

**Elucidation and Functional
Characterization of the C-terminal Adenylyl
Cyclase Activity of a Recombinant Leucine-
Rich Repeat Protein from *Arabidopsis thaliana***

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DECLARATION

I, Amogelang Seheri, declare that this thesis titled “Elucidation and Functional Characterization of the C-terminal Adenylyl Cyclase Activity of a Recombinant Leucine-Rich Repeat Protein from *Arabidopsis thaliana*, submitted to the Department of Botany at North-West University, Mafikeng Campus, for the Masters in Biology/Plant Biotechnology degree has not been submitted at this university or at any other institution elsewhere. This is my own work and all the sources used or quoted here have been properly indicated and sincerely acknowledged.

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DEFINITION OF TERMS

Adenosine Triphosphate: A nucleoside triphosphate used in cells as a co-enzyme to transport chemical energy within them for metabolism.

Adenylyl Cyclases: Enzymes that catalyze the formation of 3',5'-cyclic AMP (cAMP) with the release of pyrophosphate from adenosine triphosphate (ATP).

Cloning: A process used to create copies of DNA fragments by host expression systems.

Cyclic 3',5'-adenosine Monophosphate: A second messenger molecule important in many biological processes. The cAMP is derived from ATP and is used for intracellular signal transduction systems in many different organisms.

Cyclic Nucleotide: A single phosphate nucleoside with a cyclic bond arrangement between the sugar and phosphate groups.

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

Phosphorylation: The addition of a phosphate (PO_4^{3-}) group to a protein or any other organic molecule. Phosphorylation turns many protein enzymes 'on' and 'off', thereby altering their function and activity.

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

Protein Kinase: An enzyme that modifies other proteins by chemically adding phosphate groups to them (phosphorylation).

Recombinant DNA: A molecule formed by laboratory methods of genetic recombination to bring together genetic material from multiple sources, creating sequences that otherwise would not naturally be found in any biological organism.

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

Stratification: A process of pre-treating seeds so that they all germinate simultaneously.

Signal Transduction: A cell communication process that occurs when an extracellular signaling molecule activates a cell surface receptor.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE): A technique used in molecular biology to separate different protein molecules according to their sizes and migrational properties in a polyacrylamide gel system subjected to a strong electrical field.

Transformation: A process by which the genetic makeup of an organism is altered through the insertion of a new gene fragment (or exogenous DNA) into its genome.

Vernalisation: A pre-treatment of seeds by mimicking winter conditions in order to break their dormancy.

LIST OF ABBREVIATIONS

AC	:	Adenylyl cyclase
ATP	:	Adenosine triphosphate
BLAST	:	Basic Local Alignment Search Tool
cAMP	:	Cyclic adenosine monophosphate
cGMP	:	Cyclic guanosine monophosphate
DRP	:	Disease resistance protein
GCs	:	Guanylate cyclase
IPTG	:	Isopropyl- β -D-thiogalactopyranoside.
kDA	:	kilo Daltons
LB	:	Luria Bertani
LRRs	:	Leucine-Rich Repeat
MS	:	Murashige and Skoog
NBS	:	Nucleotide-binding site
OD	:	Optical Density
PCR	:	Polymerase chain reaction
RT-PCR	:	Reverse Transcriptase-polymerase chain reaction
TAIR	:	The Arabidopsis Information Resource
TBE	:	Tris-borate-EDTA
UV	:	Ultra-violet
YT	:	Yeast-tryptone

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ABSTRACT

Adenylate cyclases (ACs) are cellular enzymes that catalyze the hydrolysis of adenosine 5'-triphosphate (ATP) into 3',5'-cyclic adenosine monophosphate (cAMP), which has long been established to be a second messenger in cellular signalling pathways of bacteria, fungi, lower eukaryotes and animals. Second messengers such as the cAMP have a key role in linking environmental stimuli to physiological responses of various cells; although the presence of this cAMP and the enzymes that generate it has always been a topic of long disagreements and serious controversy in higher plants. In 2010, Gehring bioinformatically identified various plant molecules carrying the probable AC catalytic motif yet most of such molecules have not yet been practically and/or experimentally tested for their abilities to generate cAMP from ATP. One of such candidate molecules is the leucine-rich repeat (LRR) protein that is coded for by the At3g14460 gene. To prove that this candidate has adenylate cyclase activity, the total RNA was extracted from the 6-week old *A. thaliana* followed by amplification of the LRR gene fragment using RT-PCR with its sequence-specific primers. The coding sequence of the putative AC catalytic domain of this protein (LRR-AC) was cloned and then further expressed in *E. coli*. This was then followed by an *in vivo* activity evaluation through complementation test in which *Escherichia coli* *cyaA* SP850 mutant was used to determine the inherent AC activity of the LRR. Furthermore, various bioinformatics tools were used to elucidate and analyse the functionality of this protein in Arabidopsis and other related higher plants. Findings obtained demonstrated that the LRR-AC was a functional AC capable of catalysing the conversion of ATP into cAMP with an essential role cAMP-regulated abiotic and biotic stress response.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Many African countries are faced with a massive challenge in terms of food security because most of them experience harsh environmental conditions that reduce crop yields (Stuhmer *et al.*, 1989). By end of the century, global warming is expected to increase from the current 2.5°C to 4.38°C, further reducing food productivity. For instance, between 1981 and 2002, cereal crop yields have declined by 5 billion dollars per year. In addition to the challenge of temperature extremes, water stress and flooding associated with climatic variability, the incidence and severity of biotic stresses such as pests, diseases and the invasion of alien weed species are also likely to be greater. Crops that are at great risk include wheat and rice in South and Southeast Asia as well as maize in the Southern Africa (Varshney *et al.*, 2011). Due to their sessile nature, plants are likely to be attacked by many disease-causing organisms, causing large crop losses (Martin *et al.*, 2003). Biotic and abiotic stress factors are the main inhibitors of growth and development in plants, and are therefore, considered a major limiting factor for crop production.

In light of this, there is a critical need to find solutions for food shortage problems. Such solutions will increase agricultural productivity in order to meet and sustain the demand of food from a growing world population and also to lower the overall cost of food. One of these solutions would be to develop crop plants that are better adapted to both biotic and abiotic stress factors. Interestingly, plants have protective genes that can make them perform better under harsh environmental conditions and such genes are commonly termed disease resistant genes (Varshney *et al.*, 2011).

The development of disease-resistant plants through the manipulation of disease resistant genes is a very useful alternative to other methods such as the use of pesticides and other chemical control methods. As much as these other methods are helpful, most smallholder farmers cannot afford them because they are costly. The use of plant resistance genes has some associated benefits, which include an effective reduction of pathogen growth, minimal damage to the host plant, no input of pesticides from the farmers and most importantly, the development of new types (cultivars) of environmentally-friendly crops (Anand *et al.*, 2012).

Nonetheless, these disease resistance genes are often controlled by gene-for-gene mechanisms in which avirulence gene products encoded by pathogens are specifically recognized, either directly or indirectly, thus specifically recognizing pathogens that may infect the plant. The main focus of this research was to functionally characterize a member of these disease resistance genes in the form of a leucine-rich repeat (LRR) family protein from *Arabidopsis*, which is known to recognize pathogens and activate a plant defence mechanism known as the innate immunity (Belkhadir *et al.*, 2011).

This LRR protein is one of the several plant molecules recently annotated as possible adenylate cyclases (ACs) (Gehring, 2010). These ACs are enzymes capable of catalysing the conversion of adenosine 5'-triphosphate (ATP) into the signalling molecule 3',5'-cyclic adenosine monophosphate (cAMP). The cAMP can affect many different physiological and biochemical processes including the activity of kinases, which has firmly been recognized as an important molecule in plants and as a second messenger (Newton *et al.*, 2004). This molecule (Figure 1.1 below) has been well-characterized in both prokaryotes and eukaryotes, and has been known to play various biological roles in cell signalling and transduction pathways.

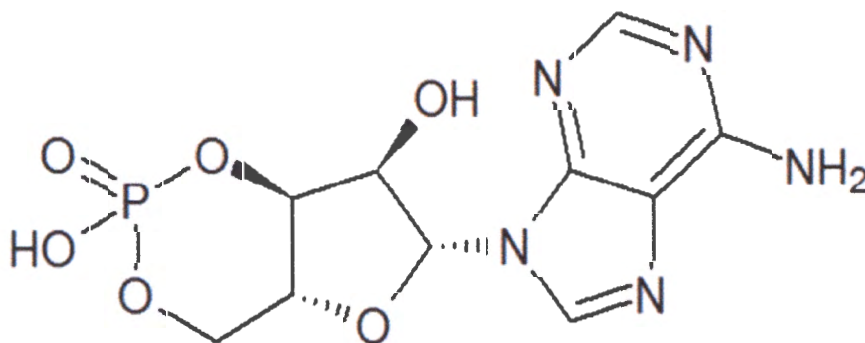


Figure.1.1: Chemical structure of cyclic adenosine-monophosphate (Alqurashi, 2013).

Surprisingly, as much as cAMP has been extensively reported in plants, the existence and/or functional roles of ACs have remained elusive (Newton and Smith, 2004; Kaplan *et al.*, 2007), and therefore, the identification of a functional AC protein in *Arabidopsis* will be a huge and notable milestone. Cyclic AMP is always released from the internal surface of the

plasma membrane into the cytoplasm and this is the major reason why it is termed second messenger (Steer, 1975).

1.2 Problem Statement

Factors such as pollution, drought, flooding, and pest invasion have had a huge impact on food security, thus resulting in related reductions in crop productivity. Even though the LRR protein from *A. thaliana* has long been shown to function in various cAMP-mediated pathogen defence mechanisms, no experiment to date has yet demonstrated an ability of this protein to generate cAMP from ATP. This is also despite the fact that the LRR protein has recently been annotated as a probable AC molecule (Gehring, 2010). Therefore, this study was set to experimentally test and demonstrate the possible AC activity of the LRR and furthermore, to better explain the probable mechanisms through which disease resistance proteins function in higher plants.

1.3 Research Aim and Objectives

1.3.1 Research Aim

The aim of the study was to determine if the annotated LRR family protein from *Arabidopsis thaliana* is a functional AC and if it is, to then further elucidate on its possible functional roles in plants, specifically with regards to the key cellular process of stress response.

1.3.2 Objectives

1. To isolate the C-terminus end of the LRR gene annotated to harbour the adenylyl cyclase center (LRR-AC).
2. To clone the LRR-AC gene fragment into a stable heterologous prokaryotic expression system.
3. To partially express the truncated LRR-AC as a recombinant product.
4. To determine the *in vivo* AC catalytic activity of the partially expressed recombinant LRR-AC protein.
5. To bioinformatically determine the functional roles of the LRR protein in *Arabidopsis* and other closely related flowering plants.

1.4 Significance of the Research Study

This study provides experimental evidence for the presence of yet another additional AC in higher plants involved disease resistance and stress response. In addition, this work can potentially provide a basis for the development of crop plants with improved resistance to diseases and other related stress factors, in order to improve yields. Such improved crop productivity will enhance food security and economic growth. The study is also significant as it has now added more literature to our current understanding of stress response and signal transduction in plants.

