Recombinant Expression of a Truncated Arabidopsis AX4-like Protein and Molecular Characterization of its Enzymatic Activities

by

HM LEKALAKE
21505004

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Supervisor : Prof O Ruzvidzo

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DECLARATION

I, Hearnes Mothakgotla Lekalake, hereby declare that the work contained in this MSc dissertation submitted to the North-West University, Mafikeng Campus (Biological Science Department, Plant Biotechnology Research Group) is my own original work and has not, previously in its entirety or in part, been submitted to any university for any degree.

STUDENT: **Hearnes M Lekalake**

Signature…………………… Date……………………..

SUPERVISOR: **Prof O Ruzvidzo**

Signature ……………………….. Date………………………

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DEDICATION

This work is dedicated to my mother, Mpho Elllen Lekalake; grandmother, Ivy Gobopamang Lekalake; my aunt, Mosadiotsile; Lekalake siblings, Agodirwe, Gaone, Boitumelo, Lebogang, Olebogeng Maureen, Omphemetse Lekalake and my friends family Harold and Neo Sepako, for standing by me through-out the period of study. I would like to extend my heartfelt love to Annalese Esterhuizen, for letting me pursue my studies despite the economic difficulties that my family was going through at the time. To all the other members of my family who contributed to my academic life in one way or the other, I say, thank you.

Never walk alone!!!!

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THE SKY IS THE LIMIT

TO GOD BE THE GLORY

Thank you all once again

DEFINITION OF TERMS

Adenylate cyclase: An enzyme that is capable of converting an adenosine 5′ triphosphate (ATP) molecule into the second messenger cyclic 3′,5′ -adenosine monophosphate (cAMP).

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

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

Clathrin: Is the main constituent of the polygonal network that forms the coat of coated vesicles and coated pits. It is responsible for the receptor-mediated endocytosis at the plasma membrane.

Guanylate cyclase: An enzyme involved in the conversion of a guanosine 5′-triphosphate (GTP) molecule into the cyclic 3′,5′-guanosine monophosphate (GMP).

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

Reverse transcription polymerase chain reaction: A technique used in molecular biology to amplify short RNA segments into a DNA product termed copy DNAs (cDNAs) using an RNA-dependent DNA polymerase enzyme.

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

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

ABBREVIATIONS

2YT	**:**	Double strength yeast-tryptone media
AC	**:**	Adenylate cyclases
AtCNGC	**:**	*Arabidopsis thaliana* cyclic nucleotide-gated channel
ATP	**:**	3′,5′-Adenosine triphosphate
BLAST	**:**	Basic Local Alignment Searching Tool
cAMP	**:**	Cyclic 3′,5′-adenosine monophosphate
DTT	**:**	Dithiothreitol
GC	**:**	Guanylate cyclase
IBMX	**:**	3-isobutyl-1-methylxanthine
IPTG	**:**	Isopropyl-β,D-thiogalactopyranoside
MSMO	**:**	Murashige and Skoog basal salt with minimal organics
OD	**:**	Optical density
RT-PCR	**:**	Reverse transcriptase polymerase chain reaction
SDS-PAGE	**:**	Sodium dodecyl sulphate poly-acrylamide gel electrophoresis
STAND	**:**	Signal transduction ATPases with numerous domains
cGMP	**:**	Cyclic 3′,5′-guanosine monophophate
ST	**:**	Sodium Tris-HCl
TAIR	**:**	The Arabidopsis Information Resource
rpm	**:**	Revolution per minute

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ABSTACT

Adenylate cyclases (ACs) are part of a group of integral membrane proteins that consist of six transmembrane segments and have two emerging catalytic domains. They are known for their role in catalyzing the conversion of adenosine triphosphate (ATP) into cyclic adenosine monophosphate (cAMP). This resultant cAMP molecule has been implicated in a number of plant processes for example signal transduction and responses to various environmental factors such as nutrient shortage and pest attacks. The premise of this research study was therefore laid upon the study of these ACs and their potential *in vivo* enzymatic activities. Based on the conserved and functionally assigned residues in the catalytic centre of annotated and experimentally confirmed nucleotide cyclases, various potential AC candidates in the *Arabidopsis thaliana* genome were annotated. Therefore in an attempt to test and determine whether this putative protein candidate has any functional AC activity, total mRNA of the 4-6 weeks old *Arabidopsis thaliana* plants was extracted and used as a template for the complementary synthesis and amplification of a 714 bp AC-like gene fragment via a specialized Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR) system. The amplified fragment was then cloned into a pTrcHis2-TOPO expression vector and the resultant recombinant expression vector eventually transformed into chemically competent *E.cloni* EXPRESSBL21 (DE3) pLysS expression host cells. Positive clones were determined by confirmatory PCR and further validated by nucleotide-specific sequencing. The 20.0 kDa C-terminus His-tagged recombinant AC-like protein was the over-expressed following an induction with isopropyl-β-D-1-thiogalactopyranoside (1 mM, IPTG) and purified over a nickel nitrilotriacetic acid (NiNTA) affinity matrix system. The endogenous and *in vitro* AC activities of the resultant recombinant AC-like protein were then tested via a cAMP-linked enzyme immunoassaying system while its inherent *in vivo* AC activity was also concurrently tested via a complementation testing system using the *cya*A SP850 mutant *Escherichia coli* cells. Results from these three independent assays collectively indicated that the AC-like protein encoded for At1g73980 gene from *A.thaliana* possesses the endogenous, *in vitro* and *in vivo* AC activities, and thus unequivocally confirming it as a *bona fide* higher plant AC molecule with a possible cAMP-mediated signalling system. One such candidate molecule in the form of an AX4-like protein was hereby cloned and partially expressed followed by its intensive functional characterization. Results from all these experimental approaches, practically showed that this putative AX4-like protein has some inherent adenylate cyclase

activities and therefore, confirming it as a functional higher plant adenylate cyclase with a possible role in cAMP-mediated signalling systems.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Crop yields have grown slowest in many parts of the developing world, most especially in Africa (Abah *et al*., 2010). Traditional methods of agriculture, the green revolution and the use of fertilizers, pesticides and irrigation systems have all notably failed to remarkably increase crop yields (Machuka, 2001). Agricultural production is currently being faced with many challenges from global warming to pest infestations (Long *et al.,* 2006). Therefore, food and nutritional security is now becoming heavily and essentially dependent onto the development of crop plants with increased resistance to both biotic and abiotic environmental stress factors (Artkinson and Erwin, 2012). With the advent agricultural modern technology and plant biotechnology can be successful used into conventional breeding programs to substantially enhance effectiveness and efficiency agricultural research and development (Abah *et al.,* 2010). Burachik, 2010 proves that agricultural application can immensely contribute towards the protection of environments as result of diminished use of unfavourable agricultural inputs such as pesticites and herbicides. In both plants and animals, there is a special group of systemic biomolecules termed adenylate cyclases (ACs), which are enzymes that catalyze the formation of 3′ 5′ cyclic adenosine monophosphate (cAMP) from adenosine triphosphate (ATP). These biological molecules have long been established as key components and second messenger molecules in many cellular signalling pathways of various organisms including plants and animals (Gehring, 2010). In the year 2010, Gehring used a 14-mer modified guanylate cyclase catalytic centre search motif with specificity for ATP binding and the C-terminal metal-binding residues to conduct a BLAST search of the Arabidopsis genome (Gehring, 2010). This motif then retrieved fourteen putative AC gene candidates of which the At1g73980 gene that codes for the AX4-like protein was one of them (Table 1.1). Apparently, since proteins that affect homeostasis in plants are a targeted candidate group for plant biotechnology, one such potential molecule and more specifically, the AX4-like protein from *Arabidopsis thaliana* was hereby studied to try and elucidate its possible activity as an AC and its probable involvement in plant stress response and adaptation mechanisms using bioinformatic tools.

1.2 LITERATURE REVIEW

1.2.1 Cyclic nucleotides and nucleotide cyclases

Cyclic AMP (cAMP) has firmly been established as an important second messenger signalling molecule in eukaryotic and prokaryotic organisms (Goodman *et al*., 1970, Gerish *et al*., 1975 and Weigant, 1978). Cyclic AMP synthesis is the result of ATP catalysis by a special group of enzymes termed adenylate cyclases (ACs). Cyclic AMP may also have a important signalling role with regard to abiotic stress responses, specifically to sodium chloride stress since voltage-independent channels in the *Arabidopsis thaliana* roots have been reported to have open probability sensitivity to micromolar concentration of the cAMP at the cytoplasmic side (Maathuis and Sander, 2001). An up-regulation of cAMP-dependent calcium permeable conductance activated by hyperpolarization was also reported in the guard cells as well as the mesophyll cells of *Arabidopsis thaliana* and *Vicia faba* plants (Lemetiri-Chelieh and Berkowzitz, 2004).

