Heterologous Expression and Functional Characterization of a Truncated Uridine Kinase Family Protein from *Arabidopsis thaliana*

By

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Supervisor: **Prof. O Ruzvidzo**

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Declaration

I, Kgomotso Faith Mohatalle, do hereby declare that the dissertation entitled, **Heterologous Expression and Functional Characterization of a Truncated Uridine Kinase Family Protein from *Arabidopsis thaliana*** has been undertaken by me for the award of Masters of Biology. I completed the study under the supervision of **Prof Oziniel Ruzvidzo, Professor of Plant Biotechnology, North-West University (Mafikeng Campus).**

I also declare that dissertation has not been submitted for the award of any Degree in this University or any other University.

Student: **Kgomotso Faith Mohatalle**

Signature  Date 24/04/2017

Supervisor: **Prof. O Ruzvidzo**

Signature Date.....

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Dedication

This research work is dedicated to my pillar of strength, my beautiful mother Letticia Mohatalle, for her undying love and support throughout my academic work, the Department of Biological Sciences, North-West University, Mafikeng Campus, my supervisors (the guptas) Prof. O Ruzvidzo and Dr. DT Kawadza for their mentorship, encouragement and motivation, my beloved family for their support, the NRF and Foodbev for their financial support, to my Plant Biotechnology Research Group, for their corporation and support and most importantly to my King, my creator, The Lord I serve. Praise!!!

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This research is the collective effort of many people, who include the Mohatalle family, my mother, the Plant Biotechnology Research group and especially my supervisor Prof. O Ruzvidzo, for his patience and continuous guidance with extraordinary enthusiasm and intellect. I would also like to extend my warm gratitude to the Biological Sciences Department of the North-West University Mafikeng Campus and the National Research Foundation for their financial assistance. Lastly, I would like to thank God for granting me such an opportunity; it was through his word. Amen

(Isaiah 41:10)

Definitions of Terms

Cloning: A process whereby copies of DNA fragments are obtained by allowing an inserted DNA fragment to be replicated several times by a plasmid.

Cloning vectors: Genetic elements into which genes can be recombined and replicated.

Adenylate cyclases: Enzymes capable of converting adenosine triphosphate (ATP) to the cyclic adenosine monophosphate (cAMP).

Guanylate cyclases: Enzymes capable of converting guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP).

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

Reverse transcriptase-polymerase chain reaction: A molecular method used to amplify a RNA segment into copy DNA (cDNA) using an RNA-dependent DNA polymerase enzyme.

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

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

Signal transduction: A process whereby an external signal is detected by a sensor that transmits it to the rest of the cellular regulatory machinery.

List of Abbreviations

AC	:	Adenylate cyclase
ATP	:	Adenosine 5′-triphosphate
BLAST	:	Basic Local Alignment Search Tool
cAMP	:	Cyclic 3′,5′-adenosine monophosphate
CRP	:	cAMP receptor protein
IPTG	:	Isopropyl-3-D-thiogalatopyranoside
MSMO	:	Murashige and Skoog basal salt with minimum organics
OD	:	Optical density
RT-PCR	:	Reverse transcriptase polymerase chain reaction
sAC	:	Soluble adenylyl cyclase
UKFP	:	Uridine kinase family protein
Ump	:	Uridine 5′-monophosphate
UPRT	:	Uracil phosphoribosyl transferase
UV	:	Ultraviolet
WAK	:	Wall-associated kinase
YT	:	Yeast-tryptone

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Abstract

Adenylate cyclases (ACs) are enzymes that convert adenine-5′-triphosphate (ATP) to cyclic 3′, 5′-adenosine monophosphate (cAMP). Virtually in nature, it has been found that cAMP has an important role in chemical signaling and as a second messenger in both animals and lower eukaryotes. The second messenger systems can be synthesized and activated by enzymes for example, the cyclases that synthesize cyclic nucleotides. These small molecules bind and activate protein kinase and other proteins, thus continuing the signaling cascade. Signaling transduction occurs when an extracellular molecules activates a specific receptor located on the cell surface or inside the cell which in turn triggers a biochemical chain of events inside the cell known as signaling cascades that eventually elicits a response. This response may alter the cell`s metabolism, shape, gene expression and ability to divide. Cyclic AMP can affect many different physiological and biochemical responses including the activity of protein kinases. Currently, the only annotated and experimentally confirmed ACs in higher plants are the *Zea mays* pollen signaling protein with a role in polarized pollen tube growth , the *Arabidopsis thaliana* pentatricopeptide repeat protein with a role in regulating gene expression for pathogen responses at the RNA level and bind the RNA in a sequence specific a modular fashion way, the *Nicotiana benthamiana* adenylyl cyclase protein responsible for the tabtoxinine-β-lactam-induced cell deaths during wildfire diseases and the *Hippeastrum hybridum* adenylyl cyclase protein involved in stress signalling. Otherwise not much is presently known about this group of molecules in higher plants as is in other organisms. Therefore, in this study, and in line with an endeavor to attempt and identify more higher plant AC candidates, we describe the cloning, partial expression and functional characterization of a putative *Arabidopsis thaliana* uridine kinase family protein (UKFP), which has recently been bioinformatically annotated as a possible AC but has not yet been practically tested and/or confirmed. Findings from this study have firmly confirmed that this putative candidate molecule is actually a *bona fide* and catalytically functional higher plant AC.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

There are a number of factors such as droughts and floods that are associated with climatic changes that ultimately and drastically affect both plants and food production. Climate change effects can be managed through the production of modified crop varieties, which have the ability to withstand such harsh conditions. There is sufficient research on the effects of climate change onto plant diseases and some reasonable progress being made. Abiotic stress has a huge impact on growth and it is responsible for severe losses in the field. The resulting growth reductions can reach up to 50% in most plant species. Moreover, biotic stress is an additional challenge inducing a strong pressure on plants and adding to the damage through pathogen or herbivore attack. Recent evidence shows that a combination of abiotic and biotic stress can have a positive effect on plant performance by reducing the susceptibility to biotic stress (Rejeb *et al.*, 2014).

Cross-tolerance between environmental and biotic stress may induce a positive effect and enhance resistance in plants and have significant agricultural implications, this was concluded based on the research that shows that certain environmental stresses have the possibility to predispose the plant in order to allow it to respond faster and in a resistant manner. Plants exposed to both of these two stresses were observed to have a better resistance as opposed to those exposed to individual stresses, a combination seem to lead to resistance (Rejeb *et al.*, 2014), more so the control of every kind of stress by specific hormones allows defence responses against defined environmental conditions.

At the genomic level, advances in technologies for the analysis of gene expressions have made it possible to begin discriminating responses to different biotic and abiotic stressors and potential trade-offs in responses (Garrett *et al.*, 2006). At the scale of an individual plant, enough experiments have to date been performed to begin appreciating the effects of climate variables on infection rates. Models of plant disease have now been developed to incorporate more climatic predictions. At the population level, the adaptive potential of plant and

pathogenic populations may prove to be one of the most important predictors of the magnitude of climate change effects (Garrett *et al.*, 2006).

Thus with the ever-growing world population, there is a greater need for a continued production of crops and crop plants, whereby biotechnology and plant molecular approaches may be the only probable solutions. In this presently reported research study, particular focus was on one of the probable signaling protein molecules from the *Arabidopsis thaliana* plant, called the uridine kinase family protein (UKFP), which may hold answers to some of these sticking and outstanding problems. Uridine kinase family protein is an enzyme that belongs to the family of transferases, which transfer the phosphorus molecule to other groups and also catalyze the reaction of ATP (adenine 5′-triphosphate) and uridine to form ADP (adenine 5′-diphosphate) and UMP (uridine 5′-monophosphate) (Gehring, 2010). In addition, this putative molecule has previously been bioinformatically annotated to contain a probable adenylate cyclase catalytic center in its architectural structure (Gehring, 2010).