1.5 Literature Review

Plants disease resistance genes are macromolecules that enable plants to withstand harsh conditions caused by biotic and abiotic stresses, and their products (proteins) form part of the non-specific immune responses. The defence is characterized by rapid calcium and ion fluxes, an extracellular oxidase burst, transcriptional reprogramming within and around the infection site, and in most cases, a localized programmed cell death, which is termed the hypersensitive response (Rajagopal *et al.*, 2005). This disease resistance gene act as specific receptors for specific pathogen recognition; they are specific determinants for the plant immune response, which involves an allele-specific genetic interaction between host resistance genes and pathogen avirulence genes and when this genetic interaction takes place, a defence response is triggered (Belkhadir *et al.*, 2004). These resistance genes contain several classes which are classified according to motifs contained within proteins they encode. As is shown in Figure 1.2 below, LRR genes are the major class, which encodes a centrally positioned nucleotide-binding site and a carboxyl terminal (C-terminal) block of leucine-rich repeats. They are well adapted genes that detect a wide range of signals (Boyes *et al.*, 1998). In most cases, the activation of downstream effectors is normally organized. The amino-terminus is necessary for protein-protein interactions with an adaptor protein while ATP hydrolysis and release of the signal is controlled by the carboxyl-terminus. The amino-terminal and carboxy-terminal domains within the LRRs appear to have different functions, in that the amino-terminal domain controls the activation of downstream effectors while specific residues that are positioned in the carboxy-terminal domain appear to be responsible for bacterial neutralization (Belkhadir *et al.*, 2004).

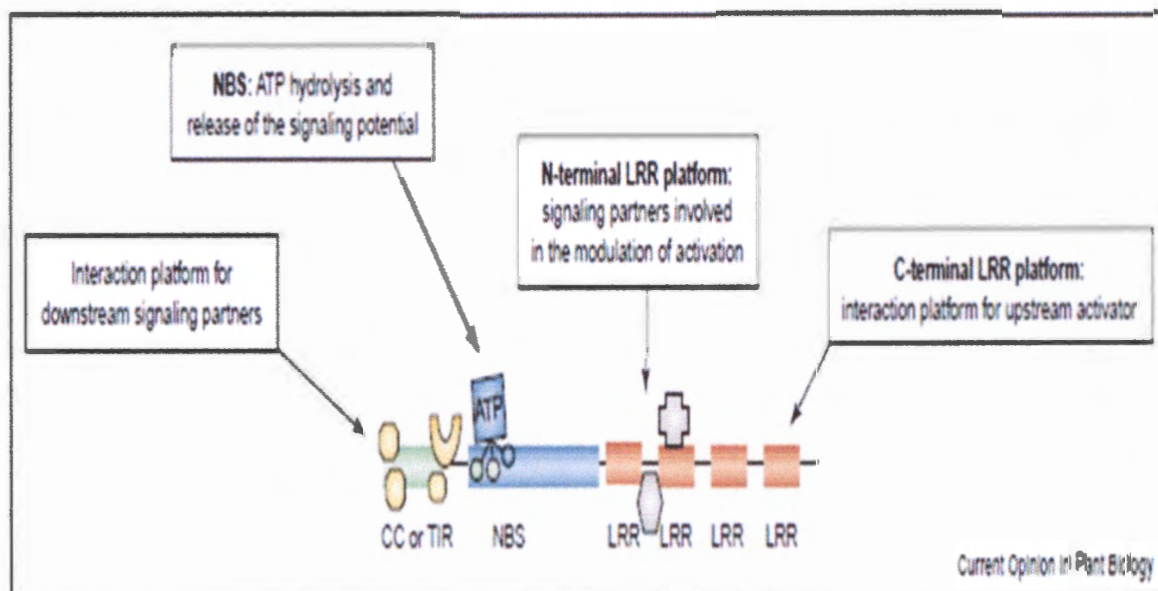


Figure 1.2: Assembly of various regulatory factors for disease resistance signalling in plants (Belkhadir *et al.*, 2004).

The LRR family proteins are responsible for ATP binding and the general functionality of resistance genes (Walker *et al.*, 1982; Saraste *et al.*, 1990). The proteins directly bind pathogens, which cause conformational changes in their N-terminal, C-terminal and LRR domains, a process which is thought to promote the exchange of ADP for ATP by the NBS domain (Boyes *et al.*, 1998). Recently, a putative Arabidopsis protein (AtLRR) that belongs to the LRR super-family has been proposed to be involved in pathogen-sensing and playing a role in defence responses and apoptosis (DeYoung and Innes, 2006). Furthermore, the same protein candidate has recently been proposed to harbour an annotated AC catalytic center in its carboxyl-terminus (Gehring, 2010). It is thus possible that LRR downstream signalling may be enabled by cAMP (Gehring, 2010).

In the 1970s, the probable activity of higher plant ACs was tested through the utilization of biochemical and histo-biochemical approaches (Moutinho *et al.*, 2001). Thereafter, the subsequent identification of higher plant ACs was then undertaken through homology searches to other annotated and/or experimentally confirmed adenylyl cyclases from lower and higher eukaryotes (Gehring, 2010). From this approach, several putative AC candidates were identified including the AtLRR (Gehring, 2010), which is studied in this work. It was hoped that a careful and detailed study of this putative protein (LRR) would provide a better insight into the functional roles of LRRs in plants and particularly in the critical cellular processes of pathogen recognition and disease resistance.

The mode of action of cAMP as a second messenger for mammalian hormones had been elucidated and accepted long before any attempts to discover cAMP and its functions in higher plants had been made. Originally, it was discovered as the intracellular mediator or activator of many peptides and catecholamine hormones in animals. Since then, cAMP has been involved in signalling systems that mediate cellular responses to imbalances of the external biosphere (Lemtiri-chlieh *et al.*, 2001) . It is only recently that the first proteomic response to cAMP in *A. thaliana* paved the way to a better understanding of the important role that this signalling molecule plays in light and temperature responses (Thomas *et al.*, 2013).

Cyclic AMP is one of the second messenger molecules that have been extensively studied in lower eukaryotes, and its metabolic status has a critical role in signalling. The action of adenylate cyclase produce cAMP from ATP, and then the cAMP is hydrolysed to AMP by the enzyme cAMP phosphodiesterase (Assmann, 1995). After stimulation, adenylyl cyclase, already functioning at a basal level in the absence of agonist, catalyses the conversion of ATP to cAMP, which is then released into the interior of the cell. The cAMP signal is later switched off by phosphodiesterases. Compared to the enormous research effort concentrated on the adenylyl cyclase system, little interest was shown initially in these enzymes (Newton *et al.*, 1999). In prokaryotes the variety of cAMP-coordinated processes have been found in both Eubacteria and Archaeobacteria. It has been found that it plays a significant role in gene expression, nitrogen fixation and not only for the classic inducible catabolic operons but also other categories. The effects of cAMP on gene expression is facilitated through interaction with an allosteric modification of a cAMP-binding protein (CPR), the CPR-cAMP complex that then binds to specific DNA sequences to either bind or activate transcription. Enteric coliforms provide a model that involves the mechanisms of dominant AC activity in regulating AC synthesis (Hanoune and Defer, 2001).

In *Escherichia coli* specifically, cAMP acts as a second messenger necessary for the synthesis of many inducible enzymes, for example, in the operation of its lactose (*lac*) operon (Assmann, 1995). In this particular system, cAMP forms a complex with the catabolite activator protein and the formed cAMP-CAP complex then binds to the promoter region of the operon. The DNA-bound cAMP-CAP complex in turn, then physically interacts with RNA polymerase and increases its affinity for the *lac* promoter, hence activating the transcriptional process of the *lac* operon (de Gunzburg, 1985). In the presence of lactose, the lactose repressor generally falls off the *lac* operator site, thus allowing RNA polymerase to

bind to the operator, leading to the transcription of the β -galactosidase, lactose permease and transacetylase genes of the operon. This process is normally aided by a background level of the β -galactosidase, which basally converts some lactose to allolactose, the inducer of the *lac* operon (Lemtiri-chlieh *et al.*, 2001). Cyclic AMP and ACs in this regard, therefore play a central role in the physiological processes of *E. coli*.

In this bacterial strain (*E. coli*), only a single AC naturally exists and is encoded for by the *cyaA* gene (Côté *et al.*, 2010), and this one and only enzyme was first isolated and purified by Yang and Epstein in 1983. Later on, it was then found that the *Bordetella pertussis cyaA* does encode for an AC toxin (Gross *et al.*, 1992). In 1992, Gross *et al.* then used a site-directed (deletion) mutagenesis and genetic recombination process that specifically was targeted at the *B. pertussis cyaA* gene and in order to produce mutants that did not have the AC activity of the toxin. Subsequently, it was then reported that an *E. coli cyaA* mutant strain, known as the SP850, had a *cyaA* mutation and therefore, lacked the AC activity (Cotta *et al.*, 1998).

This mutant strain cannot ferment carbon sources such as lactose, maltose, galactose, arabinose and glycerol, and it also grows very slowly on glucose, fructose and galactose sugars (Perlman and Pastan, 1969). The mutant is actually deficient in the *lacZ* expression because it lacks the AC needed for the cAMP production and therefore, cannot activate the catabolite activator protein, meaning that its cAMP receptor protein-dependent promoter is presumably inactive (Dole *et al.*, 2004). The mutant also possesses some resistance to kanamycin (Cotta *et al.*, 1998), is pH-sensitive for growth (Ahmad and Newman, 1988), and is a vibrant host for the recombinant expression systems of foreign proteins, especially when it is in its exponential phase (Grossman *et al.*, 1998).

Technically, the *cyaA* SP850 mutant provides a viable opportunity for testing the possible AC activities of foreign recombinant proteins via a complementation phenomenon of the mutation. In genetic analysis, a complementation test is used in order to determine if two recessive mutations that produce the same phenotype are located on the same gene (Hanoune and Defer, 2001). Thus through this same test, the *in vivo* AC activity of unknown recombinant protein molecules can then be pre-screened and the promising candidates subsequently further evaluated via some specific *in vitro* immunoassaying systems (Ruzvidzo *et al.*, 2013).

While a wild type *E. coli* strain naturally produces a deep red colour on MacConkey lactose agar and as a result of its ability to ferment lactose, the SP850 strain, due to its *cyaA* mutation, cannot utilize lactose and therefore, produces colourless colonies on the same growth medium. Apparently, when this mutant strain is transformed with any foreign gene capable of encoding for a functional AC activity, it also produces a deep red colour on the MacConkey lactose agar, which is indicative of a complementation of its AC deletion (Cotta *et al.*, 1998), leading to the production of the most needed cAMP, which in turn, then restores the normal utilization of lactose as a carbon source (Perlman and Pastan, 1969).

Based on this phenomenon, Moutinho *et al.* (2001) conducted a complementation process of the *cyaA* mutation in the *E. coli* SP850 strain with the PSiP protein from *Zea mays* and, in this study, the presence of a functional recombinant protein successfully gave rise to a selectable phenotype of the deep purple colonies. This specific finding was also further confirmed and fully validated through a similar approach by Ruzvidzo *et al.* in 2013 and Swiezawska *et al.* in 2014 using the AtPPR-AC and HpAC1 proteins respectively. In the same vein, Charania *et al.* (2009) also similarly demonstrated that the *Shewanella oneidensis* *cyaA*, *cyaB* and *cyaC* genes predicted to encode for the classes I, III and IV ACs, do indeed and actually encode for proteins with functional AC activities in the SP850 *cyaA* mutant.