Due to the significance of ACs and their enzymatic product, cAMP, plant researchers have been very keen to know if molecular signalling in plants was also similar to those of animals and lower eukaryotes. Unfortunately, the presence of ACs in plants was not quite apparent due to the low concentration levels of their catalytic product, cAMP, in plants (< 20 pmol/g wet weight) (Aston and Polya, 1978) as compared to animal values of >250 pmol/g wet weight (Butcher, Baird and Sutherland 1968). However, low levels of yet another cyclic nucleotide, cGMP (<0.4 pmol/g fresh weight), were eventually reported in plants whose molecules had been shown to have a physiological role in specific responses to virulent pathogens and defensive mechanisms, and thus however reinforcing the fact that cell signalling in plants at lower molecular levels is indeed very feasible and common-place (Meier *et al*., 2009).

Previously, some functionally tested guanylate cyclases were identified in the Arabidopsis genome using a 14-amino acid long search term deduced from an alignment of the conserved and functionally assigned amino acids in the catalytic centres of annotated and functionally confirmed guanylate cylases (Liu *et al*., 1997; Ludidi and Gehring, 2003). When applied to identify new ACs in higher plants, the BLAST search surprisingly could not return any candidate molecules with significant e-values. Hence specific changes in that motif were then made, whereby specificity for ATP binding was done with the C-terminal metal-binding residues included Figure 1.1 (Gehring, 2010). The subsequent search then returned a total of

fourteen candidates of which the AX4-like protein was one of them (figure 1.1) (Gehring, 2010). Specifically, Gehring (2010) first clearly explained that there were typically 3 annotated but functionally unconfirmed ACs (At1g26190, At1g73980 and At2g11890) and they all contained the search motif [RKS]X[DE]X(9,11)[KR]X(1,3)[DE]. Such a modified search motif is also present in the *Zea mays* AC and is also found in the Arabidopsis NBS-LRR class protein (Gehring, 2010). Consequently, the AX4-like protein has a similar domain organization and, notably, a very high homology to the *Dictyostelium discoidum* AX4-AC domain containing protein.

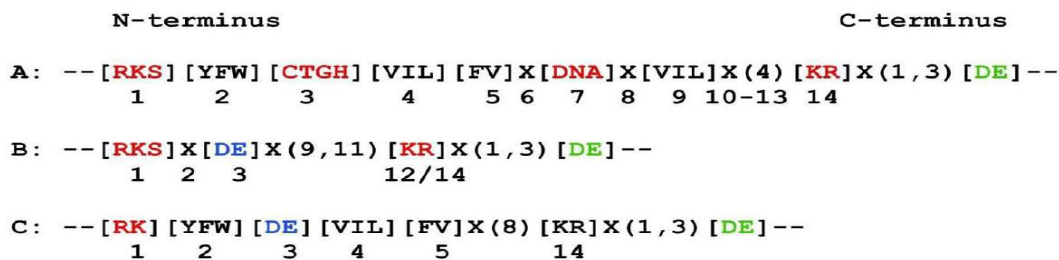


Figure 1.1: Catalytic center motifs of nucleotide cyclases. (A) Center motif of experimentally tested GCs in plants. The residue (red) in position 1 does the hydrogen bonding with the guanine, the amino acid in position 3 confers substrate specificity and the residue in position 14 stabilises the transition (GTP/cAMP), The Mg^{2+}/Mn^{2+}-binding site is C terminal (green). In the derived motifs (B and C) specific for ACs, position 3 (blue) has been substituted to [DE] to allow for ATP binding (Gehring, 2010).

Apparently due to their sessile nature, plants are easily affected by biotic and abiotic stress factors (Taki *et al*., 2005). These factors can thus drastically decrease plant growth and as such, they are considered as the major limiting factors for crop production (Dawood *et al*., 2012). However, cell signaling and communication systems do play vital and central roles in plant survival and homeostatic systems (Taki *et al*., 2005; Dawood *et al*., 2012).

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-like 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 bolded is the AX4-like gene that was studied in this work.

1.2.2 The role of cAMP in plant signal transduction

Cyclic AMP is a second messenger biomolecule that is a crucial for cell signalling and transduction systems, and whose roles and functions in animals, humans, microorganisms, and lower eukaryotes have been well documented and studied (Gancedo *et al*., 1985) but less so in plant systems (Ma *et al*., 2009). Cyclic AMP has a major impact on cellular responses against abiotic stress in prokaryotes. The bacteria *Anabaena cylindrical* has a symbiotic relationship with plants, and as a model, a documented study demonstrated some cAMP sensitivities to environmental changes in the membrane electrochemical potentials, whereby dark to light, anaerobic to aerobic conditions in the dark and pH rising from 6-8, exhibited some rapid decrease in the cAMP levels in a minute followed by a slow recovery (Ohmori, 1989). In the *Dictyostelium discoideum*, cAMP and ACs have a major influence onto the pre-spore differentiation and spore dormancy, and this is why spore differentiation has moderately been lost in AC knock-out mutant cells (Alvarez-Curto *et al*., 2007). Notably, cAMP inside the cell acts as a second messenger and triggers mutation of spores and stalk cells (Mann *et al*., 1997) and mediates osmotic resistance (Schuster *et al*., 1996) while outside the cell, the cAMP acts as a primary messenger in the form of a chemo-attractant to mediate cellular motility through a regulation of the cytoskeleton (Von Buelow *et al*., 2012).

1.3 Problem statement

The AX4-like protein, whose gene At1g73980, has recently been bioinformatically annotated to contain two putative catalytic motifs (one adenylate cyclase and one kinase) in tandem array (Gehring, 2010). Adenylate cyclases are enzymes capable of converting ATP to cAMP, kinases are, on the other hand, enzymes capable of utilizing ATP to phosphorylate other protein candidates including themselves. Both of these enzymatic systems are very vital and critical for the various plant cell signalling processes and transduction systems. Notably, the AX4-like protein has so far already been experimentally demonstrated to play significant roles in critical plant cellular processes like stress tolerance and detection of virulence agents, all of which do centrally involve mediation by the cAMP and/or protein phosphorylations. However, even though the AX4-like protein has primarily been linked to these critical cAMP/phosphorylation-dependent cellular processes, no practical evidence have yet been provided to demonstrate this protein as a functional plant AC and/or kinase. In this regard, this presented study was, therefore, set to primarily check and establish if this protein

candidate is indeed a *bona fide* higher plant AC (with its possible future study as a possible functional plant kinase) and if so, to further determine if it has any physiological and/or biochemical cellular signalling roles in plant stress response and adaptation mechanisms.

1.4 Aim of the research study

The main aim of this study was to establish if higher plants have any other functional adenylate cyclases besides the only and currently known four, and if so, whether such molecules have any physiological roles in cell signalling, particularly in adaptation to biotic and abiotic environmental stress factors. Additionally, this is also despite the fact that this molecule has recently been annotated to contain an AC catalytic centre and previously been implicated in tolerance to stress and the detection of virulence agents (Moeder *et al*., 2013).

1.5 Objectives of research study

The following specific objectives were set to attempt and address the targeted main research aim:

1. To isolate the annotated AX4-like (At1g73980) gene fragment from *Arabidopsis thaliana.*
2. To clone the annotated AX4-like gene fragment into a stable and viable heterologous prokaryotic expression system.
3. To optimize strategies for the expression processes and regimes of the annotated recombinant AX4-like protein.
4. To determine the adenylate cyclase enzymatic activity of the annotated recombinant AX4-like protein.
5. To bioinformatically determine the functional role(s) of this putative AX4-like protein in plants.

1.6 SIGNIFICANCE OF THE RESEARCH

When this project was set, the following potential significance in the agricultural and food security sectors were anticipated:

1. The project would identify and establish another possible functional AC in higher plants besides the already and only known *Zea mays* pollen signalling protein (Moutinho *et al*., 2001), *Arabidopsis thaliana* pentatricopeptide protein (Ruzvidzo *et al*., 2013), *Nicotiana benthamiana* tabtoxinine-β-lactam-induced (Ito *et al*., 2014), and the *Hippeastrum hybridum* adenyl cyclase protein (Swiezawska *et al*., 2014).
2. The project would contribute towards a better understanding of the general mechanism by which plants respond and adapt to harsh environmental conditions.
3. The project would could contribute towards the synthesis and establishment of new literature and further scholarship in the modern field of Plant Sciences.
4. Upon completion of its functional characterization, the AX4-like gene can in the future be horizontally transferred to new cultivars of agronomic importance to South Africa through genetic engineering for increased yields and ultimately, the improvement of food security in the country.