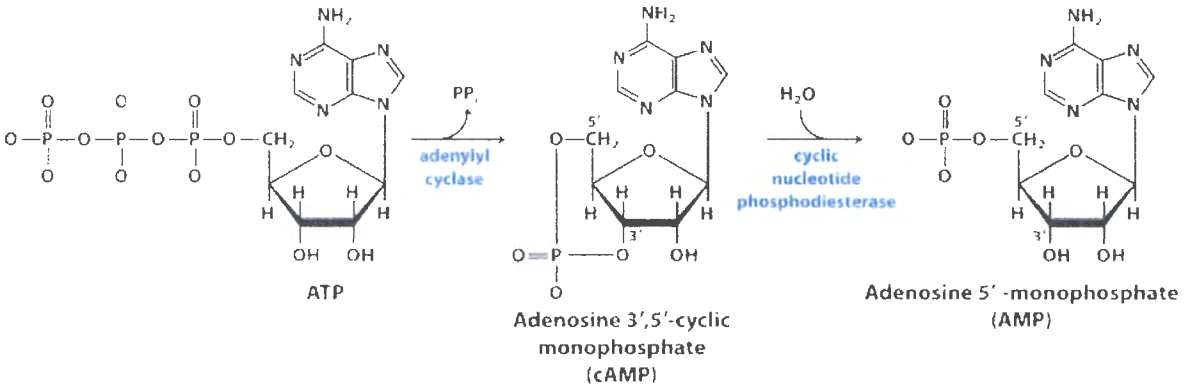

Fig.1.1 The formation of cyclic adenosine phosphate (cAMP) from adenosine triphosphate ATP by the enzyme adenylate cyclase (AC.) (Biochemistry 2002)

Adenylate cyclases (ACs) are enzymes capable of catalyzing the formation of a cyclic 3′, 5′-adenosine monophosphate (cAMP) and a pyrophosphate from the biological molecule adenosine triphosphate (ATP). Adenylate cyclases and their product cAMP are involved in the regulation of important plant processes such as cell development, morphological changes, intracellular signaling and stress response, defence processes, pyrimidine nucleoside salvage and other biosynthetic processes located in the chloroplast. The ACs are closely associated

with signaling cascades of pollen tube growths (Moutinho *et al.*, 2010) and their product, cAMP has also been shown to regulate Ca^{2+}-regulated signalings in *Nicotiana plumbaginofolia* (Makoto *et al.*, 2014).

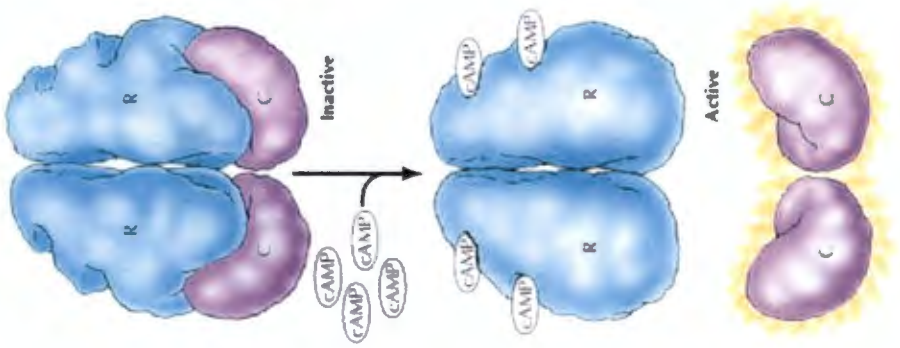

Fig 1.2: The inactive form of the protein kinase A consists of two regulatory (R) and two catalytic (C) subunits. Binding of cAMP to the regulatory subunits induces a conformational change that leads to dissociation of the catalytic subunits, which are then enzymatically active. (Cooper GM., 2000)

1.2 Literature Review

Global climate change is predicted to cause extreme temperatures and severe droughts in some parts of the world, while other parts will experience heavy storms and periodic floods (Marshall *et al.*, 2012). These conditions will have a dramatic impact on crop growth and productivity, will threaten the societal sustainability (coupled with an increasing global population), and will generate serious challenges for human welfare (Marshall *et al.*, 2012). In future, climate change is apparently expected to be problematic, resulting in dry springs and rainy summers in Northern Europe and longer dry periods in the South. Interestingly, one possible outcome of multiple stress exposure is that plants that are able to defend themselves facing one stress tend to become more resistant to other stresses and the phenomenon is called cross-tolerance, showing that plants possess a powerful regulatory system allowing them to adapt quickly to a changing environment.

Wounding, for instance, has been shown to increase salt tolerance in tomato plants. Furthermore, in tomato plants again, localized infection by *Pseudomonas syringae* pv. Tomato (Pst) induces systemic resistance to the herbivore insect known as the *Helicoverpa zea*. The association between abiotic and biotic stress is also possible, as demonstrated by the reduced infection of tomato by *Botrytis cinerea* and *Oidium neolycopersici* following the application of drought stress Ozone exposure can induce resistance to virulent *Pseudomonas*

syringae strains in Arabidopsis (Rejeb *et al.*, 2014). Recent studies show that viral infection protects plants against drought stress. Verticillium infection in Arabidopsis plants induced the expression of the Vascular-Related No Apical meristem ATAF and Cup-Shaped Cotyledon (NAC) domain (VND) transcription factor VND7. The VND7 induced *de novo* xylem formation ensuring the water storage capacity and subsequently increased plant drought tolerance. Stress combination induces different signaling pathways, which share some components and common outputs. This could help plants to minimize energy costs and create a flexible signalling network. This leads to different approaches taken in creating a flexible signalling network.

Several years ago, our understanding of metabolic cellular regulation was radically altered by the discovery of a single molecule known as the adenosine 3′,5′-cyclic monophosphate (cAMP) (Russell and Christopher, 2004). This, in turn, has also led to more discoveries of the biological signal transduction systems and the roles of kinases and protein phosphorylation/dephosphorylations in the regulation of protein and cellular activities (Russell and Christopher, 2004). The specific roles of cAMP in plants include the regulation of ion channels and ion transport (Bolwell, 1995). In *Arabidopsis thaliana*, cAMP is also known for the activation of phenylalanine ammonia lyase, cAMP-dependent signal transduction pathways and the cell cycle progression (Bolwell, 1995). Cyclic AMP also has been shown to play a role in stimulating protein kinase activities in rice (*Oryza sativa*) leaves, and more recently, to elicit stress responses and plant defense mechanisms in the same plant (Lemtiri-Chlieh *et al.*, 2011).

Although uracil phosphoribosyl transferases (UPRTs) and adenylate cyclases (ACs) play an important role in the pyrimidine salvage pathway (Rafiqul *et al.*, 2007), still there is no specific report on the characterization of either UPRT or AC genes in higher plants. Only a few studies have been conducted on the uridine salvage pathway of actively growing potato tubers while on the other hand, not much has literally been done on ACs. Adenylate cyclases are enzymes capable of converting the biological molecule adenine 5′-triphosphate (ATP) to cyclic 3′,5′-adenosine monophosphate (cAMP). In animals and lower eukaryotes, cAMP has been firmly established as an important signaling molecule, acting as a second messenger in several cellular signal transduction pathways (Ma *et al.*, 2010). However, in higher plants there are currently only four experimentally tested and functionally confirmed ACs, and these are the pollen signaling protein from *Zea mays*, which is responsible for the polarized growth of pollen tubes (Moutinho *et al.*, 2001), the pentatricopeptide repeat protein from *Arabidopsis*

thaliana, which is responsible for pathogen responses and gene expressions (Ruzvidzo *et al.*, 2013), the adenylyl cyclase protein from *Nicotiana benthamiana*, which is responsible for the tabtoxinine-β-lactam-induced cell deaths during wildfire diseases (Ito *et al.*, 2014) and the adenylyl cyclase protein from *Hippeastrum hybridum* involved in stress signaling (Swiezawska *et al.*, 2014). Otherwise besides these four, not much is known in higher plants about these ACs and/or their enzymatic product cAMP as is the case in animals and lower eukaryotes (Ma *et al.*, 2010).