There are two main reasons why it was difficult to establish the functionality of cAMP in higher plants; firstly, the levels of cAMP detected in plants appeared to be very low (<20 pmol/g fresh weight) compared to those found in animals (>250 pmol/g wet weight) and, secondly, the vagaries of assays used in plants were not conducive to reach firm conclusions. Nonetheless, specific pathogen-induced signalling at lower cyclic nucleotide concentrations has been reported in plants (Gehring, 2013).

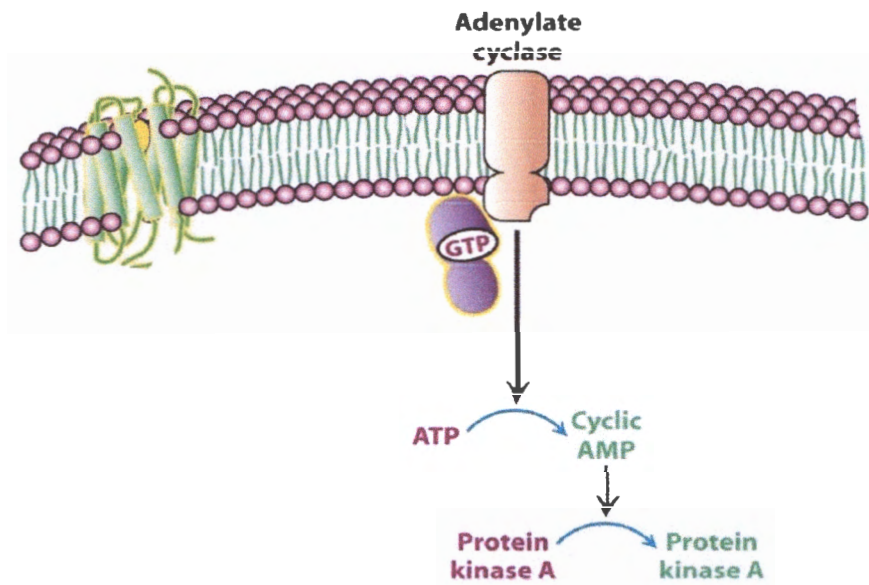


Figure 1.3: A schematic diagram showing signalling in plants. The conversion of ATP to cAMP by the enzyme AC, whereby the cAMP then activates the other enzymes (Lomovatskaya *et al.*, 2008).

The role of cAMP in plants is that it increases the influx and efflux of ions and molecules across the plasma membrane, assists in the elevation of protein kinase activities for the establishment of various downstream responses, as well as participating in responses and the development of resistance to biotic and abiotic stress factors. The detection of adenylyl cyclases in higher plants has been done through homology searches from lower and higher eukaryotes. To date, there are only six known ACs identified in higher plants and only one in lower eukaryotes. The one in lower eukaryotes was the first and only one to be characterised to date from the liverwort *Marchantia polymorpha* (Kasahara *et al.*, 2016) and it is also known that the protein PSiP from *Zea mays*, which plays a role in pollen tube growth, was found to have homology to the catalytic motif of a fungal adenylyl cyclase (Moutinho *et al.*, 2001).

The other three ACs from *A. thaliana*, which have been confirmed, have a catalytic motif related to that of guanylyl cyclases, constructed of 14 conserved amino acid residues from functionally assigned amino acids of the annotated catalytic center of eukaryote GCs, which has been modified to discover new AC candidates (Gehring, 2010). The main modification was to assign the amino acid residues that show substrate specificity to bind to ATP instead of GTP (position 3 in Figure 1.4). The predicted AC catalytic motif shown in Figure 1.4C below was used to identify the AC candidate studied in this work (Gehring, 2010).

N-terminus	C-terminus
A: --[RKS][YFW][CTGH][VIL][FV]X[DNA]X[VIL]X(4)[KR]X(1,3)[DE-- 1 2 3 4 5 6 7 8 9 10-13 14	
B: --[RKS]X[DE]X(9,11)[KR]X(1,3)[DE-- 1 2 3 12/14	
C: --[RK][YFW][DE][VIL][FV]X(8)[KR]X(1,3)[DE-- 1 2 3 4 5 14	

Figure 1.4: The catalytic motif center for nucleotide cyclases. (A) Motif centre of GCs in plants that are experimentally tested. In position 1 (red) does hydrogen bonds with the guanine residue, in position 3 the amino acid is predicted to change the substrate specificity and in position 14 the transition of (GTP/cGMP) is for stabilization. The Mg²⁺/Mn²⁺-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 (Newton *et al.*, 1999).

Principally and unlike other known eukaryotic ACs, the LRR protein possesses three annotated AC catalytic motifs (Figure 2.1), and only its C-terminal one was studied in this work since the other two motifs have already been studied and functionally confirmed in our research group. The rationale behind this structural and architectural representation is not well-known though in our own thinking, we suggested expanded functionality and evolutionary diversity among other commonly known eukaryotic ACs.

CHAPTER 2

METHODS OF INVESTIGATION

2.1 Isolation and Amplification of the Leucine-rich Repeat (LRR) Gene Fragment

2.1.1 Primer Design

The genomic and amino acid sequences of the LRR protein (At3g14660) were obtained from The Arabidopsis Information Resource (TAIR) website (www.arabidopsis.org). The annotated sequence of the putative AC domain harboured in this molecule was subsequently located and confirmed. Its forward and reverse primers were then manually designed and chemically acquired. The designing was done in such a way that the annotated AC catalytic motif of the LRR would be flanked within the two priming sites (Figure 2.1).

1	MANSYLSSCA	NVMVERINTS	QELVELCKGK	SSSALLKRLK	VALVTANPVL
51	ADADQRAEHV	REVKHWLTGI	KDAFFQAEDI	LDELQTEALR	RRVVAEAGGL
101	GGLFQNL MAG	REAIQKKIEP	KMEKVVRLL	HHVKHIEVIG	LKEYSETREP
151	QWRQASRSRP	DDLPGGRLVG	RVEDKLALVN	LLLSDDDEISI	GKPAVISVVG
201	MPGVGKTTLT	EIVFNDYRVT	EHFEVKMWIS	AGINFNVFTV	TKAVLQDITS
251	SAVNTEDLPS	LQIQVLLKKTLS	GKRFLVLDD	FWSESDSEWE	SFQVAFTDAE
301	EGSKI VLTTR	SEIVSTVAKA	EKIYQMKLMT	NEECWELISR	FAFGNISVGS
351	INQELEGIGK	RIAEQCKAGLP	LAARAIASHL	RSKPNPDDWY	AVSKNFSSYT
401	NSILFVLKLS	YDSLPPQLKR	CFALCSIFPK	GHVFDREELV	LLWMAIDLLY
451	QPRSSRRLEVD	IGNDYLGDLV	AQSFFQRLDI	TMTSFVMHDL	MNDLAKAVSG
501	DFCFRLEDDN	IPEIPSTTRH	FSFSRSQCDA	SVAFRSICGA	EFLRTLIPFN
551	SPTSLES LQL	TEKVAINPLL N	ALSGLRILSL	SHYQITNLPK	SLKGLKLLRY
601	LDLSSTKIKE	LPEFVCTLCN	LQTL LLSNCR	DLTSLPKSIA	ELINLRLLDL
651	VGTPLVEMPP	GIKKLRS LQK	LSNFVIGRLS	GAGLHELKEL	SHLRGTLRIS
701	ELQNVAFASE	AKDAGLKRKP	FLDGLILKWT	VKGS GFVPGS	FNALACDQKE
751	VLRMLEPHPH	LKTFCIESYQ	GGAFPKWLGD	SSFFGITSVT	LSSCNLCISL
801	PFVGQLP SLK	YLSIEKFNIL	QKVGLDFFFG	ENNSRGVFPQ	SLQILKFYGM
851	PRWDEWICPE	LEDGIFPCLQ	KLI IQRCPSL	RKKFP EGPLS	STEV TISDCP
901	LRAVSGGENS	FRRSLTNIPE	SPASIPMSMR	RELSSPTGNP	KSDASTSAQP
951	GFASSSQSND	DNEVTSTSSL	SSLPKDRQTE	DFDQYETQLG	SLPQQFE EFA
1001	VISARYSGYI	SDIPSTLSPY	MSRTSLVPDP	KNEGSILPGS	SSYQYHQYGI
1051	KSSVPSPRSS	EAIKPSQYDD	DETDMEYLKV	TDISHLMELP	QNLQSLHIDS
1101	CDGLTSLPEN	LTESYPNLHE	LLIIACHSLE	SFPGSHPTT	LKTL YIRDCK
1151	KLNFTESLQP	TRSYSQLEYL	FIGSSCSNLV	NFPLSLFPKL	RSLSIRDCE S
1201	FKTF SIHAGL	GDDRIALES L	EIRD CPNLET	FPQGGLPTPK	LSSMLLSNCK
1251	KLQALPVEKLF	GLTSLLSLFI	IKCPEIETIP	GGGFPSNLRT	LCISL CDKLT
1301	PRIEWGLRDL	ENLRNLEIDG	GNEDIESFPE	EGLLPKSVFS	LRI SRFENLK
1351	TLNRKGFHDAT	KAIETMEISG	CDKLQISIDE	DLPLSLCLRI	SSCSLLTETF
1401	AEVETEFFKV	LNIPYVEIDG	EIFS		

Forward primer: 5'-GCT TTC TCG ATT CAC GCA GGA CTC GGA GAC-3'

Reverse primer: 5'-GGA GAA GAT CTC GCC ATC GAT TTC CAC GTA-3'

Figure 2.1: The amino acid sequence of the *A. thaliana* LRR protein. The residues highlighted in red are the three putative AC motifs while the six black triangles indicate the various priming sites for the three AC motifs. This study focused on the C-terminal AC motif and sequences for the designed and used forward and reverse primers are provided below the amino acid sequences.

2.1.2 Seed Sterilization and Vernalization

Using aseptic techniques, about 200 *Arabidopsis thaliana* ecotype Columbia seeds were collected in a micro-centrifuge tube and washed with 500 µl of 70% v/v ethanol, vortexed at maximum speed for 5 times using a VX-200 Vortex Mixer (Lanet International Inc., New Jersey, USA) and the used solution discarded. After this, 500 µl of sterilization buffer (0.1% SDS and 5% chlorine bleach) was added to sterilize the seeds by vortexing 5 times under laminar flow conditions. This was then followed by rinsing of the seeds with 1 ml sterile distilled water and then vortexing 5 times to remove the sterilization buffer. The seeds were then suspended in 200 µl of water and vernalized for 3 days at 4°C. The vernalization process was carried out in order to induce synchronized germination for the resultant plants to grow uniformly.

