultra-rocker (Bio-Rad Laboratories., USA) until the bands were visualized.

2.15.2. Large-scale Recombinant Protein Expression

For a large scale expression 200 µL of the glycerol stocks from the expressed cultures were inoculated into a 20 mL of 2YT media (16 g tryptone, 10 g yeast extract, 5 g NaCl and 4 g glucose per L (pH 7.0), containing 100 µg/mL of ampicillin and 34 µg/mL of chloramphenicol. They were grown for an overnight at 37°C in a Labcon shaking incubator (Labex, Labdesign Engineering) at 200 rpm. In a 300 mL Erlenmeyer flask, 100 mL 2YT containing 100 µg/mL Ampicillin with 34 µg/mL chloramphenicol was sub-cultured with 1 mL overnight culture and incubated at 37°C with agitation at 100 rpm, culture was left

growing until the OD_{600} reached 0.6. After reaching that OD the culture was then induced at a final concentration of 1 mM IPTG (Sigma-Aldrich Corp., Missouri) and allowed to grow for 3-4 hours. From the culture 1 mL sample was, spun at 9200 x *g* for 5 minutes, then the supernatant was discarded and the pellet was stored at -20°C. The remaining culture was centrifuged at 15 600 x *g* for 10 minutes. The supernatant was discarded and pellet was stored at -20°C for further analysis.

2.15.3. Protein Extraction by Sonication

Cell pellet from 50 mL cells was thawed on ice and re-suspended in 5 mL of Phosphate Buffered Saline supplemented with 1 µg/mL of lysozyme (1X PBS) [NaCl 140mM (8.2 g), KCl 3 mM (0.2 g), $Na_2HPO_4.2H_2O$ 4 mM (0.6 g), KH_2PO_4 1.5 mM (0.2 g)], one mL from the cell suspension was taken out and incubated on ice for 30 minutes. After 30 minutes the cells were sonicated for 5 cycles: 30 seconds pulsed on a microfuge and 30 seconds on ice. Then the cells were centrifuged at 9200 x *g* for 5 minutes and supernatants were transferred into 1.5 mL Eppendorf tube. The obtained pellet after centrifugation was re-suspended in 1 mL 1X PBS and kept for further analysis.

2.15.4. Protein Purification Conditions under Native Non-denaturing Conditions

Protein purification was performed under native non-denaturation conditions since it appeared that the recombinant protein expressed was largely found in the soluble fraction. Bacterial cell carrying the expressed recombinant protein was pelleted by centrifugation at 9200 x *g* for 10 minutes. The pellet was re-suspended in 1 mL PBS supplemented with 10 mM Imidazole and vortexed for 1 hour then the solubilized pellet was centrifuged at 9200 x *g* for 10 minutes. The supernatant was kept as flow through (lysate) on ice. The Nickel-Nitrilotriacetic acid (Ni-NTA) slurry matrix (Lot# MG159557, Thermo Scientific., Rockford,

USA) of about 50 µL was washed with 1mL sterile distilled water in a rotary mixer for 5 minutes twice. Ni-NTA beads were equilibrated with 1 mL of PBS supplemented with 10 mM Imidazole (to selectively bind the His tag of the expressed recombinant protein) were mixed in a rotary until were ready for use. Those beads were pelleted through a low speed centrifuge for 15 seconds. The lysate was then mixed (bound) to the Ni-NTA slurry matrix in a rotary mixer for 1 hour in the -20°C. After an hour the beads were washed with 1 mL of PBS supplemented with 10 mM Imidazole three times, each wash was kept and resolved on a 12% SDS PAGE.

2.16. Activity Assaying and Functional Characterization of the Recombinant PPR Protein

2.16.1. Elution of the Purified Recombinant Protein

The bound recombinant AtPPR-AC protein from the Ni-NTA histidine tagged beads was eluted with Native Elution Buffer (30 mL PBS buffer with 250 mM imidazole and 0.5 mM PMSF (phenylmethylsulfonyl fluoride) the buffer was filter sterilized at pH 7.4. In to a 500 µL of the beads that were allowed to settle down remaining traces of the PBS buffer was removed, 1000 µL of Native elution buffer was added onto the beads then re-suspended and mixed on a rotary mixer at 4°C for 20 minutes. After mixing the beads were spun on the microfuge at a low speed to settle down and the eluent was transferred into a fresh Eppendorf and 20 µL of eluent was used for the SDS page analysis compared with the original beads before the addition of elution buffer and after their elution.