Apparently, the major reasons why AC and/or cAMP information was not readily available in plants were firstly, that the levels of cAMP detected in plants seemed to be very low as compared to those found in animals and secondly, that the vagaries of assays conducted in plants were not conducive to reach firm and final conclusions (Gehring, 2010). Notably and in 2010, Gehring managed to identify fourteen putative proteins in the Arabidopsis genome using a search motif consisting of functionally assigned amino acids in the catalytic centre of annotated and experimentally tested nucleotide cyclases (Table 3.1). The uridine kinase family protein was one of these proteins. With an exception of only one (Ruzvidzo *et al.*, 2013) none of those protein candidates has yet been experimentally and/or functionally confirmed.

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

ATG NUMBER	ANNOTATIONS
At1g26190*	Phosphoribulokinase/uridine kinase family protein
At173980	AX4 AC domain containing protein
At2g11890	G3 AC family protein
At1g25240	Epsin N-terminal homology
At1g62590	Pentatricopeptide (PPR) protein
At1g68110	Clathrin assembly protein
At2g34780	Maternal effect embryo arrest 22
At3g02930	Chloroplast protein
At3g04220	TIR-NBS-LRR class
At3g18035	Linker histone-like protein – HNO4
At3g28223	F-box protein
At4g39756	F-box protein

*The UKFP gene that was studied in this work.

Given that cAMP has previously been identified in higher plants and its biological role linked to various cell signaling and communication systems, it was therefore, important that the uridine kinase family protein be experimentally characterized to assess whether it was biologically or enzymatically functional. This would lead to an establishment of yet another additional AC protein candidate in higher plants in addition to the currently known four ACs. Furthermore, this investigation would lead to an establishment of the probable role(s) of these novel proteins in plant stress response and adaptation mechanisms.

1.3 Problem Statement

Although the uridine kinase family protein has already been shown to catalyze the molecular transfer of a phosphorus group to the other protein molecules as well as facilitating the reaction of ATP and uridine, this biological enzyme has not yet been experimentally shown to catalyze the molecular cyclization of ATP into cAMP. This is also despite the fact that this same enzyme has also been previously annotated to contain a probable adenylate cyclase catalytic center in its structural domain architecture (Gehring, 2010). Thus this research was set to attempt and test if the annotated AC catalytic motif of this putative protein is indeed functional and if so, whether this novel protein had any apparent physiological role in stress response and/or adaptation mechanisms. This was done through the cloning of this catalytic AC motif followed by a functional testing and/or characterization of its possible catalytic activity.

1.4 Research Aim

The main research aim was to establish if the uridine kinase family protein gene could code for a functional AC protein and whether such a protein has any physiological roles in cell signaling and communication systems, particularly in biotic and/or abiotic environmental stress responses and adaptation mechanisms. This was, therefore, explored by practically testing if the bioinformatically annotated uridine kinase family protein from *Arabidopsis thaliana* could function as a *bona fide* adenylate cyclase *in-vivo*.

1.5 Objectives of the Research

The following objectives were set in order to address the stated research question/aim:

1. To isolate and clone the *Arabidopsis thaliana* uridine kinase family protein (UKFP) AC gene fragment into a stable and viable heterologous prokaryotic expression system.
2. To optimize strategies for the recombinant expression of the annotated UKFP protein.
3. To determine the enzymatic activity of the truncated UKFP protein.
4. To bioinformatically determine the functional role of this annotated UKFP protein in plants.

1.6 Significance of the Research Study

A successful completion of this research study would result in the following:

1. The identification and establishment of yet another additional functional higher plant AC in addition to the only and currently known four candidate molecules.

2. A better understanding of the mechanisms which plants use to respond and adapt to stressful environmental conditions.

3. A potential contribution towards the moderate integrated management of both biotic and abiotic stressful conditions of agronomically important crops in South Africa.

CHAPTER TWO

RESEARCH METHODOLOGY

2.1 Isolation of the Uridine Kinase Family Protein Gene Fragment

2.1.1 Seed Sterilization

Arabidopsis thaliana ecotype Columbia seeds were germinated on Murashige and Skoog (MS) plate media (0.43% (w/v) of organic salts, 3% sucrose and 0.4% (w/v) tissue culture grade agar supplemented with 1 ml/l of Gamborg's vitamins, at pH 5.7). The seeds were washed three times with 70% (v/v) ethanol in a 1.5 Eppendorf tube for two minutes each wash. The alcohol used for washing was discarded and the seeds were further washed with 500 µl of the seed sterilization buffer (0.1% (w/v) sodium dodecyl sulphate (SDS), 5% (v/v) commercial bleach). The seeds were rinsed (5 times) with sterile distilled water (1 ml) and further suspended in sterile distilled water (500 µl). The seeds were then stratified at 4°C for 3 days and were then germinated in an MS petri dishes for two weeks to grow in a growth chamber, subjected to greenhouse conditions with adjustments of 23/16 day/night for periods of 8/16 hours night/day at 10 000 light lux. Sterile distilled water was used to water the germinated seedlings transplanted into a potting soil (50% (w/w) peat based soil and 50% (w/w) vermiculite), to help retain moisture a plastic cling wrap was used to cover the soil, which was then kept in the growth chamber to grow for a further 2-4 weeks. Isolation of the annotated UK gene was from these 4-6 week old Arabidopsis plants.

2.1.2 Designing and Acquisition of Sequence-specific Primers

The amino acid sequences of the uridine kinase family protein (Figure 2.1 below) were retrieved from the TAIR site and using its locus details (gene bank numbers) (http://www.arabidopsis.org), and as was described by Gehring (2010) to manually design its sequence-specific primers for its AC catalytic center. The primers were designed at specific positions and in order to flank and isolate the catalytic domain of the targeted gene fragment. The designed primer sequences were then sent for synthesis and subsequent supply by the Inqaba Biotechnological Sciences (Pretoria, RSA).

Fig. 2.1: The complete amino acid sequence of the uridine kinase family protein. This protein consists of a total of 2435amino acids. The highlighted sequences are the annotated AC catalytic center (green) while the arrows are marking both the forward and reverse priming sites.

At1g26190AC FWD
(**Fwd:** 5′-GCGGGGCGGGATCCGATGGGTCAAGACAGCAATGGA-3′)
At1g26190AC REV
(**Rev:** 5′-CGCGGCCGAATTCA GCTAGTCATAGTCCGTTAACCG-3′)

Fig. 2.1.1. The two sequence-specific primers that were manually designed to target the AC catalytic centre of the putative uridine kinase family protein.

2.1.3 Amplification of the UKFP Gene Fragment

0.1-0.2 g of the material from the leaf of a plant was harvested to obtain the UKFP gene fragment using the RNA Purification Mini Kit (Thermo Scientific GeneJET), and following the manufacturer's protocol (Thermo Scientific Inc., Massachusetts, USA). Weighed 100 mg of the leaf tissue was flash-frozen in liquid nitrogen and was then thoroughly grinded with a pestle and mortar into to a fine powder. 500 µl of plant Lysis Solution was poured into the tissue powder in an Eppendorf tube and transferred into another 1.5 ml Eppendorf tube and vortexed for 20 seconds at maximum speed. The mixture was then incubated for 3 minutes at 56°C and then centrifuged at maximum speed using LSE High Speed Microcentrifuge (Corning Inc., Amsterdam, Netherlands). Transferring the supernatant into a clean 1.5 ml Eppendorf tube helped, for the mixture to be thoroughly mixed by pipetting using a 250 µl of 96% (v/v) ethanol. This mixture was then transferred into a purification column in a collection tube and centrifuged for 1 minute at 12,000x*g*. The flow-through was discarded while the column and collection tube were re-assembled. 700 µl of Wash Buffer 1 was then added to the purification column and then centrifuged at 12,000x*g* for 1 minute. The purification column was placed in a new and clean 1.5 ml collection tube and 500 µl of Wash Buffer 2 was added to the column. The column was then centrifuged at 12,000x*g* for 1

minute. Thereafter the flow-through was discarded, and the washing step was repeated once more time. The column was then transferred into an RNAse-free 1.5 ml collection tube and ultimately the mRNA was eluted by adding 50 µl of nuclease-free water directly onto the column membrane and centrifuged at 12,000x*g* for 1 minute. The total mRNA extracted was then used as a template to generate copy DNA (cDNA). Together with the acquired sequence-specific primers, the cDNA generated was subsequently used to amplify the targeted UKFP gene fragment in a reverse transcriptase-polymerase chain reaction (RT-PCR) system through the use of the Thermo Scientific Verso 1-Step RT-PCR Reedy system following the manufacturer's protocol (Thermo Scientific Inc., Massachusetts, USA). The reaction mixtures and cycling conditions are illustrated below in Tables 2.1 and 2.2 respectively.