2.1.3 Germination and Growth of *Arabidopsis thaliana* Plants

The sterilized and vernalized seeds were then germinated on Murashige and Skoog (MS) medium (0.43% Murashige and Skoog powder, 3% sucrose and 0.8% agar at pH 5.7) for 2 weeks under sterilised growth chamber conditions (25°C/16°C day/night temperature, 16/8 hours' night/day periods, and 10,000 lux). After these 14 days, the seedlings were transferred to potting soil consisting of 1:1:1 (v/v) vermiculite: humus: potting soil mix. The transplanted seedlings were then covered with plastic wrap for 7 days to retain moisture and grown further for 4 weeks, under the following conditions in a growth chamber (25/16°C day/night temperature; range of midday irradiance of 570-650 µmol·s⁻¹·m⁻², and average day/night humidity of 30/70%).

2.1.3 Extraction of the *Arabidopsis* Total RNA

Total RNA was extracted from the 6-week old *A. thaliana* whole plants using the GeneJet Plant RNA Mini Purification Kit (# K0801) and according to the manufacturer's protocol (Thermo Scientific Inc., California, United States). In brief, the generated plants were harvested and placed in a sterilized mortar, snap-frozen with liquid nitrogen and ground thoroughly into a fine powder using a sterile pestle. Immediately, the tissue powder was transferred into a 1.5 ml micro-centrifuge tube containing 500 µl of plant RNA Lysis Solution. The mixture was vortexed for 10-20s and then incubated for 3 minutes at 56°C. The mixture was then centrifuged for 5 minutes at 12,000 x g. The supernatant was collected

and transferred into a clean micro-centrifuge tube, and then a volume of 250 µl of 95% ethanol was added and mixed by pipetting. The mixture was then transferred into a purification column inserted in a collection tube and centrifuged for 1 minute at 12,000 x g. The flow-through solution was discarded while the column and its collection tube were re-assembled. A total of 700 µl Wash Buffer 1 was added to the purification column and centrifuge for 1 minute at 12,000 x g. The flow-through and the collection tube were discarded. The purification column was then placed into a clean 2 ml collection tube and washed with 500 µl of Wash Buffer 2 through centrifugation for 1 minute at 12,000 x g. The purification column was transferred into an RNase-free 1.5 ml collection tube, and the RNA eluted by adding 50 µl of nuclease-free water to the centre of the purification column membrane before centrifuging for 1 minute at 12,000 x g. Finally, the purification column was discarded while the eluted RNA was stored at -20°C for further use.

2.1.4 Reverse Transcriptase-Polymerase Chain Reaction

The targeted LRR-AC gene fragment was isolated from the Arabidopsis genome by reverse transcriptase polymerase chain reaction (RT-PCR). Copy DNA (cDNA) for the targeted gene fragment was first synthesized from the total mRNA using this system. Consequently, the two designed sequence-specific forward and reverse primers were used in the same system to amplify the targeted and desired LRR-AC gene fragment and using a Verso 1-Step Reddy Mix RT-PCR kit and as instructed by the manufacturer's protocol (Catalogue #AB-1454/LD/A; Thermo Scientific Inc., California, USA). All RT-PCR processes were undertaken on a C1000 Thermo-cycler System (Bio-Rad Laboratories Inc., California, USA), and Tables 2.1 and 2.2 below show the reaction mixture preparations and associated cycling conditions.

Table 2.1: The respective components of a 1-step RT-PCR reaction mix for amplification of the LRR-AC gene fragment in a 50 µl volume mixture.

Reaction Component	Volume (µl)	Final Concentration
Verso Enzyme Mix	1.0	
1-Step PCR ReddyMix (2X)	25.0	1X
Forward Primer (10 µM)	1.0	200 nM
Reverse Primer (10 µM)	1.0	200 nM
RT Enhancer	2.5	
Water (PCR Grade)	14.5	
Template (mRNA)	5.0	1 ng
Total Volume	50.0	

Table 2.2: The 1-Step RT-PCR thermal cycling program intended for amplification of the LRR-AC gene fragment.

Step	Temperature (°C)	Time (mins)	Number of Cycles
cDNA Synthesis	50	15	1
Thermo-start Activation	95	15	1
Denaturing	95	0.30	45
Annealing	60	0.5	
Extension	72	1	
Final Extension	72	5	1

2.1.5 Agarose Gel Electrophoresis of the Amplified LRR-AC Gene Fragment

A total volume of 7 µl of the RT- PCR amplicon was resolved against a 100 bp Gene-Ruler DNA ladder (Thermo Scientific Inc., California, USA) on a 1% agarose gel in 1X TBE (62.5% Tris-HCl, 31.8% boric acid and 5.7% EDTA) at 80 volts and a constant current of 400 mA for 30 minutes. The gel was stained with 0.5 µg/ml ethidium bromide and then visualised under UV light using the UV 2000 Trans-illuminator system (Bio-Rad

Laboratories Inc., California, USA) and images captured by a Chemi Doc™ Imaging system (Bio-Rad Laboratories Inc., California, USA).

2.2 Recombinant Cloning of the LRR-AC Gene Fragment

2.2.1 Addition of the Poly-A Tail

The amplified LRR-AC gene fragment was first poly-adenylated before being cloned, this was done because the vector is a linear and non-directional expression system with the 5'-thymine (T) cloning overhangs, to compliment with the 3'-adenine (A) overhangs. Briefly, 40 µl of the amplicon was mixed with 1 µl *Taq* and incubated on a C1000 Thermo-cycler System (Bio-Rad Laboratories Inc., California, and USA) at 72°C for 10 minutes. The resultant reaction mixture was then kept at 4°C for further use.

2.2.2 Ligation of the LRR-AC Gene Fragment into a pTrcHis2-TOPO Vector

The poly-adenylated LRR-AC gene fragment was ligated into the pTrcHis2-TOPO plasmid expression vector system and as instructed by the manufacturer's protocol (Invitrogen, Carlsbad, USA) to generate a pTrcHis2-TOPO:LRR-AC fusion expression construct with a C-terminus His purification tag. This ligation process was carried out by mixing 4 µl aliquot of the LRR-AC gene fragment with 1 µl of the pTrcHis2-TOPO vector followed by a 5-minute incubation period at room temperature.

2.2.3 Transformation of Competent Cells with the pTrcHis2-TOPO: LRR-AC Fusion Construct

About 2 µl portion of the pTrcHis2-TOPO: LRR-AC fusion expression construct were added to 100 µl of competent TOPO®10 cells. The mixture was gently mixed followed by its incubation on ice for 30 minutes. The samples were then heat-shocked at 42°C for 30 seconds before being immediately incubated on ice for 5 minutes. A total volume of 250 µl of a room temperature SOC media (2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM Mg₂SO₄, and 20 mM glucose) were added to the cells and then incubated in an SI-600 shaking incubator (Lab Companion, Boksburg, South Africa) for an hour at 37°C at 200 rpm. This step is always included so that the cells can synthesize the antibiotic resistant enzyme, β-lactamase. Then 10 µl and 50 µl aliquots of the

cells were then spread-plated onto two 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% (v/v) glucose). The plates were then incubated at 37°C overnight.

2.2.4 Screening for the Successful Clones

2.2.4.1 Colony PCR

Colony PCR was carried out for a quick screening of transformants and/or to ensure that the desired insert was ligated. This was done by using a tip of a pipette to touch a single colony from an overnight plate and transferring it into a sterile PCR tube. PCR was then conducted according to the Standard MyTaq™ kit and protocol (Catalogue BIO-21105; Bioline, London, UK). All PCR processes were carried out on a C1000 Thermo-cycler System (Bio-Rad Laboratories Inc., California, USA), and the used reaction mixes together with their associated cycling conditions are shown below in Tables 2.3 and 2.4 respectively.

Table 2.3: Reaction components of the PCR reaction mixture to confirm the presence of the LRR in the pTrcHis2-TOPO expression vector.

Reaction Component	Final Concentration	Volume (µl)
1-MyTaq Reddy Mix (2X)	1X	17
Insert Forward Primer (20 µM)	10 µM	1
Insert Reverse Primer (20 µM)	10 µM	1
Colony mixed with water		3
Water (sdH ₂ O)		3
Total Volume		25

Table 2.4: PCR cycling conditions to confirm the presence of the LRR in the pTrcHis2-TOPO expression vector.

Step	Temperature (°C)	Time	Cycles
Initial Denaturation	95	1min	1
Denaturation	95	15 sec	25-35
Annealing	63.5	15 sec	
Extension	72	10 sec	

2.2.4.2 Plasmid Extraction

After confirming presence of the LRR-AC gene fragment in the pTrcHis2-TOPO expression vector, all colonies harbouring the pTrcHis2-TOPO:LRR-AC fusion expression construct were then retraced for plasmid extraction and to check if such plasmid constructs were carrying the cloned LRR-AC in the correct orientation. In this process, about 5 ml of 2YT broth (1.6% w/v tryptone powder, 1.0% w/v yeast extract and 0.5% w/v sodium chloride) supplemented with 50 µg/ml ampicillin and 0.5% w/v glucose was inoculated with a distinct colony picked from the selected pool. The bacterial culture was then incubated overnight at 37°C with shaking at 200 rpm in an SI-600 orbital shaker (Lab Companion, Boksburg, South Africa). The next day, the overnight culture was centrifuged at 3,400 x g for 2 minutes to harvest the cells and extract plasmid using the Zyppy™ Plasmid Miniprep Kit (Catalog number: D4036; Thermo Fisher Scientific Inc., California, USA). Briefly, the harvested cells were re-suspended in 250 µl volume of the Resuspension Solution and the resulting cell suspension transferred into a micro-centrifuge tube. A total volume of 100 µl of the 7X Lysis Buffer was added and gently mixed by inverting the tubes 4-6 times. A total volume of 350 µl of the cold Neutralization Buffer was added and mixed gently by inverting the tube 4-6 times. The solution was then centrifuged at 16,000 x g for 4 minutes and the supernatant transferred into a Zymo-Spin™ column. The column was placed into a collection tube and centrifuged for 15 seconds. The flow-through was discarded while the column was placed back into the same collection tube. About 200 µl of the Endo-Wash Buffer 1 was added to the column, which was then centrifuged for 30 seconds. This was followed by an addition of 400 µl of the Zyppy™ Wash Buffer 2 to the column, which was also centrifuged for 30 seconds. The centrifuged column was then transferred into a clean 1.5 ml micro-centrifuge tube then 30 µl of the Zyppy™ Elution Buffer added directly onto the column matrix and let

to stand for one minute at room temperature. Finally the column was centrifuged for 15 seconds to elute the plasmid DNA.

2.2.4.3 Screening of the Correct Clones

Once again, the standard MyTaq™ Kit (Catalog # BIO-25041, Bioline Inc.,) was used to conduct PCR and verify if the LRR-AC gene fragment was correctly oriented in the purified pTrcHis2-TOPO:LRR-AC plasmid construct. In this regard, a vector primer and an insert primer were used as is briefly summarized in Tables 2.5 & 2.6 below.

Table 2.5: Reaction components of the PCR reaction mixture to confirm the correct orientation of the LRR-AC gene insert into the pTrcHis2-TOPO expression vector.

Reaction Component	Final Concentration	Volume (µl)
Vector Primer (20 µM)	10 µM	1
Insert Primer (20 µM)	10 µM	1
1-MyTaq Mix, 2X	1X	25
Plasmid DNA (template)	1 ng	1
Filtered Sterile Distilled Water (fs-dH ₂ O)		22
Total Volume		50

Table 2.6: The thermal cycling conditions for the PCR assessment process of the successful cloning of the LRR-AC gene insert into the pTrcHis2-TOPO expression vector.