2.16.2. Protein De-salting and Concentration Determination of the Recombinant protein

The eluted protein obtained was unbound from the buffering salts and concentrated by pouring 3 mL of the eluent to the upper chamber of the Corning® Spin-X UF 6 mL concentrator device with a molecular weight cut off (MWCO) of 5000 (Product # 431482,

Corning Life Sciences, USA) and spun down at 2540 x *g*, at 4°C using a swing-out bucket rotor of a Hermile Z300k centrifuge (Hermle Labortechnik, Germany) and monitored until the final of the protein sample volume was at 0.2 mL. The de-salted protein fraction was washed by diluting it with 5 mL of sterile distilled water and re-spun until its final volume was again 0.2 mL. This washing step was repeated one more time before the protein concentration was determined on a nanodrop 2000 spectrophotometer (Thermo scientific, USA) and stored at -20°C.

2.16.3. Determination of the Endogenous Adenylate Cyclase Activity of the Recombinant AtPPR-AC

An overnight culture of cells confirmed to be harbouring the recombinant pCRT7/NT-TOPO: AtPPR-AC expression construct was prepared using 200 µL of glycerol stock to inoculate fresh 20 mL of 2YT media (16 g tryptone, 10 g yeast extract, 5 g NaCl and 4 g glucose per L (pH 7.0), containing 100 µg/mL of ampicillin and 34 µg/mL of chloramphenicol. The culture was grown overnight at 37°C in a Labcon shaking incubator (Labex, Labdesign Engineering) at 200 rpm. On the subsequent day, fresh 100 mL 2YT media containing 100 µg/mL ampicillin and 34 µg/mL chloramphenicol was sub cultured with 1000 µL of the overnight culture and incubated at 37°C in a shaker until the OD_{600} had reached 0.5. The culture was immediately placed on ice and splitted into four parts of 3 ml each of culture. Protein expression was induced by the addition of 1 mM IPTG into three cultures and one tube being left un-induced. From two of the three induced cultures, one culture was supplemented with 100 µM Forskolin (Sigma-Aldrich Corp., Missouri) and the other culture with 100 µM 2′, 5′-Dideoxyadenosine (Sigma-Aldrich Corp., Missouri). Cells were harvested by centrifugation at 9200 x *g* for 10 minutes and lysed in 1 mL lysis buffer 1 (Amersham Healthcare, USA) supplemented with 2 mM IBMX (Sigma-Aldrich Corp., Missouri) to inhibit

phosphodiesterases. The sample was then shaken at 100 rpm, 37°C for 30 minutes in an AGITADOR orbital shaker (Comecta, S.A. optic ivy men system) to intensify the cell lysis process. The samples were centrifuged at 16.3 x *g* for 5 minutes using Corning, LSE, High speed micro centrifuge and then the lysate was transferred into a fresh Eppendorf tube where 200 µL of the Lysis buffer 2 (Amersham Healthcare, USA) was added and mixed. The 220 µL of the mixture was transferred into a fresh Eppendorf and 11 µL of acetylating reagent (Sigma-Aldrich Corp., Missouri) was added and the mixture pulsed. The endogenous cAMP contents from the lysates were then measured by cAMP enzyme immunoassay kit (Catalog# CA201, Sigma-Aldrich Corp., Missouri) following the acetylation version as described by the manufacturer's manual. The measurements were taken using a Microplate Reader (Labtech, International Limited East Sussex, UK) at 405 nm and results obtained were subjected to statistical analysis, analysis of variance (ANOVA) samples were done in replicates.

2.16.4. Determination of the *In vitro* Recombinant AtPPR-AC Enzymatic Activity

The *in vitro* enzymatic activity of the purified AtPPR-AC recombinant protein was determined by assessing its ability to convert ATP to cAMP. To determine activity, 2.5 µg of the purified recombinant AtPPR was assessed in a 200 µL reaction containing 50 mM Tris-HCl; pH 8.0, 2 mM IBMX, 5 mM Mg^{2+} and/or 5 mM Mn^{2+}, 1 mM GTP, 1M $NaHCO_3$, 100 µM Ca^{2+}, 1 mM ATP and/or 1 mM GTP were added to all tubes. Measuring of the residual cAMP levels was done by setting a control containing all incubation components except for the AtPPR protein. Reactions were then incubated at room temperature for 20 minutes (24°C) then terminated by the addition of 1 mM EDTA as well boiling for 5 minutes. Sample tubes were then centrifuged for 5 minutes at 16.3 x *g*; and the obtained lysates then kept at -20°C or until being assayed for cAMP content using the cAMP enzyme immunoassay kit (Catalog# CA201, Sigma-Aldrich Corp., Missouri) and following the acetylation version