Table 2.1: Components of the RT-PCR reaction mixture for the specific amplification of the UKFP gene fragment in a total volume of 50 µl.

Component	Volume (µl)	Final Concentration
Verso Enzyme Mix	1	
1-Step PCR Reddy Mix (2X)	25	1X
Forward Primer (10 µM)	1	200 nM
Reverse Primer (10 µM)	1	200 nM
RT Enhancer	2.5	
Water (PCR Grade)	14.5	
Template (RNA)	5	1 ng
Total Volume	50	

Table 2.2: The reaction conditions for the step-by-step amplification of the uridine kinase family protein gene fragment.

Component	Temperature (°C)	Time	Number of cycle
cDNA Synthesis	50	15 min	1
Verso Inactivation	95	2 min	1
Denaturing	95	20 sec	44
Annealing	60	30 sec	
Extension	72	1 min	
Final Extension	72	5 min	1

2.1.4 Agarose Gel Electrophoresis of the Amplified UKFP Gene Fragment

The products of amplified RT-PCR was resolved on an agarose gel (1% (w/v)) supplemented with 0.5 µg/ml ethidium bromide. (100 bp Gene-Ruler DNA ladder, immersed in 1X TBE buffer at 80 volts and a constant current of 250 mA for 50 minutes) were conditions used to resolve all samples obtained. And the gel was then visualized under UV light using a UV 2000 Trans-illuminator System (BioRad Laboratories Inc., California, USA). Capturing of the images was done using an Imaging System known as the ChemiDoc (Bio-Rad Laboratories Inc., California, USA).

2.2 Cloning of the Amplified UKFP Gene Fragment

2.2.1 Addition of the 3′-adenines Overhangs

1 unit of *Taq* polymerase (1 µl) was then added to 40 µl of the RT-PCR product and n incubated for 10 minutes at 72°C using C1000 Thermo-cycler System (Bio-Rad Laboratories Inc., California, USA). The remaining mixture from reaction was then kept for further use on ice.

2.2.2 Ligation of the Adenylated UKFP Gene Insert into the pTrcHis2-TOPO Vector

From the UKFP gene fragment reaction mixture which has been adenylated, an aliquot of 4 µl was collected and transferred into a fresh PCR tube which contained a 1 µl of the pTrcHis2-TOPO expression vector (Figure 2.2 below) (Invitrogen, Carlsbad, USA) which were then gently mixed by swirling the pipette through the mixture. The ligated mixture was left at room temperature to incubate for 5 minutes before being used for the transformation process of the competent *Escherichia coli* expression cells.

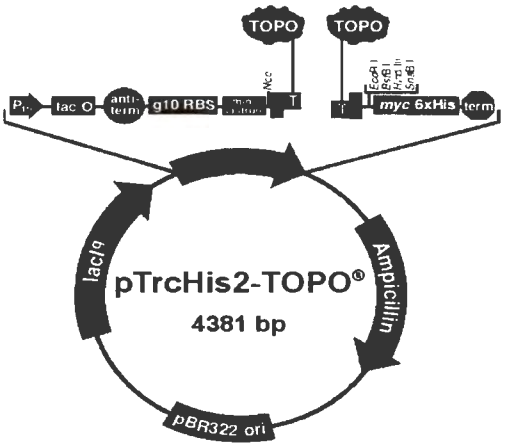

Figure 2.2: A commercially acquired pTrcHis2-TOPO vector for cloning of the UKFP PCR product: The illustration shows the expression and purification features of the plasmid which include the $\mathbf{P_{trc}}$ promoter for high level expression with forward and reverse priming sites for both restriction sites *Nco* I 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*. (Adapted from www.lifetechnologies.com).

2.2.3 Transformation of the Competent One Shot TOPO 10 *E. coli* Cells with the pTrcHis2-TOPO:UKFP Fragment Construct

Upon completion of the ligation process, a 2 µl aliquot of the ligation mixture (pTrcHis2-TOPO:UKFP fragment construct) was transferred into an ice-cold Eppendorf tube which contained 40 µl of the chemically competent One Shot TOPO 10 *E. coli* cells, followed by 30 minutes of incubated on crashed ice. The mixture was then heat-shocked 30 seconds on a dry-bath heating block at 42°C before being incubated for 5 minutes on ice. The reaction mixture was then supplemented with SOC medium (250 µl) (2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10 mM $MgCl_2$, 2.5 mM KCl, 10 mM Mg_2SO_4 and 20 mM glucose10 mM NaCl) and incubated in a shaker (200 rpm) for 30 minutes at 37°C (the incubation process is crucial such that it allows the cells to produce an enzyme known as the β-lactamase, which will detoxify ampicillin at a later stage). The mixture was then plated (80 µl and 20 µl) onto two Luria agar plates (Bertani (LB)) which consisted of (0.5% (w/v) NaCl, 1% (w/v) tryptone powder, 0.5% (w/v) yeast extract, 1% (w/v) agar, .) which has been supplemented with ampicillin (50 µg/ml)and 0.5% (w/v) glucose followed by overnight incubation of the plates at 37°C.

2.2.4 Extraction of the pTrcHis2-TOPO:UKFP Expression Construct from the Transformed Competent One Shot TOPO 10 *E. coli* Cells

From the TOPO 10 (One Shot) cells which have been transformed with the pTrcHis2:UK construct for expression, individual colonies were taken from LB plates cultured and were used in inoculating about 10 ml of (2YT) media (a double strength yeast-tryptone) containing 0.5% (w/v) yeast extract, 0.25% (w/v) NaCl and 0.8% (w/v) of tryptone powder supplemented with glucose (0.5%) and ampicillin (100 µg/ml). The inoculants were then subjected to an overnight incubation at 37°C, shaking at 200 rpm. Harvesting of the cells from the tubes was done the following day through centrifuging for 5 minutes at 6800x*g* and at room temperature.

The pellets of the cells were re-suspended using a total volume of 250 µl of the Re-suspension Solution which has been supplemented with RNase, and by pipetting them up and down until all cells have been suspended into the liquid. The mixture was then transferred into a sterilized Eppendorf tube, where a volume of 250 µl Lysis Solution was added and mixed thoroughly by inverting the tube 4-6 times or until the contents were slightly clear. From the Neutralization Solution, a volume of 350 µl was added to the mixture and then mixed thoroughly by inverting the tube 6 times. The cells were then clarified by centrifuging at maximum speed for 5 minutes and the resultant supernatant was then transferred into a provided GeneJET spin column, which subsequently, was centrifuged for 1 minute. The flow-through was discarded while the column was replaced into the same collection tube. A total volume of 500 µl of a previously prepared Wash Buffer (with 96% alcohol) was added to the spin column followed by a centrifugation at full speed for 30-60 seconds. The flow-through was discarded and the washing step was repeated twice more. The empty GeneJET spin column was further centrifuged for an extra minute to get rid of any extra chemicals and/or residual alcohol, and was then transferred into a sterile Eppendorf tube to elute the plasmid DNA. Elution was done by adding 50 µl of RNAse free water, pre-warmed at 56°C, to the center of the GeneJET spin column membrane followed by an incubation at room temperature for 2 minutes before a centrifugation at 16800x*g* for 2 minutes. The column was then discarded while the purified plasmid DNA was labeled and kept at-20°C.

2.2.5 Analysis of the Positive Clones

To confirm if the clones were positive, the normal PCR was used according to the standardized MyTaq Mix protocol (Bioline, London, UK). This step was mainly to confirm if the gene insert was successfully cloned into the pTrcHis2-TOPO vector and also to check if such a cloning system was in the correct orientation. Tables 2.3 and 2.4 below, respectively, show both the reaction mixtures and their respective thermal cycling conditions in Table 2.5.

Table 2.3: Reaction components of a PCR reaction system to confirm the successful cloning of the uridine kinase family protein gene fragment into the pTrchis2-TOPO expression vector.