Step	Temperature (°C)	Time	Cycles
Initial Denaturation	95	60 sec	1
Denaturation	95	15 sec	25-35
Annealing	45	15 sec	
Extension	72	10 sec	

2.2.4.4 Agarose Gel Electrophoresis

Amplicons from above (Tables 2.5 & 2.6) were resolved on 1% agarose gel in 1X TBE (62.5% Tris-HCl, 31.8% boric acid and 5.7% EDTA) at 80 volts for 30 minutes. The gel was stained with 0.5 µg/ml ethidium bromide and then visualised under UV light using a UV 2000 Trans-illuminator system (Bio-Rad Laboratories Inc.,) and images captured by the Chemi Doc TM Imaging System (Bio-Rad Laboratories Inc.,).

2.3 Recombinant Expression of the LRR Protein

The transformed bacterial colony generated in section 2.2.3 and confirmed to harbour the LRR-AC gene fragment in the correct orientation in sections 2.2.4.1 and 2.2.4.3, was used to inoculate 10 ml of the 2YT media supplemented with 100 µg/ml ampicillin, followed by overnight incubation at 37°C at 200 rpm. The next morning, a total of 50 ml fresh 2YT media supplemented with 100 µg/ml ampicillin and 0.5% w/v glucose were inoculated with 1 ml of the overnight culture and incubated at 37°C with shaking at 200 rpm and up until its optical density (OD₆₀₀) had reached 0.5-0.6, as measured by the Helios spectrophotometer (Merck Chemicals Ltd, California, USA). At an OD₆₀₀ of 0.5, the culture was split into two 20 ml cultures and one culture was induced to express the intended LRR-AC recombinant protein by adding 1 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma-Aldrich Corp., Missouri, USA) while the other culture was left un-induced (control). The split cultures were then incubated for a further 3 hours at 37°C at 200 rpm. After the 3 hours, 500 µl aliquots of each culture were then transferred into Eppendorf tubes and centrifuged at 8 000xg for 5 minutes to pellet the cells. The pelleted cells were then analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) for protein expression.

2.4 Activity Assaying

2.4.1 Preparation of Chemically Competent *cyaA E. coli* Cells

A loopful of *E. coli cyaA* SP850 cells (Coli Genetic Stock Center, Yale University, Connecticut, USA) was used to inoculate 10 ml of 2YT media supplemented with 15 µg/ml kanamycin followed by incubation overnight at 37°C at 200 rpm. The next morning, 1 ml of the overnight cell culture was then used to inoculate 50 ml of the pre-warmed 2YT media supplemented with 15 mg/ml kanamycin followed by incubation at 37°C at 200 rpm while

monitoring optical density at 600 nm. When an optical density of 0.5 was reached, cell growth was slowed down by immediate incubation on ice for 5 minutes. The culture was then transferred into a centrifuge tube and cells harvested at low speed (5 minutes at 4,000 x g at 4°C). The supernatant was carefully discarded while the cells were gently resuspended in an ice-cold Transformation Buffer 1 (TFB1; containing 30 mM KAc, 50 mM MnCl₂, 75 mM CaCl₂, and 15% (v/v) glycerol at pH of 5.8), and incubated on ice for 90 minutes. The cells were then pooled and collected through centrifugation at 4,000xg for 5 minutes. The supernatant was again carefully discarded while the cells were resuspended in 2 ml Transformation Buffer 2 (TFB2; containing 10 mM MOPS, 75 mM CaCl₂, 10 mM RbCl₂, 15% (v/v) glycerol at pH 8). Aliquots of 200 µl were then prepared in sterile 1.5 ml micro-centrifuge tubes and snap-frozen with liquid nitrogen before storing at -80°C for further use.

2.4.2 Transformation of the Competent *cyaA* Cells

The chemically competent *cyaA* SP850 cells prepared in section 2.4.1 above were transformed with the pTrcHis2-TOPO:LRR-AC construct extracted previously in section 2.2.4.2 and in accordance with the transformation procedure already outlined in section 2.2.3.

2.4.3 Complementation Testing of the Recombinant LRR-AC Protein

A MacConkey agar plate supplemented with 15 µg/ml kanamycin and 0.1 mM IPTG (Sigma-Aldrich Corp., Missouri, and USA) was prepared and sub-divided into 3 quadrants using a permanent marker. The first quadrant was left un-streaked (no *cyaA* cells), the second quadrant was streaked with the non-transformed *cyaA* mutant cells while the last quadrant was streaked with the *cyaA* mutant cells transformed with the pTrcHis2-TOPO:LRR-AC fusion expression construct. The plate was inverted and incubated at 37°C for 40 hours. After the incubation, all quadrants were then visually inspected for the various phenotypic characteristics. A deep red or purple colour on the transformed *cyaA* mutant cells would mean a positive AC activity for the cloned and recombinantly expressed LRR-AC protein.

2.5 Bioinformatic Expressional Analysis of the LRR Gene in *Arabidopsis thaliana*

In order to beef up our biochemical work undertaken above on the recombinant LRR-AC protein, we resorted to the bioinformatics analysis of its gene (*LRR* or At3g14460).

Bioinformatics is the application of computer-based technology to the understanding and effective use of biological data, to synthesize new predictions and/or discover new biology. In this approach, a wide range of web-based programs and softwares were utilized to elucidate the most likely mechanisms and/or molecular functions of the LRR protein in *A. thaliana* and other related flowering plants.

2.5.1 Anatomical expression analysis of the LRR protein

A microarray database was used in order to determine the expression levels of the LRR gene in different tissues of the *A. thaliana* plant (www.genevestigator.com/gv/). The information about genomic transcriptome of a selected specific gene was retrieved using the Affymetrix *Arabidopsis* genome array platform. In this case, the At3g14460 gene was used as the probe term before the uninformed values of the expression intensities of LRR in various *Arabidopsis* tissues were retrieved.

2.5.2 Developmental Expression Analysis of the LRR Gene

The development tool, AtGeneExpress (<https://www.genevestigator.com>), was used to determine the expression levels of the LRR gene in the Arabidopsis plant at the different stages of its development. The At3g14460 gene was searched against 10 different developmental stages of the plant and such stages were set from the radicle embryonic phase up to the release of mature seeds.

2.5.3 Co-Expressional analysis of the LRR Gene

In order to have a better understanding of the LRR protein, its gene was subjected to the analysis of co-expression by using the co-expression tool (<http://www.genevestigator.com>). The tool establishes an expressional profile of the LRR gene together with the other closely related genes in Arabidopsis, whose functions are already known for inference purposes. The tool makes use of signal intensities from microarray experiments to obtain the Pearson correlation co-efficient (r-value), which indicates the linear association of expressions between a reference gene (At3g14460, LRR) and all other Arabidopsis genes represented on the selection chip. The tool then calculates both the positive and negative correlations

(ranges from -1 to +1), which are measures of statistical significance, expressed as probability (P) and expectation (E) values.

2.5.4 Stimuli Specific Analysis of the LRR Gene

After retrieving a *LRR* co-expression group of 50 genes (*ECGG-50*) from section 2.5.1 above, we then used the FatiGO⁺ tool in the Babelomics suite to identify any important enhancement in functional terms associated with the *LRR:ECGG-50* (Al-Shahrour *et al.*, 2007). Then, using the stimulus tool, the expression profiles of the *NBS:ECGG-50* were screened over the ATH1:22K array Affymetrix public microarray data in the GENEVESTIGATOR version V3 (www.genevestigator.com) (Zimmermann *et al.*, 2004). The GEO (NCBI) (www.ncbi.nlm.gov/geo), the TAIR GeneExpress (www.ebi.ac.uk/microarrays-as/ac) and the NASC Arrays (www.affymetrix.arabidopsis.info/narrays/experimentbrowse.pl) were then used to download the normalized microarray data and subsequently analysed for experiments that were found to induce a differential expression, and the fold-change (log2) values 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 3

RESULTS

3.1 Generation of the Arabidopsis Plants

Sterilized Columbia ecotype seeds of *Arabidopsis thaliana* were germinated on Murashige and Skoog medium in a growth chamber and the resultant seedlings allowed to grow for 2 weeks. The seedlings were then transferred to potting soil for a further 4 weeks in the growth chamber before total RNA was extracted for subsequent isolation of the targeted LRR-AC gene fragment.



Figure 3.1: *Arabidopsis thaliana* germinated seeds developing as seedlings on Murashige and Skoog medium (left plane) and the fully grown plants growing on potting soil (right plane). The LRR-AC outlined in this study was isolated from these presented plant lines.

3.2 Isolation and Amplification of the LRR-AC Gene Fragment

Total RNA was extracted from the 6-week old Arabidopsis plants and then used to synthesize copy DNA (cDNA) via a specialized Verso™ 1-Step RT-PCR Reddy Mix system. The generated cDNA together with the manually designed primers were then used to amplify the targeted LRR gene via the same specialised RT-PCR. Using electrophoresis, the amplified gene fragment was then resolved on a 1% agarose gel as is shown in the figure below.

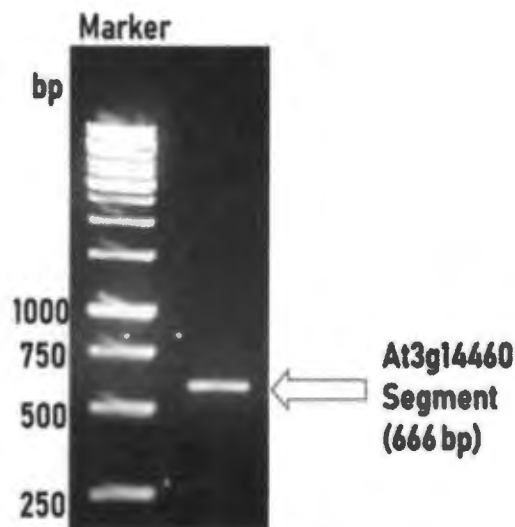


Figure 3.2: A 1% agarose gel resolution of the LRR-AC gene fragment isolated from the 6-week old *Arabidopsis thaliana* plants via a specialized Verso™ 1-Step RT-PCR Reddy Mix system. Marker represents the 1 kb low range molecular weight marker while the arrow is marking the band of the amplified and resolved 666 bp LRR-AC gene fragment.

3.3 Screening for the Correct Transformants

When the LRR gene fragment was successfully isolated from the 6-week old *A. thaliana* plants, it was then ligated into a pTrcHis2-TOPO expression vector to yield the pTrcHis2-TOPO:LRR-AC fusion construct. The resultant pTrcHis2-TOPO:LRR-AC fusion construct was then used to transform chemically competent *E. coli* One Shot TOPO 10 cells, in preparation for the subsequent partial expression and activity assaying of the expected NBS-AC recombinant protein. The transformed cells were then checked by colony PCR and using the LRR-AC primers, to see if they were carrying the generated pTrcHis2-TOPO:LRR-AC fusion construct. As is shown in Figure 3.3 below, the transformed cells were indeed carrying the cloned pTrcHis2-TOPO:LRR-AC fusion construct since the ligated LRR-AC gene fragment could be successfully re-amplified by its primers.