CHAPTER 2

MATERIAL AND METHODS

2.1 Isolation of the AX4-like gene

A bioinformatics approach was used to retrieve the *AX4* like amino acid from the Arabidopsis information resources (TAIR) located on the website http://www.tair.com (Figure 2.1).

```
   1 ATCTCTCCAA CTATATTCTT ACTATCGCTT CTGGGTTTCC CGAAAATATG
  51 GAATAATCTC CGGCGACGTT TTTCCCCAAT TTGACTCTTC TTCCGTTAGC
 101 TTCCAGATAT TCTTCTTCAT TTGCAACTTT GAGGCAATGG CGCTCGATAG
 151 TTCTGTTGCT CTGTCACCTC GCCGGCGGCA TGGCTTGTTG CGAGATCAGG
 201 TTCAGCTTAT AAAAAGAAAG GACTCTGGAA GATATGAGAT AGTTCCAATA
 251 GAGGATCCAT TGTCTTTTGA AAAAGGTTTC TACGCTGTTA TTCGTGCTTG
 301 TCAATTATTG GCTCAGAAGA ACGATGGGCT CATTTTGGTT GGGTTAGCTG
 351 GTCCTTCTGG AGCTGGCAAA ACCATTTTCA CTGAGAAGAT TCTCAATTTT
 401 ATGCCTAGTA TTGCTATCAT TAACATGGAC AACTATAATG ATGGTACTCG
 451 TGTTATCGAT GGAAACTTTG ATGATCCAAG GTTGACTGAC TATGATACAC
 501 TTCTTGACAA TATACATGGT CTAAGGGATG GAAAACCTGT TCAGGTTCCA
 551 ATATATGACT TCAAGTCGAG TTCTCGAATA GGTTACAGAA CGCTTGAGGT
 601 GCCTAGCTCT CGCATTGTTA TTCTAGAAGG CATATATGCT TTGAGTGAGA
 651 AGCTACGGCC TTTGCTAGAT CTTCGTGTCT CTGTCACTGG TGGAGTGCAT
 701 TTTGATCTTG TTAAGCGTGT TTTGCGGGAC ATTCAACGTG CTGGCCAGGA
 751 ACCTGAAGAA ATAATCCATC AGATATCTGA GACGGTTTAT CCTATGTACA
 801 AGGCGTTTAT TGAACCTGAT CTGAAGACAG CTCAGATTAA GATCCTTAAT
 851 AAGTTCAACC CATTCAGTGG CTTTCAGAAC CCAACATATA TTTTAAAGTC
 901 ATCAAAGGCT GTAACACCCG AACAAATGAA GGCAGCTCTA TCTGAAGATT
 951 TCAAGGAACG TACAGAGGAA ACTTATGACA TCTATCTGTT ACCACCAGGC
1001 GAGGATCCTG AAGCATGCCA ATCATACCTC AGAATGAGGA ACCGAGATGG
1051 AAAATACAAT CTCATGTTTG AGGAGTGGGT TACAGATCGT CCATTTATTA
1101 TATCACCCAG AATAACTTTT GAAGTTAGTG TTCGTCTTCT TGGAGGATTA
1151 ATGGCACTGG GTTATACCAT TGCAACAATC CTGAAAAGAA AGAGTCATAT
1201 CTTTGATGAT GACAAGGTAA TTGTGAAAAC TGATTGGTTG GAACAACTGA
1251 ATCGGACATA TGTACAGGTA CAAGGTAAAG ACCGTACCTT TGTCAAAAAT
1301 GTGGCAGACC AACTTGGACT GGAAGGTTCA TATGTTCCAC ATACATATAT
1351 TGAACAGATA CAGCTGGAGA GGCTTGTGAA TGATGTTTTG GCTTTGCCAG
1401 ATGACTTAAA AACAAAACTT AGCTTAGATG ATGATACAGT TTCTAGCCCT
```

```
1451 AAAGAAGCCC TCTCAAGAGC TTCTGTTGAT AGTAGAATGA AATATCTTCA
1501 CGGCGGCGTA TCAAAGTCTT ACACAAACCC AAGACACAAA GTCTTGCCTA
1551 ACTTGACAAG ACTTGCTGTT AACAATAGAA TGTTAGATGC CAGAGCCCCT
1601 GCTTCACCGG CCACTCTTCC AAATCAGGGT TTTATCACTC AACTTTCAGA
1651 CCAAATATCA ACACTGAACG AGAGGATGGA TGAATTCACA TCCCGCATTG
1701 AAGAGTTGAA TTCCAAGATC CCGAACAGGA TAGCTCCTTC AGGTAGTCAA
1751 CATAACTTGG CTTTACCAAT CGAAAACGGT AATGGGTCTG TCTTATCATT
1801 CTCCGCATCC GCATCTCAGC TCGTAAGGGA ATCTCCTCTG ATGGAAGAGG
1851 TCGTACTGGT TGCTCGTGGA CAGCGTCAGA TAATGCATCA AATGGACACA
1901 CTGAGTAATC TTCTTCGGGA GTATGTTGGA GAAAAGACTC GCATAGAGAG
1951 ACTCGACAGC AGCAGAACAA ACAGTACAAC ACAAAACCTC GAATCCTCTA
2001 CTGTACCGAT TCTTCTTGGT TTGGCCATTG GCTGTGTGGG CATCTTTGCC
2051 TACAGTCGTC TGAAATAGTT GCTCTAACTG TGGAAACCCA GGCTTCGTGA
2101 AATAGTGGCA ATGATGTAAA TAACATGGTT TGGGATACAC ACTGGACAAA
2151 TTCAGTCTTG ACAATGTAAC ATGTGCCAAG AATAAAAGAC TGATTCGTTT
2201 CTTACCCTTT C
```

Fig 2.1: DNA sequence of the *AX4* like protein. The protein consists of 2201 nucleotide and harbours an adenylate cyclase catalytic centre (highlighted in GREEN) and both its FORWARD and REVERSE priming sites are highlighted in YELLOW.

2.1.2 Seed sterilization and vernalization

About 150 *Arabidopsis thaliana* seeds were placed in a sterile 1.5 mL microcentrifuge tube and were then washed 5 times by vortex (VX-200 Vortex Mixer, Labnet International Inc., New Jersey, USA) in 500 µL of 70% ethanol. The materials were moved to the laminar flow where the seeds were further washed in sterilization buffer (50% commercial bleach and 0.1% sodium dodecyl sulphate). This was followed by a successive rinsing (5 times) in 1 mL of filter-sterilized distilled water and vortexing for 30 seconds. Thereafter, 500 µL of the filter-sterilized water were added to the seeds that were then vernalized at 4℃ for four days and to break dormancy.

2.1.3 Seed germination

After vernalisation, the *Arabidopsis thaliana* seeds were germinated on sterile petri dishes containing Murashige and Skoog media (0.43% MS media, 0.30% sucrose and 0.8% agar, pH at 5.7) for 14 days on a grow chamber (Lab Companion GC -300TL Growth Chamber

System, Jeio Tech, Seoul, Korea). The plants were on an average temperature range of 23/16 ºC day/night for periods of 8/16 hours night/day at 10, 000 light lux. The seedlings were then transplanted onto sterile potting mix containing peat based soil, humus and vermiculite at a ratio of 1:1:1 respectively, and then allowed to grow for a further 2-4 weeks. The plants were on an average temperature range of 23/16 ºC day/night for periods of 8/16 hours night/day at 10, 000 light lux in a growth chamber (Lab Companion …)

2.1.4 Designing and acquisition of the sequence-specific primers

The amino acid sequences of the At1g73980 gene fragment encoding the putative AX4-like protein was retrieved from the TAIR web page (http://www.arabidopsis.org). Then forward primer 5′-TCA CCC AGA ATA ACT TTT GAA GTT AGT GTT-3′ and reverse primer 5′-AGA TGC GGA TGC GGA GAA TGA TAA GAC AGA-3′ flanking the AC catalytic motif were designed based on the AX4-like gene sequence and then sent to Inqaba Biotech for chemical synthesis and subsequent supply.