as described by the manufacturer's manual. The measurements were taken at 405 nm in triplicates using the Microplate Reader (Labtech, International Limited East Sussex, UK) and results were subjected to statistical analysis, analysis of variance (ANOVA).

2.16.5. Complementation of *cya*A for Functional Activity using MacConkey Agar

*E. coli cya*A cells obtained from the Coli Genetic Stock Center (Yale University, Connecticut,USA), were prepared to become chemically competent as is described in section 2.11 but replacing 34 µg/mL chloramphenicol with 15 µg/mL kanamycin. The competent *cya*A cells were then divided into three portions. The first portion was transformed with the pCRT7/NT-TOPO: AtPPR-AC expression construct as already outlined in section 2.12. The second portion was transformed with empty pCRT7/NT-TOPO plasmid vector while the last portion was left un-transformed. A MacConkey agar plate supplemented with 15 µg/mL kanamycin and 0.1 mM IPTG (Sigma-Aldrich Corp., Missouri) was prepared and then plate was sub-divided into 4 quadrants using a permanent marker. The first quadrant was then streaked with c*ya*A mutant cells transformed with pCRT7/NT-TOPO:AtPPR-AC expression construct, the second quadrant streaked with *cya*A mutant cells transformed with the empty pCRT7/NT-TOPO plasmid vector, and the last quadrant was streaked with the non-transformed *cya*A mutant cells. The fourth quadrant was left un-streaked and the plate was then inverted and incubated at 37°C for 40 hours. After the incubation, all the quadrants were then visually inspected for various phenotypic characteristics.

CHAPTER THREE

3.0. Results and Interpretation

3.1. Amplification of the PPR gene by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR):

The cDNA of the AC region of the PPR gene (At1g62590) was isolated and amplified from *A. thaliana* RNA via RT-PCR using specific forward and reverse primers(Fig 3.1A). The amplified fragment was then cloned into pCRT7/NT-TOPO: AtPPR-AC and transformed into *E. coli* BL21 (DE3) pLysS cells, which was confirmed by colony PCR using the same set of primers as for RT-PCR (Fig 3.2B).