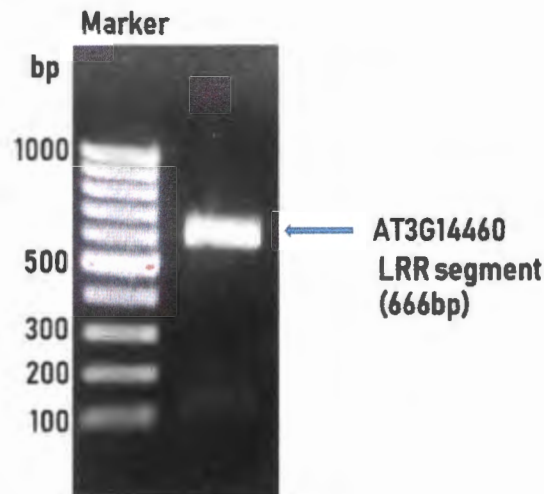


Figure 3.3: A 1% agarose gel resolution of the LRR-AC gene fragment after amplification by colony PCR of the *E. coli* One Shot TOPO 10 cells harbouring the pTrcHis2-TOPO:LRR-AC fusion construct. Marker represents the 1 kb low molecular weight marker while the arrow is marking the band of the re-amplified and resolved 666 bp LRR-AC gene fragment.

3.4 Screening for the Correct Recombinants

After the *E. coli* One Shot TOPO 10 cells were confirmed to have been transformed with the correct cloned pTrcHis2-TOPO:LRR-AC fusion construct, the fusion construct was then isolated, followed by its close analysis to check if it was carrying the LRR-AC gene fragment in the correct frame orientation. The checking involved using conventional PCR in the presence of one LRR-AC primer and a pTrcHis2-TOPO vector primer. As is shown below (Figure 3.4), a representation of a pTrcHis2-TOPO:LRR-AC fusion construct harbouring the LRR-AC gene fragment in the correct in-frame orientation is presented.

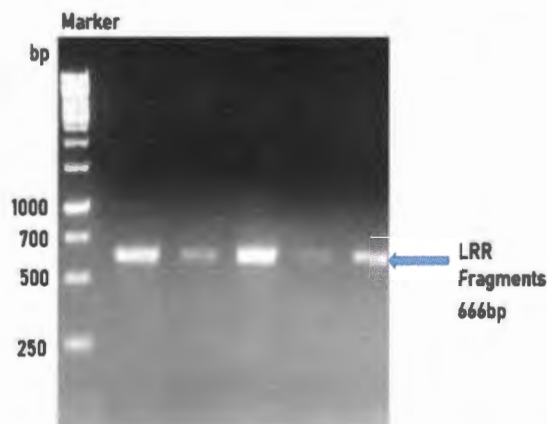


Figure 3.4: A 1% agarose gel resolution confirming correct orientation of the LRR-AC gene fragment in the pTrcHis2-TOPO expression vector. Marker represents the 1 kb low molecular weight marker the arrow is marking the respective bands of the 666 bp LRR-AC gene fragment amplified by its own reverse primer and the pTrcHis2-TOPO vector forward primer.

3.5 Partial Expression of the Recombinant LRR-AC Protein

After identifying the correct colony harbouring the successfully cloned LRR-AC gene fragment, the ability of this gene fragment to express a recombinant protein was then checked by inducing the transformed colony with IPTG. As is shown below in Figure 3.5, a recombinant protein product of approximately 24,420 kDa was expressed demonstrating a successful expression of the desired and targeted LRR-AC recombinant protein.

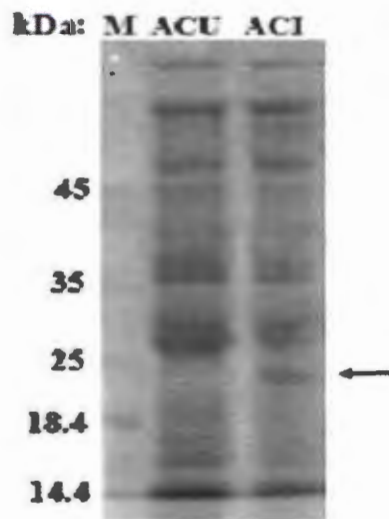


Figure 3.5: Partial expression of the LRR-AC protein was initiated through an induction of the transformed cells with 1 mM IPTG at an OD₆₀₀ of 0.6. The resultant protein fragments were then resolved on a 12% poly-acrylamide gel, where lane 1 (M) contains a low molecular weight marker; lane 2 (ACU), the un-induced cell culture and lane 3 (ACI), the induced cell culture. The arrow marks the position of the partially expressed LRR-AC recombinant.

3.6 Determination of the *In Vivo* Activity of the Recombinant LRR-AC Protein

In order to determine the *in vivo* AC activity of the cloned and partially expressed recombinant LRR-AC gene fragment, a complementation test was conducted using the *E. coli* *cyaA* SP850 mutant host strain, which systematically lacks the inherent AC activity. In this test, the mutant cells were transformed with the pTrcHis2-TOPO fusion expression vector harbouring the LRR-AC gene fragment, followed by an analysis of the LRR-AC's ability to generate cAMP on a differential and selective MacConkey media as is shown in Figure 3.6 below.

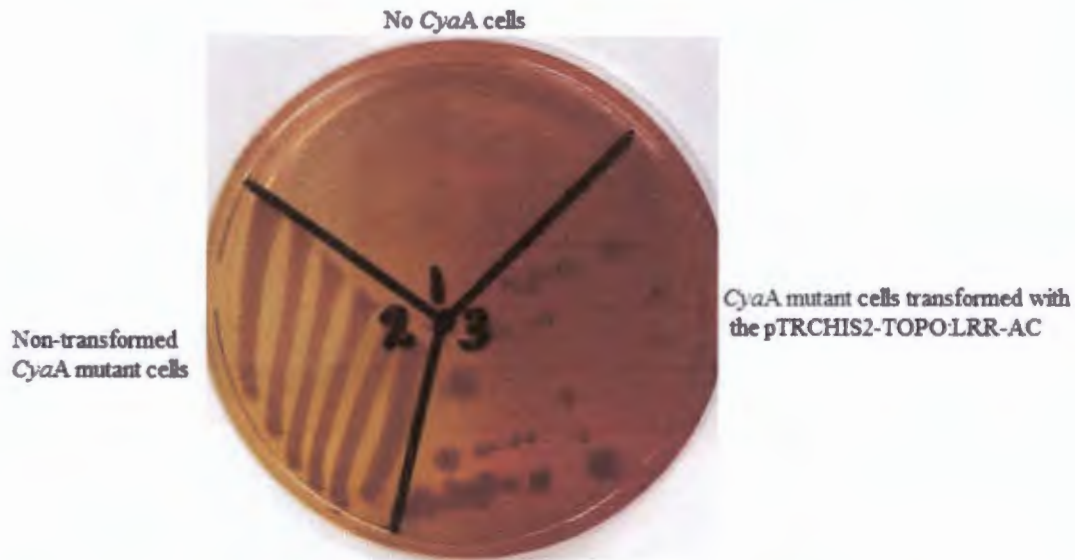


Figure 3.6: Different *cyaA* cells were plated onto a MacConkey agar plate supplemented with 15 $\mu\text{g/mL}$ kanamycin and 0.5 mM IPTG, and divided into three sections with a permanent marker and incubated for 4 days at 37°C. Section 1 of the plate contains no cells, section 2 contains the non-transformed *cyaA* mutant cells while section 3 contains the *cyaA* mutant cells transformed with the pTrcHis2-TOPO:LRR-AC fusion expression construct. Cells in sections 2 are non-lactose fermenters and produce yellowish colonies while cells in section 3 have picked a deep purple phenotype – a characteristic signifying the ability to ferment lactose.

3.7 Determination of the Anatomical Expression Profile of LRR

An anatomical expression analysis of the LRR protein shows that it is expressed differentially expressed in various tissues of the *A. thaliana* plant, and mostly in the pollen grains, (www.arabidopsis.org). However, the analysis also conversely indicates that the LRR protein is lowly expressed in the ovules.

Dataset: 105 anatomical parts from data selection: AT_AFTV_ATM1-0
Showing 1 measure(s) of 1 gene(s) on selection: AT-0

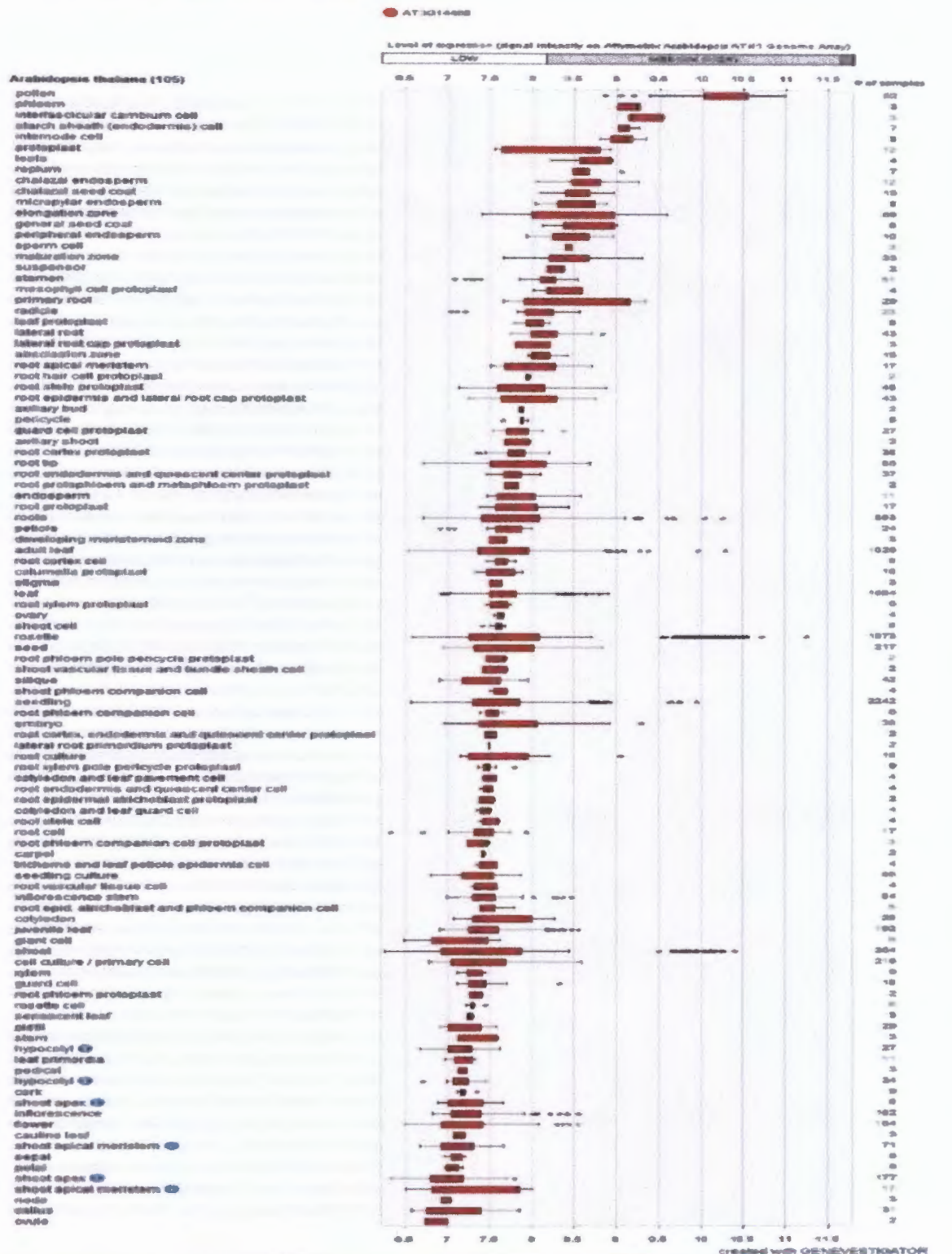


Figure 3.7: Expression intensity levels of the LRR AC protein in various tissues of the *A. thaliana* plant. The LRR protein shows that it is systematically expressed across the various parts of the plant but most expressed into the pollen grains and also least expressed in the ovules.