2.1.5 Extraction of Total RNA from *Arabidopsis thaliana*

The extraction of total RNA was conducted according to the manufacturer’s protocol of the Thermo Scientific Gene Jet RNA Purification Mini Kit #K0801, An amount of 0.10 g of leaf tissue was harvested from three week old *A. thaliana* plants. Quickly, the leaf material was immersed into liquid nitrogen and ground to a fine powder using a pestle and mortar. The mixture was then decanted into an RNase-free, 2 mL microcentrifuge tube containing 500 µL of Plant RNA Lysis Solution and was vortexed for 10 seconds to mix thoroughly. The mixture was incubated for 3 minutes at 56℃ and then centrifuged for 5 minutes at 14000 rpm. Immediately, a supernatant of 450 µL was collected and then transferred to a clean microfuge tube. 250 µL of 96% of ethanol was added, and then mixed by pipetting. The prepared mixture was transferred to a purification column inserted in a collection tube and centrifuged for 1 minute at 11000 rpm. Thereafter, the flow-through was discarded and the column and collection tube were reassembled. 700 µL of Wash Buffer 1 was added to the purification column and centrifuged for 1 minute at 11000 rpm; then the flow-through and collection tube were discarded. The purification column was placed into a clean 1.5 mL collection tube and 500 µL of Wash Buffer 2 were added to the purification column and then centrifuged for 1 minute at 11000 rpm. The flow-through was discarded and the column and

collection tube reassembled. The step above was repeated with the column centrifuged for 1 minute at a maximum speed of 14000 rpm. The collection tube and flow-through were discarded and the purification column transferred to an RNAse free 1.5 mL collection tube. Finally and to elute the RNA, 50 μL of nuclease-free water was added directly to the purification column membrane and centrifuged for 1 minute at 11000 rpm. The purification column was discarded and the collected RNA where immediately flash freeze in liquid nitrogen and stored at -80℃ and until use.

2.1.6 Isolation and amplification of the targeted AX4-like gene from *Arabidopsis thaliana*

The reverse transcriptase- polymerase chain reaction (RT-PCR) was used to target the desired AX4-like gene from the Arabidopsis genome, whereby 100 mg of the leaf tissue was harvested from the 6 weeks old *Arabidopsis thaliana* plants followed by isolation of the total mRNA using a Thermo Scientific Gene Jet Plant RNA purification mini kit and according to the manufacturer's protocol. The total mRNA extracted from the *Arabidopsis thaliana* leaves was used as template to generate copy DNA (cDNA). The copy DNA (cDNA) and the two acquired sequence-specific primers were then simultaneously used to amplify the targeted AX4-like gene fragment on a C1000 Thermocycling system (Bio-Rad laboratories Inc., California, USA) using the Thermo Scientific Verso 1-step RT-PCR Reddy Mix kit (Fermentas International Inc., Burlington, Canada) and as was instructed by the manufacturer. The used reaction mixtures and their associated cycling condition are shown below in Tables 2.1 and 2.2, respectively.

Table 2.1: Components of the RT-PCR reaction mixture for the amplification of the AX4-like gene fragment in a 50 µl reaction volume.

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

Table 2.2: Reaction conditions for the 1-Step RT-PCR thermal cycling program for the specifc amplification of the AX-4 like gene fragment.

	Temperature	Time	Number of Cycles
cDNA Synthesis	50°C	15 min	1
RT Inactivation	95°C	15 min	1
Denaturation	95°C	20 sec	35-45
Annealing	60°C	30 sec	
Extension	72°C	1 min	
Final Extension	72°C	5 min	1

2.1.7 Agarose gel electrophoresis of the amplified AX4-like gene fragment

A 1% agarose gel supplemented with 0.5 µg/ml ethidium bromide was used to resolve the above amplified RT-PCR product. All samples were resolved against a 100 bp GeneRuler DNA ladder and was immersed in a 1X TBE buffer at 80 volts and 250 mA current for 50 minutes. The gel was then visualized under a 2000 UV trans-illuminator system (Bio-Rad laboratories). The final image was captured using a ChemiDoc Imaging System (Bio-Rad Laboratories).

2.2: Cloning of the amplified AX4-like gene fragment

2.2.1: Addition of the 3′-adenine overhangs

A total volume of 1 µl 1 unit *Taq* polymerase was added to 40 µl of the RT-PCR product reaction mixture and then incubated at 72°C for 10 minutes in a C1000 Thermo cycler System (Bio-Rad Laboratories…). The resultant mixture was then kept on ice till further use.

2.2.2 Ligation of the putative AX4-like gene insert into a pTrcHis2-TOPO vector

An aliquot of about 4 µl was collected from the adenylated AX4-like gene fragment reaction mixture and then transferred to a fresh PCR tube before 1 µl of the pTrHis2-TOPO expression vector (Invitrogen, Carlsbad, USA) was added. The ligation mixture was then incubated at room temperature for 5 minutes before its subsequent use for transformation of the competent *E coli* expression cells.

2.2.3 Transformation of the *E coli* One Shot TOPO 10 competent cells with the pTrcHis2-TOPO: AX4-like fusion expression construct

Immediately after the ligation process, about 2 µl of the TOPO 10 cells were added into an ice-cold microcentrifuge tube containing 40 µl of the One Shot chemically competent *E coli* cells. The reaction mixture was gently mixed and incubated on ice for 30 minutes. The cells were heat-shocked for 30 second at 42ºC without shaking, and immediately, the tube was transferred onto ice for 5 minutes. Subsequently, the reaction mixture was supplemented with 250 µl of SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, and 10 mM Mg_2SO_4 and 20 mM glucose) and incubated in a shaking

incubator for 30 minutes at 37ºC. This incubation step helps the cells to produce the β-lactamase enzyme that then degrades ampicillin during the selection pressure process. The mixture was then plated as 80 µl and 20 µl onto Luria Bertani (LB) agar plates (1% (w/v) agar, 1% (w/v) tryptone powder, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl) supplemented with 100 µg/ml ampicillin and 0.5% glucose. The plates were then incubated overnight at 37ºC.

2.2.4 Extraction of the pTrcHis2-TOPO:AX4-like plasmid construct from the transformed One shot TOPO 10 *E coli* cells

Single colonies of cells were picked up from the previously grown LB plates and incubated in 10 ml double strength yeast-tryptone (2YT) media (0.8% (w/v) tryptone powder, 0.5% (w/v) yeast extract, 0, 25% (w/v) NaCl) supplemented with 100 µg/ml ampicillin and 0.5% of glucose and incubated overnight at 37ºC while shaking at 200 rpm. The bacterial culture was harvested by centrifuge at 6 800x*g* in a microcentrifuge for 5 minutes at room temperature and the supernatant was discarded. Plasmid extraction was performed using the GeneJET plasmid mini prep kit (Thermo Fisher Scientific…). The pelleted cells were resuspended into 200 µl of the Suspension Solution supplemented with RNase by pipetting up and down or until no cells clumps had remained and the cell suspension was transferred into a microcentrifuge tube. A volume of 250 µl of the Lysis Solution was added and mixed thoroughly by inverting the tubes 4-6 times and until the solution had become viscous and slightly clear. Approximately 350 µl of the Neutralization Solution was added and promptly mixed thoroughly by inverting the tube 4-6 times (mixing thoroughly y after addition of the Neutralization Solution is important to avoid the localized precipitation of bacterial cell debris). The lysed cells were the centrifuged for 5 minutes at 16300x*g* to pellet out the cell debris and chromosomal DNA. The supernatant was transferred to the supplied GeneJET spin column by pipetting and the solution was then centrifuged for 1 minute at the same speed. The flow through was discarded and the column was placed back into the same collection tube. About 500 µl of the Wash Solution (diluted with 96% ethanol prior to its first use) was added to the GeneJET spin column and centrifuged for 60 seconds. The flow-through was discarded and the column was placed back into same collection tube. The wash procedure was repeated one more time. The flow-through was discarded and the empty column centrifuged for an additional 1 minute to remove any residual Wash Solution. The GeneJET spin column was then transferred into a fresh 1.5 ml microcentrifuge tube and 50 µl

of the Elution Buffer was added to the centre of the spin column membrane. This was followed by an incubation of 2 minutes at room temperature and then centrifuged for 2 minutes at 163 00x*g*. The column was ultimately discarded while the purified plasmid DNA was stored at -20ºC further use.

2.2.5 Analysis of the positive clones

Positive clones were confirmed using the normal PCR system and in accordance with the standard MyTaq Mix protocol (Bioline, London, UK) firstly, to confirm if the AX4-like gene insert was successfully cloned into the pTrcHis2-TOPO expression vector and secondly, to ascertain if such a cloning was in the correct orientation. Tables 2.3 and 2.4 below, show both the reaction mixtures for cloning and orientation, respectively, while their associated thermal cycling conditions are shown in Table 2.5.

Table 2.3: Reaction components of a standard PCR reaction mixture to confirm the successful cloning of the AX4-like gene insert into the pTrcHis2-TOPO expression vector.

Component	Volume
Template (RNA)	1 µl
Insert Primers (20 µM each)	1 µl
My Tag Red Mix. (2X)	25 µl
Water	Up to 50 µl

Table 2.4: Reaction component of a standard PCR reaction mixture to confirm the correct orientation of the cloned AX4-like gene insert in the pTrcHis2-TOPO expression vector.