Component	Volume (µl)
Template (Plasmid DNA)	1
Insert Primers (20 µM each)	1
MyTaq Reddy Mix (2X)	25
Water (sdH_20)	Fill up to 50

Table 2.4: Reaction Components of a PCR reaction system to confirm the correct orientation of the uridine kinase family protein gene fragment into the pTrcHis2-TOPO expression vector.

Component	Volume (µl)
Template (Plasmid DNA)	1
Insert and Vector Primers (20 µM each)	1
MyTaq Reddy Mix (2X)	25
Water (sdH_2o)	Up to 50

Table 2.5: The thermal cycling reaction program for the step-by-step assessment profile of the successful cloning system of the uridine kinase family protein gene fragment into the pTrcHis2-TOPO expression vector.

Step	Temperature (°C)	Time	Cycles
Initial Denaturation	95	1 min	1
Denaturing	95	15 sec	25-35
Annealing	65	15 sec	
Extension	72	10 sec	

2.2.6 Agarose Gel Electrophoresis of the Successfully Cloned Uridine kinase Family Protein Gene Fragment

The products from the PCR in both Tables 2.3 and 2.4 above were then resolved on 1% agarose gel stained with 0.5 µg ethidium bromide and immersed in a TBE buffer(1X) for 40 minutes at 80 volts and a current of 250 mA. The samples were ran alongside a 100 bp and 1 kb GeneRuler DNA ladders as markers and viewed under the UV 200 light Trans-illuminator System (Bio-Rad Laboratories Inc., California, USA) and a ChemiDoc Imaging System (Bio-Rad Laboratories Inc., California, USA) wa used to capture the images. A double amplification of the targeted gene fragment (Tables 2.3 and 2.4) would mean its successful cloning into the pTrcHis2-TOPO expression vector.

2.3 Partial Expression of the Recombinant Uridine Kinase Family Protein

2.3.1 Transformation of the Chemically Competent *E. cloni* EXPRESS BL21 (DE3) pLysS Cells with the pTrcHis2-TOPO:UKFP Expression Construct

Upon successful cloning of the uridine kinase family protein gene fragment and confirmation of its site orientation in the pTrcHis2-TOPO expression vector, its obtained expression construct (the pTrcHis2-TOPO:UKFP) was used in the transformation of some chemically competent expression cells known as *E. cloni* EXPRESS BL21 (DE3) pLysS cells. This was done in a sterilized Eppendorf tube following the supplier's protocol (Lucigen, Wisconsin, USA), whereby a 1 µl of the pTrisHis2-TOPO:UKFP expression construct which was aseptically added to 40 µl of the ice-cold chemically competent *E. cloni* EXPRESS BL21 (DE3) pLysS cells and the mixture was then incubated on ice for a period of 30 minutes. Thereafter, the mixture was heat-shocked on a dry bath for 45 seconds at 42°C and immediately placed on ice for 2 minutes. The mixture from the reaction was then supplemented with 960 µl of the Expression Recovery Medium and incubated in a shaker (200 rpm) for 1 hour at 37°C. The suspension was then used to spread-plate some agar plates (LB) supplemented with chloramphenicol (34 µg/ml) and ampicillin (100 µg/ml). The plates were finally subjected to an overnight incubation at 37°C.

2.3.1 Recombinant Protein Expression

Some 2YT media (10 ml) supplemented with 34 µg/ml chloramphenicol, 100 µg/ml ampicillin and 0.5% (w/v) glucose, was inoculated with the transformed *E. cloni* EXPRESS BL21 (DE3) pLysS cell colony harboring the pTrcHis2TOPO:UKFP expression construct in a 50 ml falcon tube. The falcon tube was then incubated overnight at 37°C, shaking at 200 rpm. On the subsequent day, 200 µl of the overnight culture was then used to inoculate 20 ml fresh 2YT media containing 34 µg/ml chloramphenicol, 100 µg/ml ampicillin and 0.5% (w/v) glucose. The culture was then incubated at 37°C, shaking at 200 rpm and until an optical density of 0.6 was reached and as was measured by using the Hekios Spectrophotometer (Merck, Gauteng, RSA). Immediately, two tubes were used to split the culture media in which each contained a total volume of 5 ml. the other tube containing culture media(5 ml) was induced by adding 1 mM of the isopropyl-β-D-thiogalactopyranoside (IPTG) to express the intended UKFP recombinant protein (SigmaAldrich Corp., Missouri, USA) while the other culture was left un-induced (control). The split cultures were then shaken in an incubator (200 rpm) for 3 hours at 37°C. Thereafter, the cultures were then centrifuged at for 5 minutes 8 000x*g* to pellet the cells. The pelleted cells were then analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.4 Activity Assaying

2.4.1 Determination of Endogenous AC Activity of the Recombinant UKFP Protein

Preparation of the overnight culture of cells confirmed to be harbouring the recombinant pTrcHis2-TOPO:UKFP expression construct was through the use of their glycerol stock (200 µl) in inoculating fresh 2YT media (10 ml) which has been supplemented with chloramphenicol (34 µg/ml) and ampicillin (100 µg/ml). The culture was left to grow in a shaking incubator at 200 rpm at 37°C. The following day, a fresh 20 ml of the 2YT media containing chloramphenicol (34 µg/ml) and ampicillin (100 µg/ml) was inoculated with 1 ml of the overnight culture and incubated at 37°C in a shaker at 200 rpm and for its OD_{600} to reach 0.6. The culture was immediately placed on ice and split into four portions each containing a total volume of 3 ml.

Adding the 1 mM IPTG into three of the tubes containing culture media was mainly to induce protein expression while one tube left without the IPTG so it may be used as a control (un-induced). From two of the three induced cell cultures, one culture was supplemented with 100 µM of the AC activator, forskolin (Sigma-Aldrich Corp., Missouri, USA) while the other culture was supplemented with 100 µM of the AC inhibitor, 2',5'-dideoxyadenosine (Sigma-Aldrich Corp., Missouri, USA). All of the four cell cultures were then incubated at 37°C for a further 2 hours before harvesting the cells through centrifugation for 10 minutes at 9 200x*g*. The harvested cells were then lysed in 1 ml Lysis buffer 1 (Amersham Healthcare Inc., California, USA) supplemented with 2 mM 3-isobutyl-1methylxanthine (IBMX) (Sigma-Aldrich Corp., Missouri, USA) to inhibit phosphodiesterases. In actual sense, the samples were shaken at 100 rpm, at 37°C for 30 minutes in on orbital shaker to intensify the cell lysis process. The samples were then centrifuged for 5 minutes at maximum speed and their lysates transferred into fresh Eppendorf tubes which contained 200 µl of the Lysis buffer 2 (Amersham Healthcare Inc., California, USA) was subsequently added and mixed.

Thereafter, a total volume of 220 µl from the mixture was then transferred into a fresh Eppendorf tube containing 11 µl of the acetylating reagent (Sigma-Aldrich Corp., Missouri, USA) the mixture was then gently mixed through pulsing. The cAMP-linked enzyme immunoassaying kit (Catalog #: CA201) kit was used in measuring the content of the endogenous cAMP from each of the lysates generated and following the acetylation version of its protocol according to the manufacturer's manual (Sigma-Aldrich Corp., Missouri, USA). The measurements were taken at 405 nm using a Microplate Reader (Labtech International Limited, East Sussex, UK) and all results obtained were then resolved in sets of triplicate and subjected to the statistical analysis of variance (ANOVA).

2.4.2 Complementation Testing of the Recombinant UKFP Protein

Amongst the two portions of the chemically competent *cya*A mutant *E. coli* cells (SP850), one of the portion was transformed with the pTrisHis2-TOPO:UKFP expression construct while the other portion with left as a control (un-transformed). A MacConkey agar plate supplemented with 0.1 mM IPTG and 15 µg/ml kanamycin (Sigma-Aldrich., Missouri, USA) was also prepared and segmented into 3 quadrants. The first quadrant was left untouched, while the second one was streaked with the non-transformed mutant *cya*A cells and the third one was streaked with the mutant *cya*A cells transformed with the pTrcHis2-TOPO:UKFP

expression construct. The streaked plate was then incubated for 40 hours at 37°C followed by observations on its various phenotypic characteristics. A magenta red or deep purple colour on the transformed *cya*A mutant cells would indicate positive functionality *in vivo* AC activity for the cloned and partially expressed uridine kinase family protein.