3.8 Determination of the Developmental Expression of LRR

Furthermore, to obtain a wide range of expression profile for the LRR protein, different stages of plant development were assessed using the development tool to screen the microarray data using At3g14460 as the search probe. The acquired data generated 10 developmental stages during which the LRR protein is differentially expressed and indicating that the protein is highly expressed when the plant is fully matured and least expressed during the 16-leaf rosette stage.

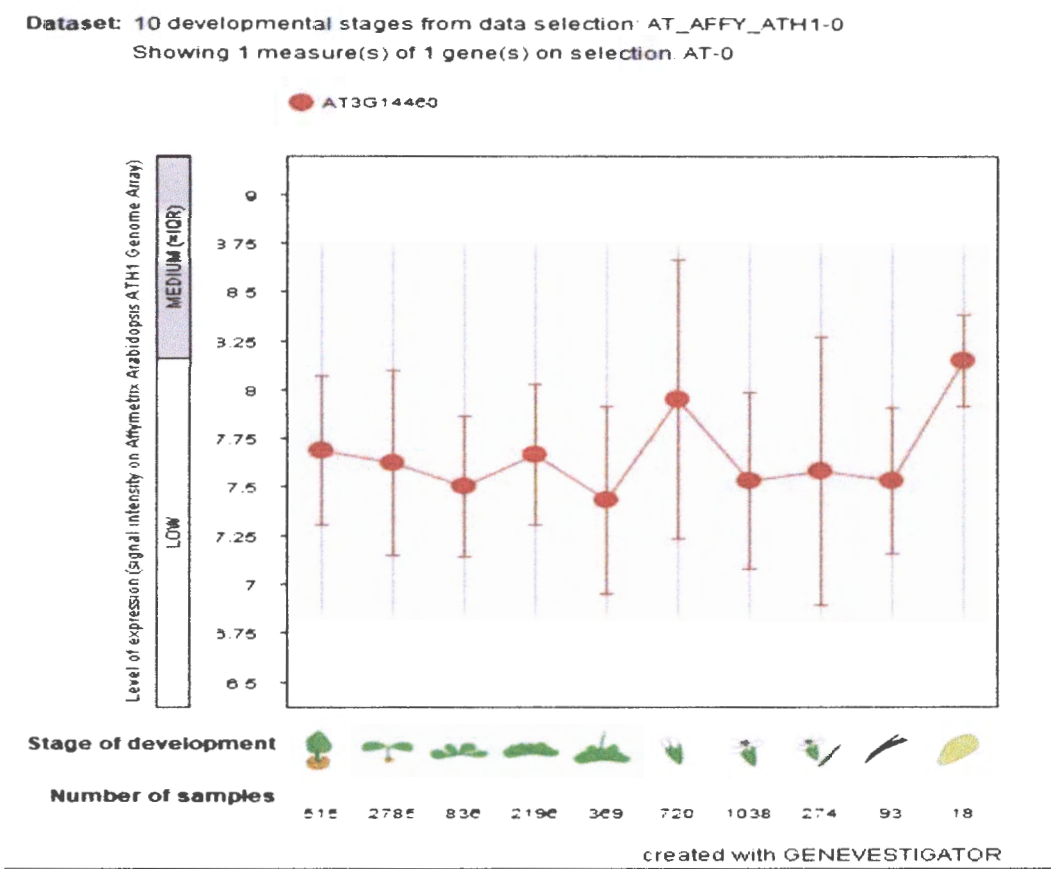


Figure 3.8: Expression profile of the LRR protein during the various developmental stages of the *A. thaliana* plant. Data indicates the different developmental stages from the time when the radicle develops from seed coat up to the time when mature seeds are release from the pod. The profile reflects that the LRR protein is most expressed during the matured stage of the plant and least expressed during the 16-leaf rosette stage.

3.9 Determination of the Co-Expression profile of LRR

In order to understand the expressional profile of the LRR gene in *Arabidopsis thaliana*, a desktop analysis of the publicly available microarray data was performed. Out of the 322 diverse transcriptome experiments, the LRR gene was found to be highly co-expressed with

numerous other genes in the Arabidopsis genome, with the top most 25 genes having a Pearson's correlation coefficient (*r*-value) of between 0.82 and 0.84 (Table 3.1 below). These 25 top most co-expressed genes (hereafter referred to as the LRR Expression Correlated Gene Group (*LRR: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 expressional analysis of their profiles.

Table 3.1: A list of top most 25 other known proteins that are co-expressed with the LRR protein in the *Arabidopsis thaliana*.

Rank	<i>r</i> value	Locus	Description or Annotation
1	0.84	At1g77890	DNA-directed RNA polymerase II protein
2	0.84	At1g03190	Protein binding
3	0.84	At5g01490	Cation exchanger 4
4	0.84	At2g31990	Exostosin family protein
5	0.84	At3g07600	Heavy metal transport/detoxification superfamily protein
6	0.84	At1g70170	Matrix Metalloproteinase
7	0.84	At3g05930	Germin-like protein (GLP8)
8	0.84	At5g25940	Early nodulin-like protein
9	0.83	At3g08870	Concanavalin A-like lectin protein kinase family protein
10	0.83	At1g24140	Matrixin family protein
11	0.83	At3g51180	Zinc finger C-x8-C-x5-C-x3-H type family protein
12	0.83	At4g04960	L-type lectin receptor kinase vii.1
13	0.83	At4g25070	Caldesmon-like protein
14	0.83	At4g12120	Protein transporter
15	0.83	At1g61560	Mildew resistance locus O 6 protein
16	0.83	At3g26910	Hydroxyproline-rich glycoprotein family protein
17	0.83	At1g51920	Transmembrane protein
18	0.83	At3g09010	Protein kinase superfamily protein
19	0.83	At5g11290	Transmembrane protein, putative (DUF247)
20	0.83	At5g44990	Glutathione S-transferase family protein
21	0.83	At2g17750	NEP1-interacting protein
22	0.82	At2g23770	Encodes a putative LysM-containing receptor-like kinase
23	0.82	At1g72950	Disease resistance protein (TIR-NBS class)
24	0.82	At5g61010	Exocyst subunit EXO70 family protein E2
25	0.82	At5g67350	Hypothetical protein

3.10 Determination of the Stimulus-specific Expression of LRR

After establishing the co-expressional profile of the *LRR* with the *ECGG-25*, this gene set (*LRR:ECGG-25*) 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-expressional analysis, the heat maps generated from this microarray expressional analysis revealed that the transcriptional processes of both the *LRR* and its associated *ECGG-25* gene complement are generally and collectively induced in response to a wide range of biotic and abiotic stress factors (Figure 3.9 below).

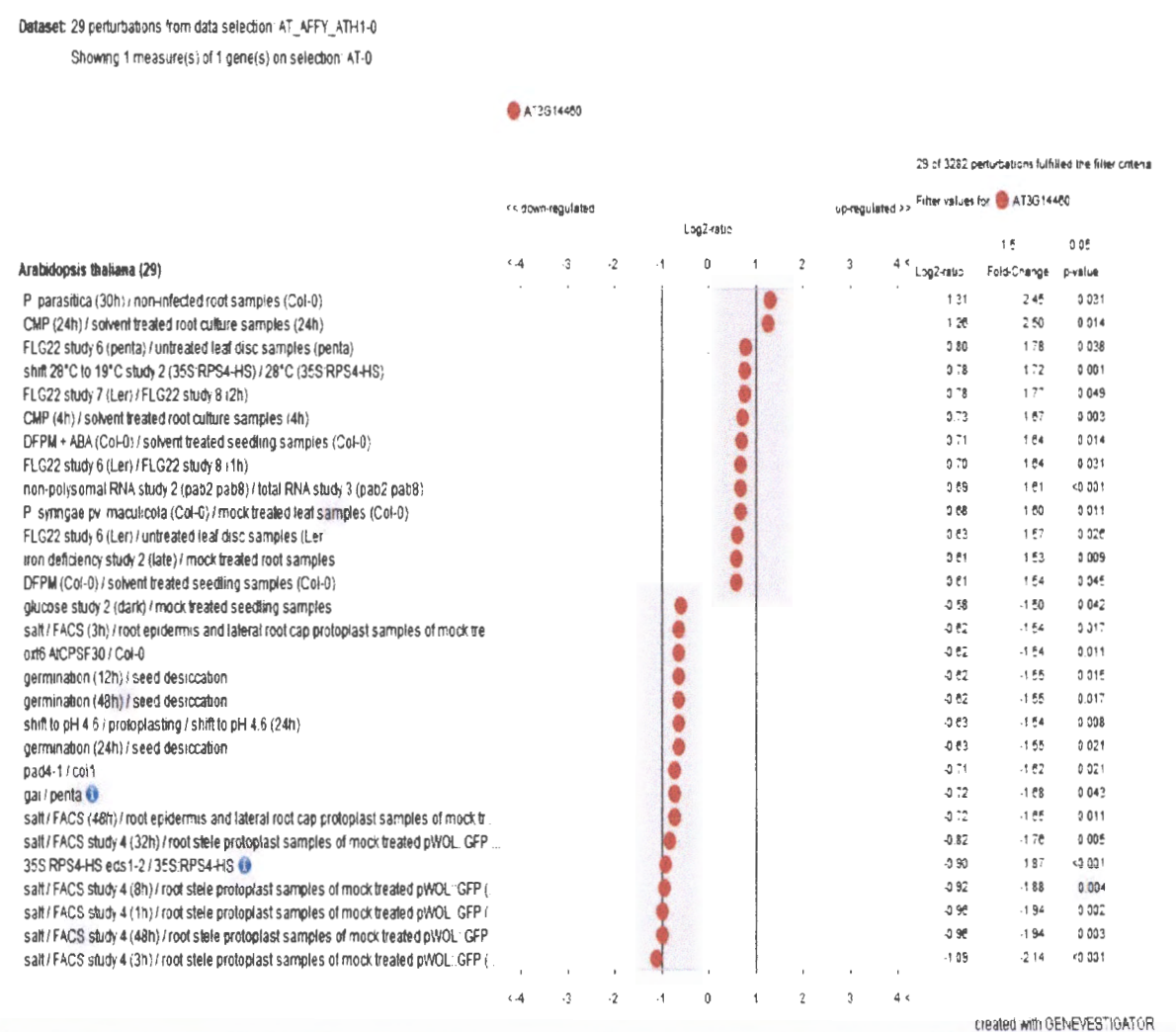


Figure 3.9: Expression profile of the LRR protein in *Arabidopsis thaliana* in response to a wide range of stimulus-specific conditions. The profile shows that the protein is strongly induced in response to a wide range of biotic and abiotic factors. Some of those factors include *Phytophthora parasitica* and *Pseudomonas syringae* (biotic) and salinity and high temperature (abiotic).