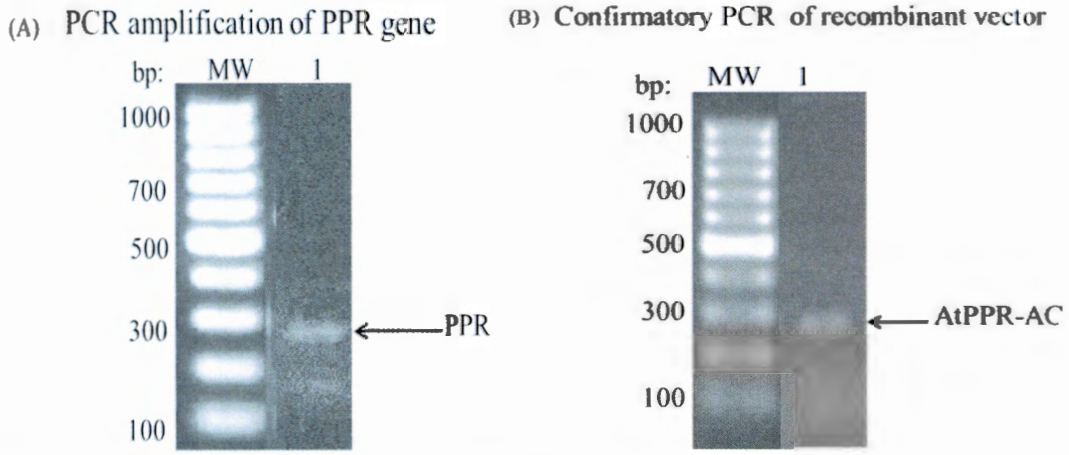

Figure: 3.1. Amplification of the AtPPR-AC gene by RT-PCR and Confirmation of its Cloning Success. **(A)** A 0.8 % agarose gel showing the AtPPR-AC gene (At1g62590) amplified from *A. thaliana* total RNA via RT-PCR. Lane 1 -100 bp MW ladder (Catalog# SM1143-Fermentas International Inc., Burlington, Canada); lane 2 shows the amplified AtPPR-AC gene fragment. **(B)** An agarose gel showing the PCR product after colony PCR of *E. coli* BL21 (DE3) pLysS cells containing pPCRT7/NT-TOPO. Lane 1 -100 bp MW ladder (Catalog# SM1143-Fermentas International Inc., Burlington, Canada); lane 2 shows the amplified AtPPR-AC gene fragment

3.2. Expression and Purification of the Recombinant AtPPR-AC Protein

The cloned AtPPR-AC gene was expressed in BL21 (DE3) Star pLysS cells as a fusion product with a His-tag. The expression was enabled by the addition of 1 mM IPTG to the transformed cells while part of the culture was left un-induced and acting as a control (Figure 3.2 A). The expressed recombinant protein was then purified from other bacterial proteins via a Ni-NTA affinity matrix (Figure 3.2 B).

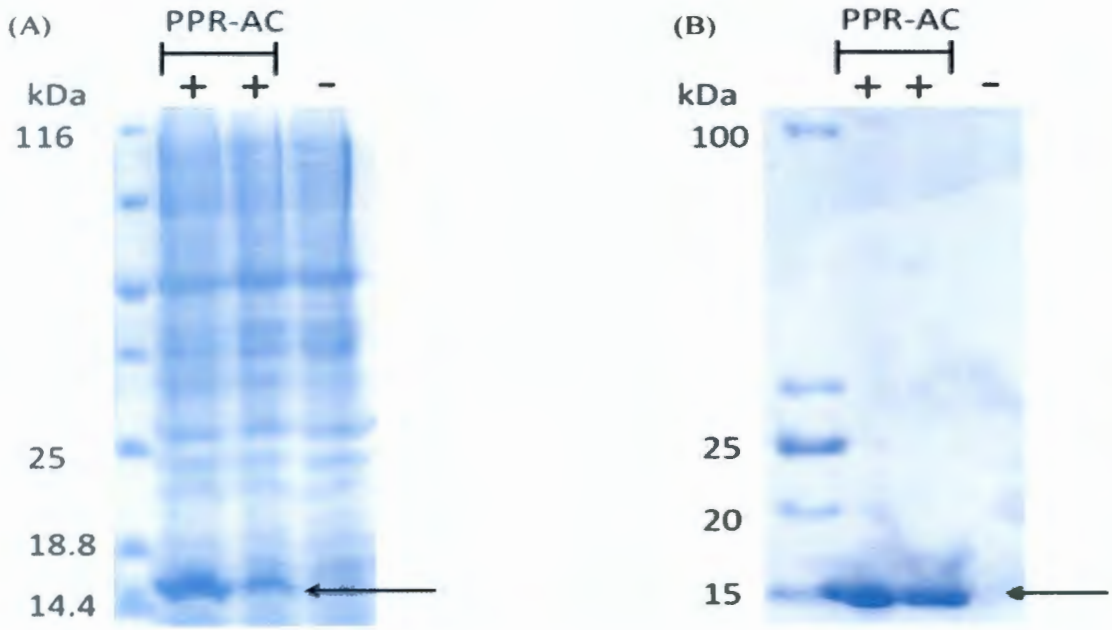

Figure 3.2: Expression and Purification of a Recombinant AtPPR-AC Protein. (A) An SDS-PAGE of protein fractions expressed in BL21 (DE3) star plysS cells transformed with the pCRT7/NT-TOPO: AtPPR-AC fusion construct where lane 1 is the unstained low molecular weight marker (Catalog# SM0431 Fermentas International Inc., Burlington, Canada), lane 2 and 3 (+) represents the induced culture with IPTG and, while lane 4 (-) is the un-induced culture without IPTG. **(B)** An SDS-PAGE of the purified AtPPR-AC ,where lane 1(M) represents the low range unstained marker (Catalog# 26632 Thermo Scientific., USA), lane 2 and 3 (+) showing a purified recombinant fusion construct, lane 4 represents (-) the control where there was no protein bound to the matrix. The arrows indicate the expressed and purified AtPPR-AC.