2.4.3 Bioinformatic Analysis of the *UKFP* Gene

2.4.3.1 Co-expressional Analysis

The Arabidopsis co-expressional tool, (ACT) (www.arabidopsis.leeds.ac.uk/ACT/), was used to establish the co-expressional profile of the *UKFP* gene and its other related genes in the Arabidopsis plant. This web-based tool allows for the identification of genes whose expressional patterns are correlated and based on an *Arabidopsis thaliana* microarray data set obtained from the Nottingham Arabidopsis Stock Centre (NASC). This ACT provides the correlation relationship results accompanied by estimates of their statistical significance, expressed as probability (P) and expectation (E) values (Jen *et al.*, 2006). The co-expression analysis was performed across all the available microarray experiments using the At1g26190 as the driver gene.

2.4.3.2 Stimulus-specific Microarray Expressional Analysis

Following the retrieval of a *UKFP* co-expression group of 25 genes (*ECGG25*), the stimulus tool was then used to screen the expression profiles of the *UKFP-ECGG25* complement over the whole ATH1:22K array Affymetrix public microarray data in the GENEVESTIGATOR V3 version (www.genevestigator.com) (Zimmermann *et al.*, 2004; Grennan, 2006). The normalized microarray data were downloaded from the GEO (NCBI) (www.ncbi.nlm.gov/geo/), the TAIR GenExpress (www.ebi.ac.uk/microarray-as/ac/) and the NASC Arrays (www.affymetrix.arabidopsis.info/narrays/experimentbrowse.pl), and subsequently analysed for experiments that were found to induce a differential expression of this gene complement. For each experiment found to induce a differential expression, the fold-change (log2) values were then calculated. Subsequently, expression values were then generated using a Multiple Array Viewer program from the Multi-Experiment Viewer (MeV) software package (Version 4.2.01) (The Institute for Genomic Research (TIGR)).

CHAPTER THREE

RESULTS AND INTERPRETATIONS

3.1 Generation of Arabidopsis Plants

Arabidopsis thaliana seeds were sterilized and germinated on solidified Murashige and Skoog basal medium in a growth chamber (Figure 3.1a). The germinated seedlings were then transplanted into planting potting mix soil (Figure 3.1b). Leaf material was then harvested from the 6-week old plants for the isolation of total RNA and subsequent isolation of the targeted uridine kinase family fragment gene.

(A) (B)

Figure 3.1: Generation of the *Arabidopsis thaliana* plants. (A) Newly germinating *Arabidopsis thaliana* seedlings on Murashige and Skoog growth medium in a growth chamber. (B) Fully matured *Arabidopsis thaliana* plants in a growth chamber from where leaves for the extraction of total RNA were obtained.

3.2 Amplification of the Uridine Kinase Family Protein Gene Fragment

Total RNA was isolated from the 6-week old *Arabidopsis thaliana* leaf tissue material. Copy DNA for the uridine kinase family gene was then synthesized and used together with the specifically designed sequence-specific primers to synthesize and amplify the targeted uridine kinase family gene fragments in a 1-step RT-PCR system. The amplified PCR fragment was then resolved on 1% agarose gel and as is shown in the figure below.

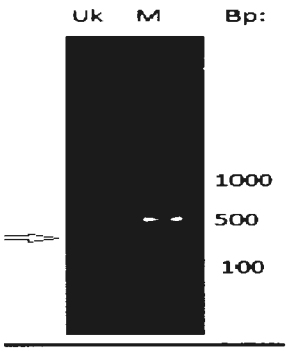

Figure 3.2: Amplification of the uridine kinase family protein gene fragment. An agarose gel resolution of the 360 bp UKFP gene fragment amplified by RT-PCR. M represents a 100 bp marker while the arrow marks the amplified and desired uridine kinase family protein gene fragment.

3.3 Confirmation of the Cloning Success of the UKFP Gene Fragment

After the UKFP gene fragment has been successfully isolated (Figure 3.2) above it was ligated into the pTrcHis2-TOPO expression vector, its cloning success was then checked and verified by standard PCR using a specialized MyTaq reaction system. Confirmation of the cloning process was done by nucleotide-specific sequencing at the Inqaba Biotechnological Sciences (Pretoria, RSA). The successfully cloned and molecularly confirmed UKFP gene fragment is shown below (Fig 3.3).

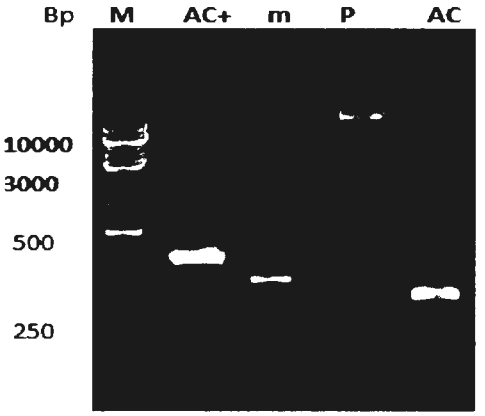

Figure 3.3: Confirmation of the Cloning Success of the UKFP Gene Fragment. The UKFP gene fragment ligated into the pTrcHis2-TOPO expression vector was also amplified with its own sequence-specific primers as well as by one of its own primers and one of the vector primers and was then rsolved on a 1% (w/v) agarose gel system. Lane 1 (**M**) represents the 1 kb GeneRuler ladder (Thermo Scientific Inc., Burlington, Canada), while the second lane (**AC+**) represents the UKFP gene fragment amplified with its own forward primer and the vector reverse primer, the third lane (**m**) represents a 100 bp Gene-Ruler ladder (Thermo Scientific Inc., Burlington, Canada), the forth lane (**P**) represents the pTrcHis2-TOPO:UKFP gene expression construct, and the last lane (AC) represents the UKFP gene fragment amplified with its own sequence-specific primers.

3.4. Recombinant Expression of the Uridine Kinase Family Protein

The uridine kinase family protein gene fragment amplified by RT-PCR was ligated into a pCRT7/NT-TOPO plasmid vector to form a pTrcHis2-TOPO:UKFP expression construct. To transform the competent *E. cloni* EXPRESS BL21 (DE3) pLysS cell this expression construct was then used, followed by a partially expressing the putative recombinant protein targeted as shown in Figure 3.4 below.

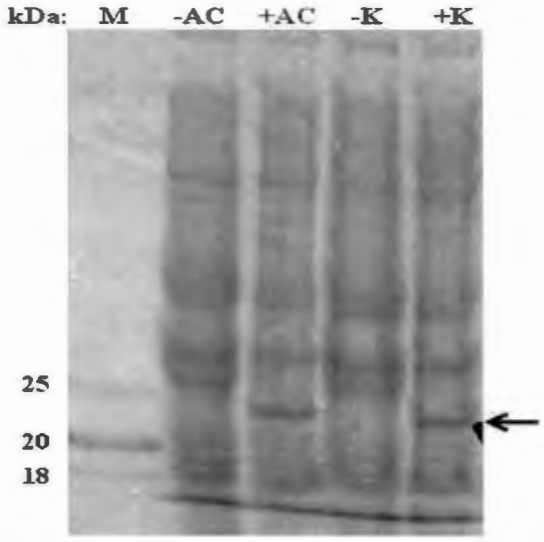

Fig 3.4: Expression of the recombinant uridine kinase family protein. (A) An SDS-PAGE of the uninduced (-AC/K) and induced (+AC/K) recombinant uridine kinase family protein fragments, where M is the low molecular weight marker and the arrow is marking the partially expressed UKFP recombinant protein.