Component	Volume
Template (RNA)	1 µl
Insert/Vector Primers (20 µM each)	1 µl
My Tag Red Mix. (2X)	25 µl
Water	Up to 50 µl

Table 2.5: The reaction thermal cycling conditions for a step-by-step assessment profile of the successful cloning and correct orientation of the AX4-like gene insert into the pTrcHi2-TOPO expression vector.

Step	Temperature	Time	Cycles
Initial Denaturation	95°C	1 minute	1
Denaturing	95°C	15 seconds	25-35
Annealing	60°C	15 seconds	
Extension	72°C	10 seconds	

2.2.6. Agarose gel electrophoresis of the positively cloned AX4–like gene fragment

The PCR products of both Tables 2.3 and 2.4 were resolved and analyzed as is already outlined in section 2.1.7. A double amplification of the targeted AX4-like gene insert in both reaction samples (Tables 2.3 and 2.4) would confirm successful and positive cloning of the AX4-like gene fragment into the pTrcHis2-TOPO expression vector.

2.3. Partial expression of the recombinant AX4-like putative protein

2.3.1 Transformation of the chemically competent *E. cloni* EXPRESS BL21 (DE3) pLysS cells with the putative AX4-like expression construct

Once the cloning process of the AX4-like gene insert into the pTrcHis2-TOPO expression vector was positively confirmed, its resultant expression construct (pTrcHis2-TOPO:AX4-like) was used to transform some chemically competent *E. cloni* EXPRESS BL21 (DE3) pLysS expression cells. This transformation was carried out in a sterile Eppendorf tube and in accordance with the pTrcHis2-TOPO TA Expression protocol and instructions (Lucigen, Wisconsin, USA). Briefly a 1 µl of the pTrcHis2-TOPO:AX4-like expression construct was aseptically added to 40 µl of the chemically competent *E. cloni* BL21 (DE3) pLysS cells on ice. The mixture was then incubated on ice for 30 minutes before being heat-shocked for 45 seconds at 42ºC and immediately placed on ice for 2 minutes. The reaction mixture was then supplemented with 250 µl of the Expression Recovery Media (Lucigen…) and incubated at 37ºC in an orbital shaker at 250 rpm for 60 minutes. The reaction mixture was then plated onto LB agar plates supplemented with 100 µg/ml ampicillin and 34 µg/ml chloramphenicol. The plates were then incubated overnight at 37ºC.

2.3.2 Recombinant expression

Pilot protein expression exercises were run and stable clones were verified by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The transformed *E. coli* BL 21 (DE3) pLysS cells harboring the pTrcHis2-TOPO:AX4-like expression construct were used to inoculate 10 ml of the double strength yeast-tryptone 2YT (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 on an orbital shaker (New Brunswick Scientific, New Jersey, USA) at 200 rpm at 37°C. Expression of the recombinant protein was then induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma-Aldrich Corp., Missouri, USA) to a final concentration of 1 mM when the optical density (OD_{600}) of the cell culture had reached 0.6. Both the un-induced and induced culture cells were then left to shake in an incubator (200 rpm) at 37ºC for an additional 3 hours. The cultured cells were then pelleted out through centrifugation at 8 000×*g* for 5 minutes and the pelleted cells were then analyzed by SDS-PAGE.

2.3.3 Sodium Dodecyl Sulfate Poly-acrylamide Gel Electrophoresis

SDS-PAGE was used to check the recombinant protein expression. The protein was resolved on a 12 % running gel (30% Acrylamide stock, 0.8% SDS stock,3M Tris Buffer, 10% Ammonium Peroxodisulfate, Tetra Methyl Ethylene Diamine, sterile distilled water , pH 8.8) and 5 % stacking gel (30 % Acrylamide stock, 0.8 % SDS stock, 1M Tris Buffer, 10 % Ammonium peroxodisulfate, Tetra Methyl Ethylene Diamine, sterile distilled water) at 200 V for 60 minutes using the Wealtec Elite 300 Plus Electrophoresis System (Waltec,USA). The gel was stained with Coomassie staining solution (10% v/v absolute ethanol, 10% v/v µL absolute methanol, 10% v/v absolute acetic acid and 0.5% v/v Commassie stain), de-stained using a de-staining solution (10% v/v absolute ethanol, 10% v/v µL absolute methanol, 10% v/v absolute acetic acid) and was shaken on the Ultra-Rocker (Bio-Rad Laboratories ..) at 50 rpm for 15 minutes to facilitate both the staining and de-staining processes. Then the expressed recombinant protein was then observed as a band on the gel using a ChemiDoc™ MP Imaging System, (Bio-Rad Laboratories…).

2.4 Activity assaying of the recombinant AX4-like protein

2.4.1 Determination of the endogenous AC activity of the putative AX4-like protein

An overnight culture of cells confirmed to be harbouring the recombinant pTrcHis2-TOPO:AX4-like expression construct was prepared using 200 µl of its glycerol stock to inoculate 20 ml of fresh 2YT media supplemented with 100 µg/ml of ampicillin and 34 µg/ml of chloramphenicol. The culture was grown overnight at 37ºC in a shaker at 200 rpm. On the following day, fresh 20 ml of the 2YT media containing 100 µg/ml ampicillin and 34 µg/ml chloramphenicol was sub-cultured with 1 ml of the overnight culture and incubated at 37ºC in a shaker until the OD_{600} had reached 0.5. The culture was immediately placed on ice and split into four parts of 3 ml each. Protein expression was induced by the addition of 1 mM IPTG into three cultures and one tube being left un-induced (control). From two of the three induced cultures, one culture was supplemented with 100 µM forskolin (Sigma-Aldrich Corp…). Forskolin acts primarily by activating the enzyme adenylate cyclase, which results

in increased cyclic adenosine monophosphate (cAMP) in cells and the other culture with 100 µM 2′,5′-dideoxyadenosine (Sigma-Aldrich Corp…). Dideoxyadenosine inhibitor of adenylate cyclase, which results in a decrease cyclic adenosine monophosphate

Cells were then harvested by centrifugation at 9200x*g* for 10 minutes and lysed in 1 ml lysis buffer 1 (Amersham Healthcare, California, USA) supplemented with 2 mM IBMX (Sigma-Aldrich Corp…) to inhibit phosphodiesterases. The samples were then shaken at 100 rpm, at 37ºC for 30 minutes in an orbital shaker to intensify the cell lysis process. The samples were then centrifuged at 16.3x*g* for 5 minutes using a Corning, LSE, High speed micro centrifuge and the lysate transferred into a fresh microcentrifuge tube, where 200 µl of the lysis buffer 2 (Amersham Healthcare, California, USA) was added and mixed. A total of 220 µl of the mixture was transferred into a fresh Eppendorf and 11 µl of acetylating reagent (Sigma-Aldrich Corp…) were then added and the mixture pulsed. The endogenous cAMP contents from the lysates were then measured by a cAMP-linked enzyme immunoassay kit (Catalog #: CA201; Sigma-Aldrich Corp., Missouri, USA) following the acetylation version and as described by the manufacturer’s manual. Measurements or readings were then taken using a Microplate Reader (Labtech, International Limited, East Sussex, UK) at 405 nm and all obtained results were then subjected to a statistical analysis using the analysis of variance (ANOVA), and whereby all samples were analysed in triplicates (n = 3).

2.4.2 Complementation testing of the putative AX4-like protein

The complementation test was performed in order to check for the *in vivo* AC functionality of the truncated AX4-like gene fragment. This was done by dividing some *E. coli cya*A mutant competent cells into two portions. The first portion was transformed with the pTrcHis2-TOPO:AX4-like expression construct, while the second portion was left un-transformed (control). A MacConkey agar plate supplemented with 15 µg/ml kanamycin and 0.1 mM IPTG (Sigma-Aldrich Corp…) was prepared and then sub-divided into 3 segments using a permanent marker. The first segment was left un-streaked (no *cya*A cells), the second segment was streaked with the non-transformed *cya*A mutant cells while the last segment was streaked with the *cya*A mutant cells transformed with the pTrcHis2-TOPO:AX4-like expression construct. The plate was then inverted and incubated at 37ºC for 40 hours. After the incubation period, all segments were then visually inspected for various phenotypic characteristics. A reddish or deep purple colour on the transformed *cya*A mutant cells would mean a positive AC activity for the cloned and expressed recombinant AX4-like putative protein.

2.4.3 Bioinformatic expressional analysis of the *AX4-like* gene

2.4.3.1 Co-expressional analysis

In order to establish the co-expressional profile of the *AX4-like* gene with the other related *Arabidopsis thaliana* genes, the co-expression tool (http://www.arabidopsis.leeds.ac.uk) (Hruz *et al.*, 2008) was used. The co-expressed analysis was performed across all available experiments using At1g73980 as the driver gene and leaving the gene list limit blank to obtain a full correlation list. This tool utilizes hybridization signal intensities from microarray experiments to calculate a Pearson correlation co-efficient (r-value), which is a scale-invariant measure of expression similarity that expresses the strength and direction of the linear relationship between the reference gene (GOI, *AX4-like* in this case) and all other Arabidopsis genes represented on the selected chip. The tool calculates and returns both negative and positive correlations (ranging from –1 to +1), associated probability (p), and expectation (e) values, which are a measure of the statistical significance.