3.3. Determination of the Endogenous Activity of the Recombinant AtPPR-AC Protein

The endogenous adenylate cyclase activity was assessed from the expressed recombinant PPR gene with the un-induced and the induced (IPTG) (Figure 3.3 A) as well as the induced

+ forskolin, and induced + 2′, 5′-dideoxyadenosine (Figure 3.3 B). Enzyme immunoassay was used to determine the quantitative levels of cAMP produced by each expressional and differently treated systems.

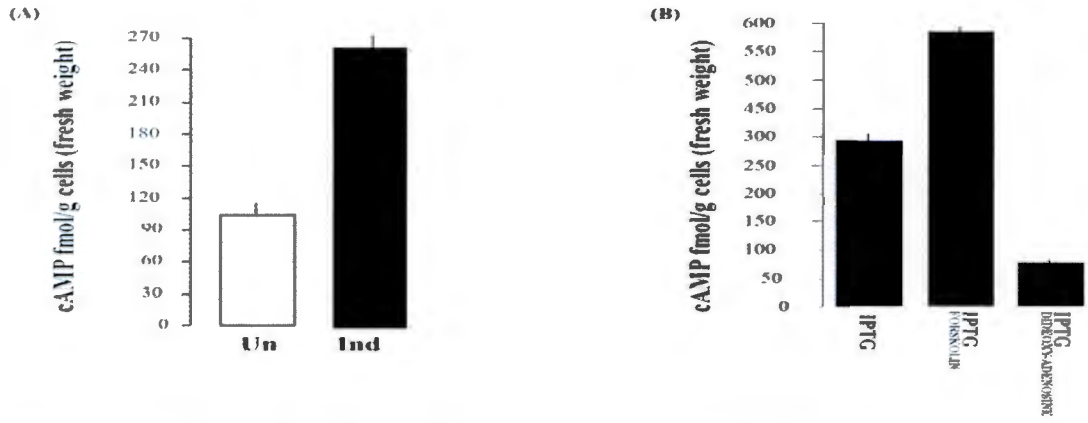

Figure 3.3: Determination of the Endogenous Activity of the Recombinant AtPPR-AC Protein. **(A)** cAMP levels generated by un-induced (Un) and induced (Ind) *E. coli* BL21 (DE3) pLysS cells harbouring the AtPPR-AC gene. **(B)** cAMP levels generated by induced cell cultures in the presence of either Forskolin or dideoxy-adenosine. All the levels of cAMP were determined using cAMP Enzyme immunoassaying system (catalogue number CA201, Sigma, Missouri, USA), where error bars represents the standard errors (SEM) of the means of three independent and representative assays.

3.4. Determination of the *In Vitro* Activity of the Recombinant AtPPR-AC Protein

The recombinant protein was first purified and tested for *in vitro* adenylate cyclase activity using the enzyme immunoassay system. The protein activity was also determined in the presence of other reaction components such as 5 mM Mn^{2+}, 100 µM Ca^{2+}, 1 mM GTP and 50 mM CO_3^{2-} in order to see their effects on the protein's AC catalytic activity (Figure 3.4).