3.5. Determination of the Endogenous Activity of the Recombinant UKFP protein

To determine the levels of cAMP generated by the recombinant *E. coli* BL21 (DE3) pLysS harbouring the UKFP gene under different growth conditions, the cells were cultured up until their OD_{600} was 0.5. One portion was treated with 1 mM IPTG only whilst the other one was left untreated and, in addition; another portion was treated with both 1 mM IPTG and 100 μM forskolin (the AC inherent activator) while the last portion was treated with 1 mM IPTG and 100 μM dideoxyadenosine (the AC potent inhibitor). The generated cAMP was then extracted from the cells and measured by a cAMP-specific enzyme immunoassay kit (Catalogue #: CA201; Sigma, Missouri, USA) based on its acetylation protocol.

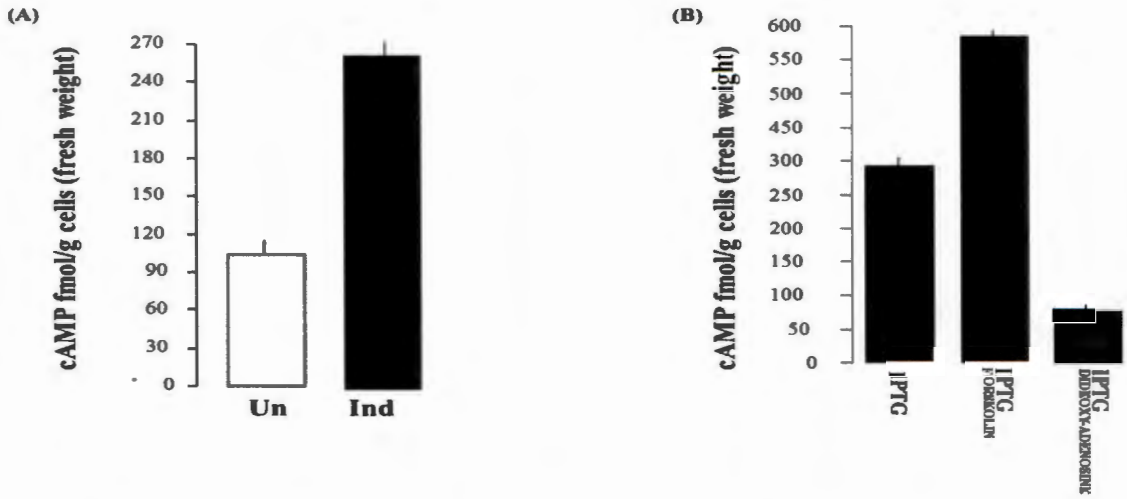

Figure 3.5: Determination of the endogenous adenylate cyclase activity of the recombinant UKFP protein. **(A)** cAMP levels generated by the un-induced (control) and induced *E. cloni* EXPRESS BL21 (DE3) pLysS cells harbouring the UKFP gene. **(B)** cAMP levels generated by the induced recombinant cell cultures in the presence of either forskolin or dideoxyadenosine. In determining the levels of cAMP, the cAMP enzyme-immunoassaying system (Catalogue #: CA201, Sigma, Missouri, USA) was utilized where error bars represent the standard errors of the means (n=3).

3.6. Complementation Testing of the Recombinant Uridine Kinase Family Protein

The figure below shows an assessment of the recombinant uridine kinase family protein's ability to convert non-lactose fermenting SP850 *cya*A *E. coli* cells transformed by this gene fragment into lactose-fermenting wild types. The outcome was then visually assessed on MacConkey agar (Figure 3.6) whereby the white/yellowish color of mutant cells changed into the deep magenta purple colour of wild type cells as a result of their ultimate transformation with the pPCRT7/NT-TOPO:UKFP expression construct.

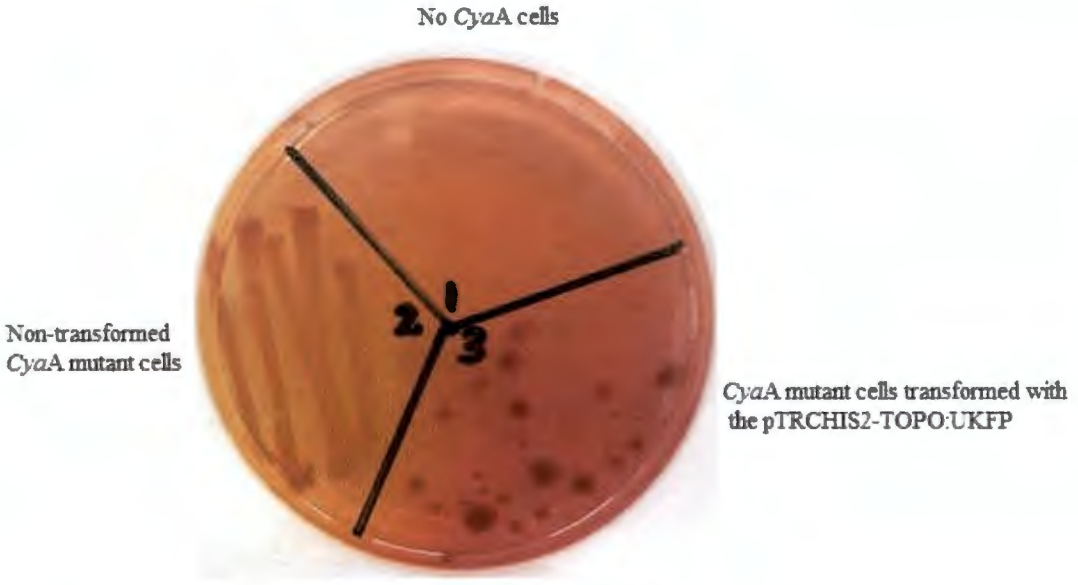

Figure 3.6: Determination of the *in-vivo* adenylate cyclase activity of the recombinant uridine kinase family protein. Different *E. coli cya*A cells were plated onto two different quadrants of a MacConkey agar plate which had been supplemented with 0.1 mM IPTG and incubated at 37°C for 40 hours. The first quadrant of the plate contains no cells, while the second quadrant contains the *E. coli cya*A mutant cells which have not been transformed, while the third quadrant contains the *E. coli cya*A mutant cells transformed with the pCRT7/NT-TOPO:UKFP expression construct. Cells in quadrant two do not ferment lactose thus white or yellowish colonies are being produced. Cells in quadrant three have picked a deep purple phenotype , which is a characteristic signifying the ability to ferment lactose.

3.7. Co-expressional Analysis of the *UKFP* Gene in *Arabidopsis thaliana*

In order to understand the expressional profile of the *UKFP* gene in *Arabidopsis thaliana*, a desktop analysis of the publicly available microarray data was performed. Out of the 322 diverse transcriptome experiments, the *UKFP* gene was found to be highly co-expressed with numerous other genes in the Arabidopsis genome, with the top 25 genes having a Pearson's correlation coefficient (r-value) of between 0.717 and 0.904 (Table 3.7 below). These 25 top most co-expressed genes (hereafter referred to as the UKFP-Expression Correlated Gene Group (*UKFP-ECGG25*)) were considered here because their correlational values were relatively high and their number also provided a moderately representative sample size for the subsequent stimulus-specific and functional expressional analysis of their profiles.

Table 3.1: The top 25 genes whose expressional profiles are directly correlated with the *UKFP* gene (At1g26190).

Locus	GO terms	*r* value	Annotation
AT1G26190		1.000	Uridine kinase family Family Protein
AT1G75040	DR, SAR	0.904	Pathogenesis-related protein 5 (PR-5)
AT5G10760		0.842	Aspartyl protease family protein
AT2G31880	Ph, CC, K	0.802	LRR transmembrane protein kinase
AT4G23150	Ph, K	0.796	Protein kinase family protein
AT4G04500	Ph, K	0.792	Protein kinase family protein
AT4G23140	Ph, K	0.781	Receptor-like protein kinase 5 (RLK5)
AT1G21250	Ph, CC, K	0.775	Wall-associated kinase 1 (WAK1)
AT1G66880	Ph, K	0.774	Serine/threonine protein kinase
AT5G02290	K	0.748	Similar to protein kinase APK1A
AT2G37710	K	0.748	Lectin protein kinase
AT5G60900		0.745	Lectin protein kinase family protein
AT4G23260	K	0.741	Protein kinase family protein
AT3G56400	DR, SAR, CC	0.734	WRKY70 family transcription factor
AT2G14610	DR, SAR, CC	0.726	Pathogenesis-related protein 1 (PR-1)
AT4G29810	CC, K	0.725	Mitogen-activated protein kinase kinase
AT3G08870	Ph, K	0.725	Lectin protein kinase
AT1G35710	Ph, CC,K	0.721	LRR transmembrane protein kinase
AT1G34420	Ph, K	0.720	Leucine-rich repeat family protein
AT2G32800	Ph, K	0.719	Protein kinase family protein
AT1G74710	DR, SAR	0.719	Isochorismate synthase 1 (ICS1)
AT3G13950		0.719	Expressed protein
AT2G02360		0.718	F-box family protein
AT2G24850		0.718	Aminotransferase
AT4G22260		0.718	Alternative oxidase
AT5G12890		0.717	UDP-glucosyl transferase family protein

Abbreviations for the indicated GO terms:
DR = defense response; CC = cell communication; K = kinase activity; SAR = systemic acquired resistance; Ph = phosphorylation.