2.4.3.2 Stimulus-specific microarray expressional analysis

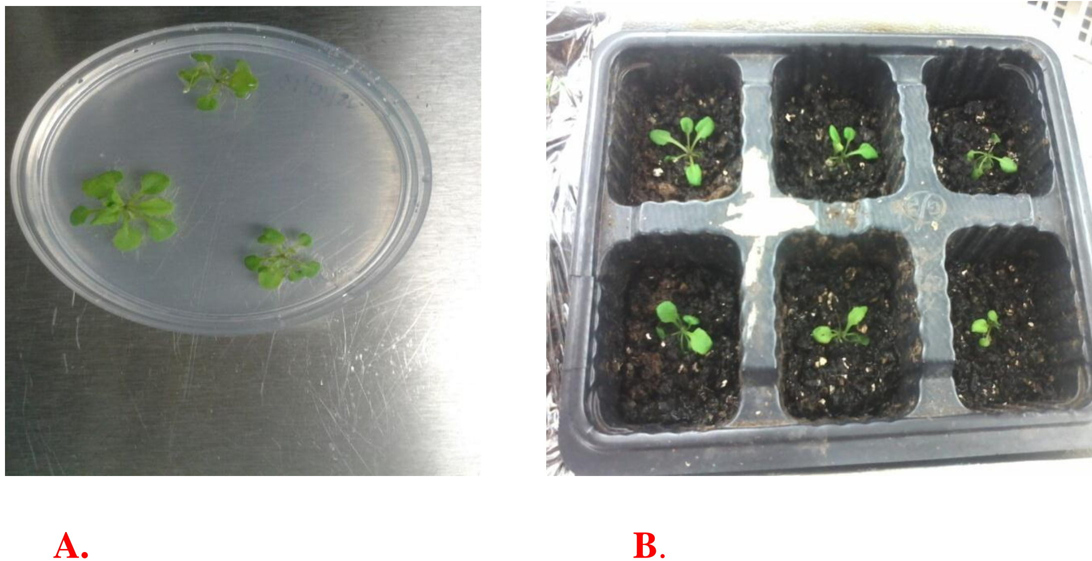
After retrieving an *AX4-like* co-expressional group of 25 genes (*ECGG25*), the expression profiles of the *AX4-like:ECGG25* were then initially screened over all of the available ATH1:22K array Affymetrix public microarray data in the Genevestigator V3 version (https://www.genevestigator.com) using the stimulus/pertubations tool (Zimmermann *et al*., 2004). In order to obtain greater resolution of gene expression profiles, the normalized microarray data were subsequently downloaded and analyzed for experiments that were found to induce differential expression of the genes. The data were downloaded from the following repository sites: GEO (NCBI) http://www.ncbi.nlm.nih.gov/geo/), NASCArrays (http://affymetrix.arabidopsis.info/narrays/experimentbrowse.pl) and TAIR-ATGenExpress (http://www.ebi.ac.uk/microarray-as/ac/). The array data were then analyzed and fold-change (log2) values calculated for each experiment. Expression values were then generated using the Multiple Array Viewer program from the Multi-Experiment Viewer (MeV) software package (vesion 4.2.01) created by The Institute for Genomic Research (TIG).

CHAPTER THREE

RESULTS AND DISCUSSION

3.1.1 Regeneration of the *Arabidopsis thaliana* plants

Arabidopsis thaliana ecotype Columbia seeds were surface-sterilized and germinated on solidified Murashige and Skoog medium (0.43% MS media, 0.30% sucrose and 0.8% agar, pH at 5.7) in a growth chamber system GC-300 (Lab Companion…). The germinated seeds were then transplanted onto planting potting mix. Leaf material was then harvested from 6 week-old plants for the subsequent isolation of total RNA.



A. B.

Figure 3.1: Germination and growth of the *Arabidopsis thaliana* plants. (A) Germination of seeds in Murashige and Skoog growth medium. (B) Six week-old *Arabidopsis thaliana* plants in potting mix soil.

3.1.2 Isolation and amplification of the AX4-like gene fragment

After designing the sequence-specific primers, total RNA was extracted from the 6 week-old *Arabidopsis thaliana* leaves and the resultant total RNA was then used as a template for the synthesis of an intron-free cDNA of the desired AX4-like gene fragment using a 1-step RT-PCR system. The amplified gene fragment was resolved on a 1% agarose gel and the band size of 714 bp was noted corresponding to the expected AX4-like gene fragment and as is shown below.

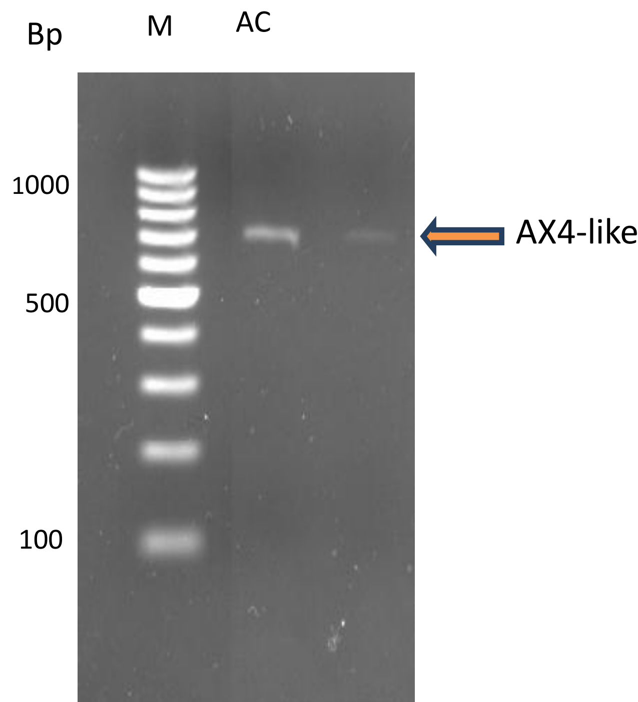


Figure 3.2: Amplification of the AX4-like gene fragment. An agarose gel resolution of the 714 base pair AX4-like gene fragment amplified from the total RNA extracted from the 6-week old *Arabidopsis thaliana* in a 1-step RT-PCR system. M represents the molecular weight marker while the arrow marks the amplified AX4-like gene fragment.

3.1.3 Recombinant expression of the AX4-like protein

In order to facilitate the partial expression of the desired recombinant AX4-like protein, the transformed BL21 (DE3) pLysS *E. coli* cells were induced with 1 mM IPTG and an OD_{600} of 0.6.

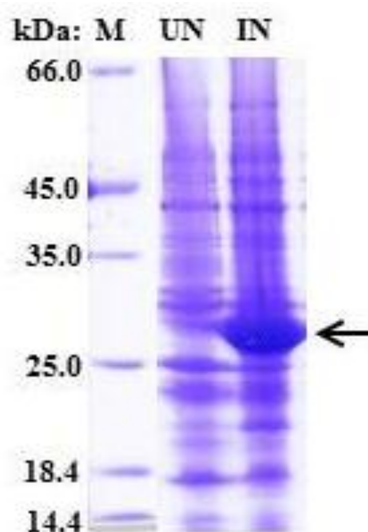


Figure 3.3: Recombinant expression of the AX4-like protein. The recombinant protein fragment was expressed in chemically competent BL21 (DE3) pLysS *E. coli* cells through their induction with 1 mM IPTG. The expressed protein was then resolved by SDS-PAGE on a 12% poly-acrylamide gel, where, M is the molecular weight marker, while UN and IN, represent the un-induced and induced cell cultures, respectively arrow marks the expressed recombinant AX4-like protein.

3.1.4 Determination of the endogenous AC activity of the recombinant AX4-like protein

To determine the levels of cAMP generated by the recombinant *E. coli* BL21 (DE3) pLysS harbouring the AX4-like gene fragment 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. In addition, another portion was treated with both 1 mM IPTG and 100 µM forskolin while the last portion was treated with 1 mM IPTG and 100 µM dideoxyadenosine. The generated cAMP was then extracted from the cells and its levels measured with a cAMP-specific enzyme immunoassay kit (Catalogue Number CA201, Sigma, Missouri, USA) based on its acetylation protocol.