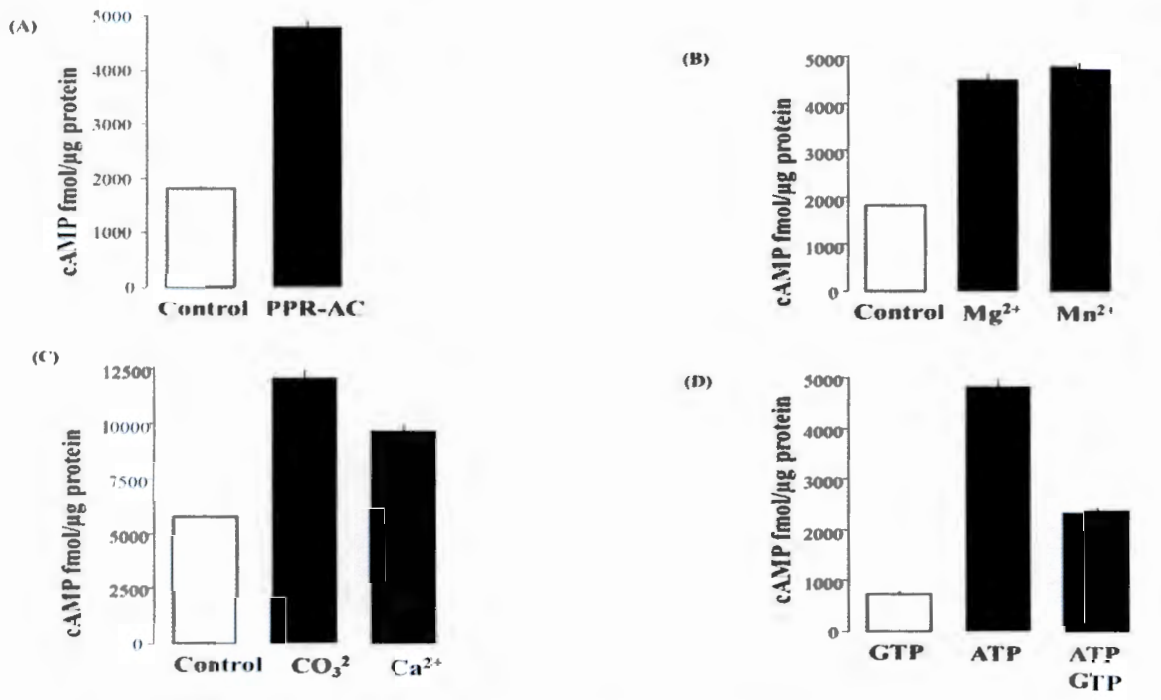

Figure 3.4: Determination of the *In Vitro* Activity of the Recombinant AtPPR-AC Protein. (**A**) *In vitro* testing of AC activity of the recombinant AtPPR-AC, where an empty bar represents the control with no protein while the solid bar represents activity in presence of AtPPR-AC. (**B**) Recombinant AtPPR-AC activity in the presence of 5 mM Mg^{2+} or 5 mM Mn^{2+}. (**C**) Recombinant AtPPR-AC activity in the presence of CO_3^{2-} or Ca^{2+} ions. (**D**) Recombinant AtPPR-AC activity in the presence of ATP, GTP or ATP and GTP. Error bars represent the standard errors (SEM) of the means of three independent and representative assays.

3.5. Determination of the *In Vivo* Activity of the Recombinant AtPPR-AC Protein

The figure below shows an assessment of the AtPPR-AC's ability to convert non-lactose fermenting SP850 *cya*A *E. coli* cells transformed by this gene into lactose-fermenting wild type cells. The outcome was then visually assessed on MacConkey agar (Figure 3.5) whereby the white/yellowish colour of mutant cells change into the deep magenta purple colour of wild type cells when transformed with pPCRT7/NT-TOPO.

Figure 3.5: Determination of the *In Vivo* Activity of the Recombinant AtPPR-AC Protein. Different *E. coli cya*A cells were plated onto MacConkey agar supplemented with 0.1 mM IPTG and divided into four quadrants with a permanent marker and incubated for 40 hours at 37°C. Section 1 of the plate contains no cells, section 2 contains non-transformed *E. coli cya*A mutant cells, section 3 contains *E. coli cya*A mutant cells transformed with the empty pCRT7/NT-TOPO vector while section 4 contains *E. coli cya*A mutant cells transformed with the pCRT7/NT-TOPO: AtPPR-AC expression construct. Cells in sections 2 and 3 are both non-lactose fermenters and produce white or yellowish colonies. Cells in section 4 have picked a deep purple phenotype – a characteristic signifying the ability to ferment lactose.

CHAPTER FOUR

4.0. Discussion, Conclusion and Recommendations

4.1. Discussion

The pentaricopeptide gene forms one of the largest gene families found in the *A.thaliana* genome, comprising 466 genes (Aubourg *et al.*, 2000; Small and Peeters, 2000). A previous study has implicated the PPR gene as harbouring a possible nucleotide cyclase catalytic centre, namely adenylate cyclase (Gehring, 2010). Previous studies have also shown that the pentatricopeptide repeat gene family is associated with post-transcriptional processes such as RNA splicing, RNA editing, RNA processing and RNA translation mainly in cellular organelles (Nakamura *et al.*, 2004, Kotera *et al.*, 2005, Schmitz-Linneweber *et al.*, 2005). Interaction of PPR with the RNA/DNA may affect, in a cascade of processes, post-transcriptional gene silencing or translational repression which as a result, may interfere with the synthesis of gene expression and/or signalling pathways (Tang *et al.*, 2003, Chen *et al.*, 2004). PPR proteins are central regulators of RNA metabolism in organelles (Schmitz-Linneweber and Small, 2008) since they interact with the RNA via the PPR motif and so, it is probably possible that its downstream cellular signalling pathways may be mediated by cAMP (Stern *et al.*, 2010).