3.8. Stimulus-specific Microarray Expressional Profile of the *UKFP-ECGG25*

In order to determine the biological processes in which the *UKFP-ECGG25* is involved in *Arabidopsis thaliana*, this gene set (*UKFP-ECGG25*) was subjected to an *in silico* global expression analysis of the specific experimental conditions that were able to induce a differential expression of all the genes contained in this set. The histogram generated from this microarray expression analysis revealed that the transcriptional processes of the *UKFP* and its associated *ECGG25* complement are generally and collectively induced in response to a wide range of biotic infectious stress factors (Figure 3.7 below).

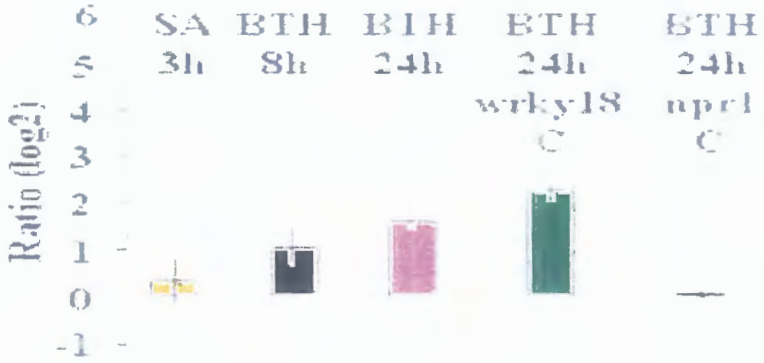

Figure 3.8: The induction profile of the *UKFP* gene in response to salicylic acid (SA) and its synthetic analogue, benzothiadiazole S-methylester (BHT). The gene was significantly induceable in response to both the SA and its functional analogue, BHT. Mutant *wrky18* is SA/BHT-induceable (Ulker *et al.*, 2004) and was used as a positive control while mutant *npr1* is SA/BHT-non-inducible (Cao *et al.*, 1994) and was used as a negative control.

CHAPTER FOUR

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

4.1 Discussion

Uridine kinase family protein in the *Arabidopsis thaliana* is a truncated protein, which falls under the uridine kinase super-family and also known as the phosphoribulokinase family, which has been tiered to be its close match. The protein is annotated to have various functions in kinase activity, phosphotransferase activity and adenylate cyclase activity, and is also speculated to be involved in various biosynthetic processes, cAMP biosynthesis and other metabolic processes. The adenylate cyclase activity was the major interest of this research work. Various efforts have been made to demonstrate the cAMP-dependent protein kinase activity in a series of plant tissues, including the germinating barley seeds, green peppers, cabbage leaves, shell beans and the *Euglena gracilis*, but were all unsuccessful.

An AC catalytic core motif has been found to be contained in this gene, but the gene has not yet been experimentally tested to show if indeed it is capable of cyclizing the AMP molecule into cAMP (Gehring, 2010). Therefore, in order to assess if the AC catalytic centre in the uridine kinase family protein of the *Arabidopsis thaliana* (Figure 3.1) had any enzymatic activity/function, the targeted uridine kinase family protein gene fragment was successfully isolated (Figure 3.2), cloned (Figure 3.3) and partially expressed (Figure 3.4). The partially expressed recombinant UKFP protein was then tested for its ability to endogenously generate cAMP in bacterial expression system (Figure 3.5). Findings from this assessment proposed that this putative molecule was either itself a *bona fide* AC protein capable of generating cAMP from ATP or simply another functional higher plant molecule capable of stimulating other resident ACs (*E. coli* ACs in this case) to produce cAMP from ATP. Additionally, by virtue of it being metabolically sensitive to both forskolin (Wuttke *et al.*, 2001) and dideoxyadenosine (Moutinho *et al.*, 2001) (Figure 3.5), further suggested that this recombinant protein this molecule as a transmembrane AC (tmAC) candidate (Kamenetsky *et al.*, 2006).

An *in vivo* assessment of the AC-containing protein domain of this putative protein molecule was also further undertaken through a functional complementation testing. This test was

performed using an SP850 mutant *cya*A strain that is deficient in endogenous adenylate cyclase activity and, therefore, cannot ferment lactose (Moutinho *et al.*, 2001). When this strain is grown on MacConkey agar, white/yellowish colonies are produced as compared to the magenta deep purple colonies produced by its wild-type counterpart (Moutinho *et al.*, 2001, Ruzvidzo *et al.*, 2013). Therefore, and in order to test if the AC activity of the UKFP protein could rescue this mutant strain, cells of this mutant were transformed with the pTrcHis2-TOPO:UKFP expression construct followed by assessment of their colony phenotypes on MacConkey agar supplemented with 0.1 mM IPTG (Figure 3.6). As is shown in the Figure 3.6, the transformed cells stained magenta deep-purple, signifying their rescuing aspect by the expression construct from a mutant state into wild a type and thus validating the recombinant uridine kinase family protein as a functional AC.

Further results from the co-expressional analysis of the *UKFP* gene in the *Arabidopsis thaliana* system firmly showed that this annotated gene is mainly expressed in the cytosolic region of the chloroplast tissues of the Arabidopsis plant, alongside other genes that are principally involved in defense responses, systemic acquired resistance, cell communication, the kinase and phosphorylation activities (Table 3.1). Furthermore, a stimulus-specific microarray expressional profiling of this putative protein essentially indicated that it is generally and specifically induced by a variety of biotic infectious stress factors (Figure 3.7).

Therefore, by summing up all the findings from this study, it is thus apparent to state that the UKFP protein (studied in this work) is an important higher plant signalling molecule with a central role in responses to a wide range of biotic infectious stress factors (Figure 3.7), and whose mechanism of action is principally mediated by the second messenger, cAMP. Thus this whole study therefore, has practically confirmed the UKFP protein as a *bona fide* and functional higher plant adenylate cyclase molecule besides the currently known *Zea mays* pollen signalling protein (Moutinho *et al.*, 2001), *Arabidopsis thaliana* pentatricopeptide repeat protein (Ruzvidzo *et al.*, 2013), *Nicotiana benthamiana* adenylyl cyclase protein (Ito *et al.*, 2014), and the *Hippeastrum hybridum* adenylyl cyclase protein (Swiezawska *et al.*, 2014). Furthermore, following the pentatricopeptide protein (Ruzvidzo *et al.*, 2013), this AC candidate has thus become the second ever higher plant AC to be identified in *Arabidopsis thaliana*.

4.2 Conclusion

This study has succeeded to practically determine the Arabidopsis uridine kinase family protein as a *bona fide* and functional higher plant adenylate cyclase.

4.3 Recommendations

Work undertaken in this study can comfortably allow for the proposition of the following two recommendations:

- Since the uridine kinase family protein has been successfully characterised, as a functional higher plant AC, it therefore serves as a preliminary foundation for the added functional testing and further characterization of the other remaining bioinformatically annotated Arabidopsis proteins (Gehring, 2010; Ruzvidzo *et al.*, 2013).
- Inclusion of the work undertaken here onto other researches in different plant databases to serve as a basis for any work based on cAMP signalling particularly in abiotic and biotic stress responses and adaptation mechanisms.

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