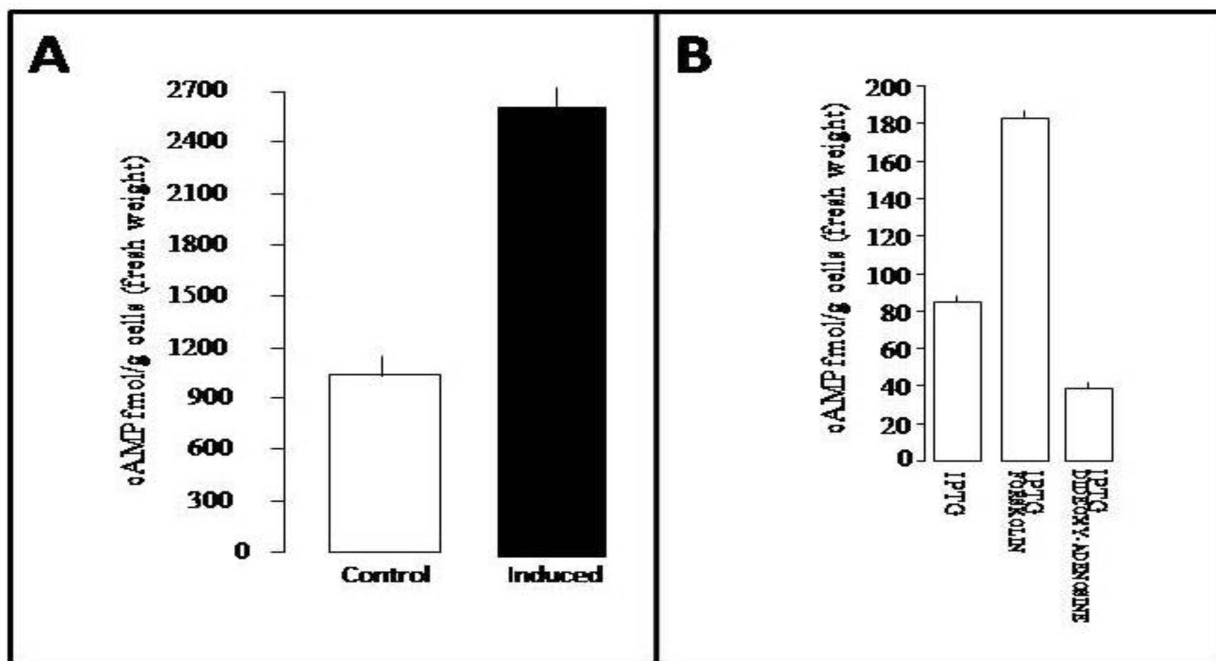


Figure 3.4: Determination of the endogenous adenylate cyclase activity of the recombinant AX4-like protein. **(A)** cAMP levels generated by un-induced (control) and induced BL21 (DE3) pLysS *E. coli* cells harbouring the AX4-like gene fragment is middle one better that dideoxyadenosine. **(B)** cAMP levels generated by induced recombinant cell cultures in the presence of forskolin is the highest and dideoxyadenosine is the smallest on the bar chart. All cAMP levels were determined using the cAMP enzyme immunoassaying system (Catalogue Number CA201, Sigma, Missouri, USA) where error bars represent the standard errors of the means (n=3). (Wuttke *et al*., 2001) (Moutinho *et al*., 2001)

3.1.5 Determination of the *in vivo* AC activity of the recombinant AX4-like protein

A MacConkey agar plate was prepared and then divided into three separate sections with a permanent marker pen. Section 1 of the plate was left un-inoculated while section 2 of the plate was inoculated with the non-transformed SP850 *cya*A mutant cells and section 3 was inoculated with the SP850 *cya*A mutant cells transformed with the pTrcHis2-TOPO:AX4 like fusion construct. The plate was then incubated at 37°C for 40 hours before being analyzed for some various but specific and differential phenotypic traits.

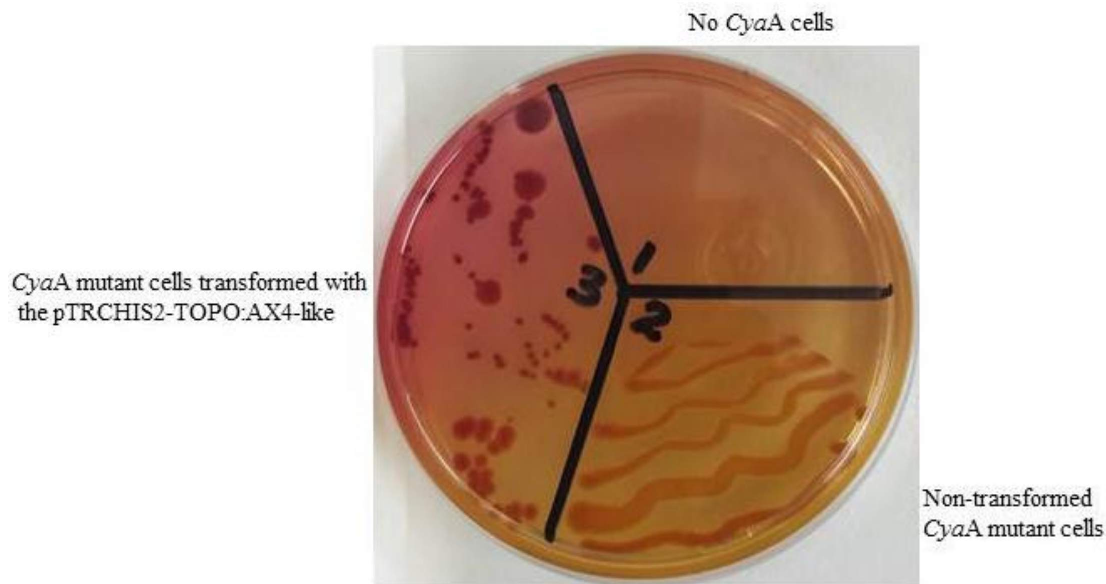


Figure 3.5: Determination of the *in vivo* adenylate cyclase activity of the recombinant AX4-like protein through a complementation test. Section 1 of the above MacConkey agar plate contains no cells, section 2 contains the non-transformed mutant cells and section 3 contains the mutant cells transformed with the pTrcHis2-TOPO:AX4-like fusion construct. Cells in sections 2 are non-lactose fermenters and therefore, produce white or yellowish colonies while cells in section 3 have picked a magenta/deep purple phenotype signifying a lactose-fermenting phenotype.

3.1.6 Co-expressional analysis of the *AX4-like* gene in *Arabidopsis thaliana*

In order to determine the co-expressional levels of the *AX4-like* gene with the other *Arabidopsis thaliana* genes, an expression correlational analysis across a large number (322) of the diverse microarray experiments represented on the ATH1-22K full genome microarray chip was performed. This analysis revealed that the *AX4-like* gene is highly co-expressed with a number of other genes in the *Arabidopsis thaliana* genome, with the top 25 genes having a Pearson's correlation coefficient (r-value) ranging between 0.810 and 0.913 (Table 3.1 below). The top 25 most co-expressed genes (hereby referred to as the AX4-like-Expression Correlated Gene Group (*AX4-like:ECGG25*) were considered here since their correlational values were high and their number being fairly high enough to be a good representative sample size for the subsequent stimulus-specific and functional analysis steps.

Table 3.1: Top 25 genes whose expression profiles are directly correlated with the *AX4-like* gene (At1g73980).

Locus	GO terms	*r* value	Annotation
AT1G73980		1.000	AX4 AC domain containing protein
AT5G65600		0.913	Legume lectin family protein kinase (PK)
AT4G21390		0.907	S-locus lectin PK family protein
AT3G21230		0.895	4-coumarate-CoA ligase 5
AT2G35980	DR, RBS, ROO	0.868	NHL10 (NDR1/HIN1-LIKE-10)
AT3G09410		0.867	Pectin acetylesterase family protein
AT1G15520	RBS, PM	0.866	Pleiotropic Drug Resistance (PDR) transporter
AT1G61560	DR, RBS, ROO	0.865	Mildew Resistance Locus O -6 (MLO6)
AT3G11840		0.862	U-box domain-containing protein
AT1G76070		0.861	Similar to syringolide-induced protein 14-1-1
AT3G26830	RBS, ROO	0.859	Phytoalexin deficient 3 (PAD3) protein
AT3G43250		0.858	Cell cycle control protein
AT1G51890		0.858	Leucine-rich repeat PK, Serine/threonine PK
AT3G63380		0.856	Calcium-transporting ATPase, PM-type
AT1G28190		0.855	Expressed protein
AT3G53600		0.854	Zinc finger (C_2H_2 type) family protein
AT5G64890		0.844	Elicitor peptide 2 precursor (PROPEP2)
AT4G39030	DR, RBS, ROO	0.841	SID-1/ EDS5
AT1G29690		0.841	CAD1, neg. reg. SA-med. Pathway
AT3G25780	DR, RBS, ROO	0.830	Allene Oxide Cyclase 3 (AOC3), JA-biosynthesis
AT1G22400		0.829	UDP-glucoronosyl/UDP-glucosyltransferase
AT2G15390		0.828	Xyloglucanfucosyltransferase, putative (FUT4)
AT3G52400	DR, RBS, ROO	0.828	Syntaxin of Plants(SYP)-122 (SYP122) PM
AT4G18170		0.820	WRKY28 transcription factor
AT5G05730	RBS, ROO	0.819	Anthranilate Synthatase Alpha subunit-1
AT4G33430	**DR, RBS, ROO**	**0.810**	**BAK1**

Abbreviations for the indicated GO terms:
DR = defence response; RBS = response to biotic stimulus; ROO = response to other organism; PM = plasma membrane.