Typical localisation of most adenylate cyclases including the PPR has been documented to be primarily at the plasma membrane and in this study, part of the PPR's intracellular domain which harbours the AC catalytic centre was targeted and cloned. In order to target the catalytic AC centre in the PPR sequence, we first retrieved the PPR (At1g62590) gene sequence from TAIR and determined its expressible cDNA sequence. We designed primers that specifically amplified a 50 amino acid region of At1g62590 containing the AC region (Figure 2.1B). Sequence specific primers were then used to amplify and isolate the targeted

AtPPR-AC gene fragment. When resolved on a 1% agarose gel, the expected fragment size of 280 bp was obtained (Figure 3.1A). After digestion the AtPPR-AC insert was ligated into pCR®T7 TOPO®-NT vector. This expression construct was transformed into BL21 (DE3) STAR pLysS cells and confirmation of ligation was verified by colony PCR (Figure 3.1B). Maximal expression of the AtPPR-AC recombinant protein was then undertaken by inducing the cell cultures at OD_{600} of 0.5 with 1 mM IPTG and the expressed recombinant protein resolved by SDS-PAGE. As expected, a recombinant fusion tagged product of approximately 14.56 kDa was obtained (Figure 3.2A).

To assess if the adenylate cyclase catalytic centre in the AtPPR-AC protein had enzymatic activity, an endogenous assaying of the cAMP levels generated without cell induction, with cell induction as well as cell induction in the presence of AC modulators (Ehsan *et al.*, 1998, Volotovski *et al.*, 1998) 100 µM forskolin or 100 µM dideoxyadenosine was undertaken. As is shown in Figure 3.3A, cells induced with 1 mM IPTG had their cAMP generation increased by a 2.7-fold factor as compared to the un-induced cells. Additionally, the treatment of induced cells with 100 µM forskolin further increased the level of cAMP generation by a factor of 2.07 (Figure 3.3B). On the other hand, the treatment of induced cells with 100 µM dideoxy-adenosine significantly reduced the levels of cAMP by a factor of 4.8 (Figure 3.3B). In a related previous study where a PSiP coding region of a pollen-specific putative AC from *Agapanthus umbellatus* (*Liliaceae*) was cloned into bacterial cells, treatment of cells with 1 mM IPTG increased the cAMP levels by a factor of 3.0 while treatment of induced cells with 100 µM of the AC inducer – forskolin, increased the levels of cAMP generation by a factor of 1.83 (Moutinho *et al.*, 2001). Furthermore, the application of 100 µM of the AC inhibitor - dideoxyadenosine to the growing pollen tubes transiently caused a temporary growth arrest accompanied by a reduction of cAMP concentration by a

factor of 1.8 (Moutinho *et al.*, 2001). These previous findings are similar to what we observed.

In summary it could be speculated at this point that the *A. thaliana* AtPPR-AC recombinant protein is capable of directly converting ATP to cAMP or else it is a functional plant molecule capable of stimulating other resident adenylate cyclases (*E. coli* ACs in this case) to produce cAMP.

Therefore, in order to further test if the recombinant AtPPR-AC really had any functional adenylate activity, the protein was purified via Ni-NTA affinity matrix (Figure 5.2B) followed by its assessment for *in vitro* AC activity (Figure 3.4). A relatively high level of AC activity (2.57 times more than the control) for the AtPPR-AC was shown (Figure 3.4A) with no specific biased preference for either Mg^{2+} or Mn^{2+} as cofactor ions for its activity (Figure 3.4B). As previously reported (Tesmer *et al.*, 2000; Geng *et al.*, 2005), some adenylate cyclases may exhibit no inherent preference of either Mg^{2+} or Mn^{2+} metal ions as cofactors for enzymatic activity. However presence of either of these metal ions, the adenylate cyclase activity does increase in a dose-dependent manner (Tesmer *et al.*, 2000; Geng *et al.*, 2005).