3.1.7 Stimulus-specific microarray expressional profile of the *AX4-like:ECGG25*

After establishing the co-expressional profile of the *AX4-like* with the *AX4-like:ECGG25* table 3.1, this gene set was further subjected to an *in-silico* global expression analysis in which specific experimental conditions that could induce differential expression of all contained genes were identified. In line with the co-expression analysis, the heat maps generated from this microarray expressional analysis revealed that the transcriptional processes of both the *AX4-like* and its associated *AX4-ECGG25* gene complement are generally and collectively induced in response to the biotic pathogenic bacterial stress factors (Figure 3.6 below).

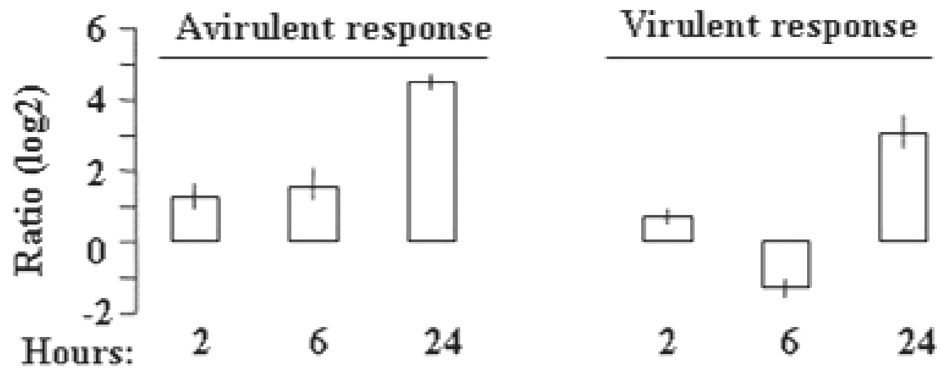


Figure 3.6: Induction of the *AX4-like* gene expression in response to *Pseudomonas syringae* infection. The pathogen induced a significant expressional response of the *AX4-like* gene in the infected plants, which was more pronounced with its avirulent than its virulent strain (Ward *et al*., 1991).

CHAPTER 4

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

4.1 Discussion

Cyclic AMP (cAMP) has been firmly established as an important second messenger signaling molecule in eukaryotic and prokaryotic animals (Goodman *et al*., 1970; Gerish *et al*., 1975; Weigant, 1978). The connection between the cyclic AMP signaling and pathology in higher plants was initially doubted due to the low levels of cAMP detected (<20 P mol/g wet weight) (Aston and Polya, 1978) as compared to those found in animals (>250 p mol/g wet weight) (Butcher and Sutherland, 1968). However and recently, Gehring (2010) has successfully managed to bioinformatically identify fourteen candidate genes from the *Arabidopsis thaliana* genome bearing various AC catalytic motifs as models to reveal the possible presence of ACs in higher plants (Table 1.1). Amongst these fourteen genes, the AX4-like (At1g73980) was part of them.

Therefore and in order to establish whether the At1g73980 gene was indeed a conceivable higher plant adenylate cyclase, the At1g73980 gene was isolated (Figure 3.2), cloned partially expressed (Figure 3.3) and furthermore, assessed for its possible AC functions. Principally and as part of its concerted functional assessment, its vector–insert construct (pTrcHis2:AX4-lik) was used to transform some chemically competent *E. cloni* EXPRESS BL21 (DE3) pLysS cells followed by an assessment of its functional ability to generate cAMP from ATP within these bacterial cells. Findings from this assessment showed that indeed the partially expressed recombinant AX4-like protein could transiently increase the levels of cAMP in those bacterial cells, whereby such an increase was metabolically sensitive on forskolin and decrease on the dideoxyadenosine (Figure 3.4) as inhibitor of cAMP. From this outcome, it could therefore be firmly concluded that the cloned and partially expressed AX4-like recombinant was either itself a *bona fide* AC capable of generating cAMP from ATP or is simply another functional higher plant molecule capable of stimulating other resident ACs (*E. coli* ACs in this case) to produce cAMP. Additionally, by virtue of it being metabolically sensitive of forskolin (Wuttke *et al*., 2001), it proposes this recombinant molecule as a transmembrane AC (tmAC) (Kamenetsky *et al*., 2006).

In order to confirm that the cloned and partially expressed AX4-like recombinant protein was indeed a functional AC, an *in vivo* assaying of this protein's activity was 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 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). Therefore and in order to test if the AC activity of the AX4-like protein could rescue the mutant cells, these cells were transformed with the pTrcHis2 TOPO:AX4-like expression construct followed by an assessment of their colony phenotypes on MacConkey agar supplemented with 0.1 mM IPTG (Figure 3.5). As is shown in the figure, the transformed cells stained deep-purple, signifying the rescuing aspect of the mutant cells into wild types and thus validating the AX4-like protein as a functional AC.

Furthermore, observations made from our bioinformatics analysis of the *AX4-like* gene firmly indicated that this putative gene is predominantly expressed in the cytosolic leaf tissues of the *Arabidopsis thaliana* alongside other genes that are specifically involved in defense response, response to biotic stimuli, response to other organism and the plasma membrane processes (Table 3.1). Apparently, by considering the fact that all the above mentioned four physiological processes in which the AX4-like gene is centrally involved, require the participation of second messengers like cAMP and/or cGMP (Ward *et al*., 1991) yet the cloned version of the truncated AX4-like gene form has since been practically demonstrated in this study to possess a functional adenylate cyclase activity (Figures 3.4 and 3.5), it is thus conceivable to conclude that the AX4-like protein (studied in this work) is an important plant signalling molecule with a principal role in responses to biotic pathogenic bacterial stress factors (Figure 3.7), and whose mechanism of action is typically mediated by the second messenger, cAMP.

Therefore, by summing up all of our findings, it is permissible to conclude that the AX4-like protein from *Arabidopsis thaliana* is a *bona fide* higher plant AC in addition to the currently known *Zea mays* pollen protein (Mountinho *et al*., 2001), *Arabidopsis thaliana* pentatricopeptide protein (Ruzvidzo *et al*., 2013), *Nicotiana benthamiana* adenyl cyclase protein (Ito *et al*., 2014) and the *Hippeastrum hybridum* adenyl cyclase protein (Swiezawska *et al*., 2014).

4.2 Conclusions

Findings from this study firmly ascertain the AX4 like protein domain of the At1g73980 gene that harbours the annotated AC motif as an AC with functional activity. In other words, the findings indirectly but firmly confirm the At1g73980 gene from *A. thaliana* as a functional AC molecule and thus becoming the firth ever candidate to be identified in higher plants after the only and currently known pollen protein from known *Zea mays* pollen signalling protein (Moutinho *et al*., 2001), *Arabidopsis thaliana* pentatricopeptide protein (Ruzvidzo *et al*., 2013), *Nicotiana benthamiana* tabtoxinine-β-lactam-induced (Ito *et al*., 2014), and the *Hippeastrum hybridum* adenyl cyclase protein (Swiezawska *et al*., 2014). These findings therefore provide a novel and elegant outcome with very huge and massive significance to both our scientific literature and academic scholarship in the whole broader domain of modern science.

Recommendations

Findings from this study permit for two possible and practically feasible recommendations:

- Firstly, since the AX4-like protein has been firmly confirmed as a functional adenylate cyclase, it is essential that its exact physiological roles in cell communication and signal transduction are further investigated so that its exact mode of action, particularly in processes like plant stress response and adaptation mechanisms, is properly and firmly ascertained. This would be more pronounced if the purified form of the protein as compared to its crude extracts is used.
- Secondly, since this molecule AX4-like was bioinformatically identified together with the other thirteen putative Arabidopsis molecules (Gehring, 2010) and this study now has confirmed the AX4-like molecule as a functional higher plant adenylate cyclase, it is therefore imperative that the other thirteen annotated molecule that are still outstanding are also tested to see if they are also indeed *bona fide* higher plant adenylate cyclases.

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