In addition, the results obtained in Figure 3.4C indicate that the recombinant PPR protein can cyclize ATP into cAMP in the presence of both Ca^{2+} and CO_3^{2-} ions as its functional modulators. The increased activity as a result of the presence of calcium ions might possibly be due to the responsive activities of the cyclic nucleotide-gated ion channels ($CNGC_S$), which are crucial for plant development through signalling pathway involved in cyclic nucleotides (Talke *et al.*, 2003). This trend was not unfamiliar since calcium has previously

been implicated as a second messenger that triggers physiological changes in response to external environmental stimuli such as oxidative stresses and phytohormones that will at the end result in signalling transduction (Ma and Berkowitz, 2007, McAinsh and Pittman 2009). In support of this observation, a previous study has reported that transient changes in cAMP levels were always accompanied by an increase in $[Ca^{2+}]$, suggesting that the two signalling pathways are interconnected (Malho *et al.*, 2000). The recombinant PPR protein had also shown an elevation in cAMP generation in the presence of carbonate ions thus suggesting the positive stimulatory effects of these ions on the enzymatic activity of protein. Once more, this finding was not surprising since carbonate is known to increase cAMP generation in a pH-independent manner, which is noted to directly modulate the enzyme (Yanqui *et al.*, 2000).

Substrate specificity for the recombinant AtPPR-AC protein between ATP and GTP as possible substrates was also assessed by testing its ability to generate cAMP from either of the two substrates (Figure 3.4D). As is shown in the figure, the generated cAMP levels from ATP were more than 5.5 times the levels generated from GTP and thus indicating the strict specificity of the AtPPR-AC for ATP. Furthermore, when these two substrates were provided to the protein in equimolar concentrations, the overall activity was somewhat reduced by a factor of 0.5, probably as a result of competitive binding to the enzyme between these two structural analogues. Conceivably, in all class III ACs including AtPPR-AC, thus specificity for the binding of the adenine moiety involves Lys938 and Asp1018 which were also observed in AtPPR-AC catalytic centre and thus a very high preference for ATP specificity over GTP (Sunahara *et al.*, 1998).

From all these *in vitro* findings, it can be concluded that the AtPPR-AC is a functional AC. To further validate this result, an *in vivo* assessment of the AtPPR-AC activity was undertaken through a functional complementation test. This test was performed using an SP850 mutant *E. coli cya*A strain that is deficient in endogenous adenylate cyclase and therefore cannot catabolize lactose (Ullmann and Danchin, 1983; Moutinho *et al.*, 2001). When this strain is grown on MacConkey agar, it produces white/yellowish colonies as compared to the magenta deep purple colonies produced by its wild-type counterpart (Moutinho *et al.*, 2001). In order to test if the activity of the AtPPR-AC could rescue the mutant cells, these cells were transformed with the pCRT7/TOPO-AtPPR-AC expression construct followed by assessment of the colony phenotypes on MacConkey agar supplemented with 0.1 mM IPTG (Figure 3.5). As shown in the figure 3.5, section 3 the transformed cells stained deep-purple, signifying the rescuing of the mutant cells and thus validating AtPPR-AC as a functional AC.

4.2. Conclusions

Findings from this study ascertains PPR as a functional higher plant adenylate cyclase and thereby becoming one of the identified higher plant adenylate cyclase after the *Zea mays* pollen protein (Moutinho *et al.*, 2001). These findings therefore provided the basis for further characterisation and exploration of the exact physiological roles played by the AtPPR-AC in higher plants by assessing its possible crosstalking between cAMP and NO, Ca^{2+} and/or other signaling molecules.

4.3 Recommendations

Findings from this study allow for these possible and practically feasible recommendations.

- Firstly, since this molecule (PPR) was bioinformatically identified together with other eight putative molecules (Gehring, 2010) and in this study, it has been confirmed to be a higher plant adenylate cyclase, it is therefore suggested that the other eight putative molecules also be tested as possible higher plant adenylate cyclases.
- Secondly, since the AtPPR-AC has been confirmed as a functional adenylate cyclase, it is imperative that its exact physiological role in cell signal transduction and particularly, in plant stress response and adaptatiom mechanisms be further investigated.
- Further assays will involve the activities of the recombinant AtPPR-AC protein in living plant tissues (protoplasts) followed by some bioinformatic correlational expression analysis of the AtPPR-AC gene level with respect to other plant stress-related genes

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