CHAPTER 4

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

4.1 Discussion

Biological components such as cAMP and cGMP are very vital signalling molecules in higher plants (Martinez-Atienza *et al.*, 2007). Naturally, plants are always faced with different environmental challenges, which may be abiotic and/or biotic stress factors, because they are sessile, resulting in decreases in food productivity as has already been outlined in Chapter 1 (Alqurashi, 2013). Recently, the improvement of stress response in plant crops has been made through the discovery of complex response molecules such as the (ACs). These are enzymes capable of catalysing the conversion of ATP into cAMP (Gehring, 2010). The produced cAMP would in turn regulate various cellular signal transduction pathways, including the activity of kinases (Robison *et al.*, 1968). Apparently, the knowledge of cAMP in plants unlike in eukaryotes, has been very low though the understanding of the importance of cAMP signalling in plants is being put in place (Thomas *et al.*, 2013). Previously, the establishment of cAMP and its associated signalling systems in higher plants has been very difficult because of the inherent difficulty to detect this molecule in plants (<20 pmol/g fresh weight) as compared to animals (>250 pmol/g wet weight). More so, this was also because of the vagaries of assaying systems used in plants, which were not conducive to attest firm conclusion (Wong, 2013).

As much as the identification of ACs and its resultant product, cAMP, has been somewhat very elusive in plants, the first ever higher plant AC to be identified was the *Zea mays* pollen signalling protein (PSiP), capable of producing cAMP, which in turn acts as a second messenger with a role in the polarized growth of pollen tubes (Moutinho, 2001). Subsequently, the second AC molecule was also identified in the form of an *A. thaliana* pentatricopeptide repeat containing protein (AtPPR-AC) responsible for the restoration of male sterility and biogenesis of chloroplasts (Ruzvidzo *et al.*, 2013). Later on, the third AC in the form of a *Nicotiana benthamiana* adenylyl cyclase (NbAC), which participates in the tabtoxinine- β -lactam-induced cell death during the occurrence of wildfire diseases in *Nicotiana benthamiana* was also identified (Ito *et al.*, 2014). The fourth AC in the form of a novel *Hippeastrum hybridum* adenylyl cyclase protein (HpAC1), involved in plant stress

signalling, was also identified (Swiezawska *et al.*, 2014). The next plant AC to be identified was the *A. thaliana* K⁺-uptake permease 7 (AtKUP7), capable of restoring an AC deficient *cyaA* mutation in the SP850 *E. coli* mutant host strain and converting ATP into cAMP *in vitro* (Al-Younis *et al.*, 2015). The last candidate to be identified is the *A. thaliana* clathrin assembly protein (AtCIAP) with a role in pathogen defense and actin cytoskeletal remodeling during endocytic internalization (Chatukuta *et al.*, 2018). Therefore, in an effort to try and identify yet another additional higher plant AC and possibly further elucidating on its probable functional roles in plant stress responses, this study targeted an LRR protein coded for by the At3g14460 gene in *A. thaliana* (<http://www.arabidopsis.org>; <http://www.expasy.org>). This protein is believed to be a plant disease resistance protein (Leach and Shah, 2002) and has also recently been annotated to contain a putative AC catalytic center (Gehring, 2010), even though its exact functional activity and/or role has not yet been experimentally determined. Thus, in an effort to try and establish whether or not this protein is a *bona fide* AC, its truncated version harbouring the putative AC center (LRR-AC) was extensively studied.

To begin with, the amino acid sequences of the LRR protein were first retrieved from the TAIR website followed by designing and acquisition of sequence-specific primers for the targeted and desired LRR-AC gene fragment (Figure 2.1). Alongside that, *A. thaliana* plants were generated and grown until they were mature (Figure 3.1), followed by isolation of the total RNA. After that, both the extracted total RNA and the designed sequence-specific primers of the LRR gene fragment were simultaneously used to amplify the targeted LRR-AC gene fragment using RT-PCR. As is shown in Figure 3.2, the targeted and desired amplicon of approximately 666 bp was amplified, signifying the successful isolation. The amplified fragment was then cloned into a pTrcHis2-TOPO vector to produce a pTrcHis2-TOPO:LRR-AC construct. The resultant construct was then subsequently screened to ensure that the cloned gene fragment was correctly orientated in the vector. This confirmation process was essential because the pTrcHis2-TOPO vector is a linear molecule with unidirectional 5'-T overhangs, and therefore it was crucial to ensure that ligation was successful. Firstly, colony PCR on cells transformed with this pTrcHis2-TOPO:LRR-AC construct was performed using the LRR-AC sequence-specific primers so as to check if the desired LRR-AC gene fragment was indeed present in the construct. As is shown in Figure 3.3, the targeted LRR-AC gene fragment was successfully re-amplified. Secondly, the pTrcHis2-TOPO:LRR-AC construct was used as a template with the vector forward and the

LRR-AC reverse primers so as to check and confirm the correct in-frame of the ligated insert. As is shown in Figure 3.4, this PCR setting managed to re-amplify the LRR-AC gene fragment and thus confirming its correct in-frame orientation in the pTrcHis2-TOPO vector. Otherwise if the orientation was not correct, then any amplification with this set of primers was not going to be achieved. More so, the yielded gene fragment was this time around slightly larger than the original LRR-AC band (amplified by its own primers) because part of the pTrcHis2-TOPO vector was also included. As an optional check, the confirmed pTrcHis2-TOPO:LRR-AC construct was also sent over to the Inqaba Biotechnological Sciences (Pretoria, RSA) for chemical sequencing, and validation (results not shown).

The successfully generated pTrcHis2-TOPO:LRR-AC construct was then taken into *E. coli* One Shot TOPO 10 cells (transformation), for expression of the LRR protein. As is shown in Figure 3.5, a recombinant protein product of approximately 24.420 kDa (which is the expected size) was expressed, signifying the successful expression of the desired and targeted LRR-AC protein. After expression, the confirmed pTrcHis2-TOPO:LRR-AC construct was then used to transform competent mutant *cyaA E. coli* cells followed by their growth on a differential MacConkey agar, and their phenotypes assessed and compared to the non-transformed mutant *cyaA E. coli* cells (controls). As is shown in Figure 3.6, all the untransformed cells lacking the vector construct (controls) appeared yellowish due to their inability to ferment lactose while all those cells carrying the vector constructs appeared deep reddish. This signified that the transformed cells had been functionally rescued back into wild type forms by the LRR-AC protein, which in this case, was capable of generating the most needed cAMP for the fermentation of lactose sugar by the rescued cells. In this regard, this outcome therefore conceivably confirmed that the LRR-AC protein was indeed a *bona fide* functional AC. Incidentally, this outcome also positively and directly concurs with findings from other previous and related experiments by Moutinho *et al.* (2001), Ruzvidzo *et al.* (2013), Swiezawska *et al.* (2014), Al-Younis *et al.* (2015), and Chatukuta *et al.* (2018); and thereby establishing the LRR-AC protein as the sixth ever higher plant AC to be identified via this biochemical approach.

After confirmation of the AC catalytic activity of the recombinant LRR-AC protein, bioinformatics (a wide range of web-based software's computer-based programs) was used to further analyze the probable functionality of the full-length LRR protein in *A. thaliana* and other related flowering plants. The anatomical expression analysis of the LRR protein was used to determine the expression levels of the LRR gene in different tissues of the *A. thaliana*

plant, whereas the developmental tool was used to determine the expression levels of the LRR gene during the different stages of development in the same plant. From the generated anatomical data, it was shown that the LRR protein is highly expressed in the pollen, phloem and interfascicular cambium tissues but at the same time, least expressed in the ovule, node and shoot apical meristematic tissues (Figure 3.7). On the other hand, the anatomical data showed that of the 10 derived developmental stages (seed germination to production of mature seeds), the LRR is highly expressed when the plant is fully matured and least expressed during the 16-leaf rosette stage (Figure 3.8).

Furthermore, the co-expressional analysis tool (Zimmermann *et al.*, 2004) was used to determine those other proteins whose expression is closely related to that of the LRR in *A. thaliana*. In this regard, the LRR protein was found to be highly co-expressed with numerous other proteins in the Arabidopsis genome, with the top most 25 proteins having a Pearson's correlation coefficient (r-value) of between 0.82 and 0.84 (Table 3.1). A number of such co-expressed proteins were also found to be annotated as disease resistance proteins, whose function is essentially controlled and mediated by the second messenger cAMP, and thus implying LRR as a disease resistance candidate. When the stimulus-specific microarray expression profiling of the LRR protein was undertaken, the outcomes shows that the protein is strongly induced by both the abiotic and biotic stress factors (Figure 3.9). The profile specifically showed that the protein is strongly induced by environmental factors such as iron deficiency and high temperatures (abiotic), and bacterial pathogens that include *Phytophthora parasitica* and *Pseudomonas syringae* (biotic).

Meanwhile, by considering the fact that both the abiotic and biotic stress responses are natural plant processes essentially regulated by cAMP (Kieber *et al.*, 1993); yet the cloned LRR-AC could demonstrate a functional AC activity (Figure 3.6) while at the same time, it has been linked to disease resistance proteins (Table 3.1) and induced by both abiotic and biotic stress factors (Figure 3.9), it is thus conceivable to conclude that the LRR-AC protein is an important plant signalling molecule with a central role in responses to stress, and whose mechanism of action is principally mediated by the second messenger, cAMP.

4.2 Conclusion

Findings from this work practically confirm the LRR-AC protein from *A. thaliana* as a *bona fide* functional higher plant AC with a role in cAMP-mediated abiotic and biotic stress

responses. The LRR protein, therefore, becomes the seventh ever AC molecule to be identified in higher plants after the currently known *Z. mays* pollen protein (Moutinho *et al.*, 2001), *A. thaliana* pentatricopeptide protein (Ruzvidzo *et al.*, 2013), *N. benthamiana* adenylyl cyclase protein (Ito *et al.*, 2014), the *H. hybridum* adenylyl cyclase protein (Swiezawska *et al.*, 2014), *A. thaliana* K⁺-uptake permease 7 protein (Al-Younis *et al.*, 2015) and *A. thaliana* clathrin assembly protein (Chatukuta *et al.*, 2018).

4.3 Recommendations

1. Although this study has managed to successfully identify the seventh ever higher plant AC molecule in the form of the LRR protein from *A. thaliana*, however, the study was only based on the *in vivo* and bioinformatic assays. Therefore, a further biochemical characterization of this protein as a purified molecule needs to be undertaken in order to determine its exact mode of action and specifically, in relation to plant stress responses and adaptation mechanisms.
2. Additionally, further studies are also recommended to focus on the analysis of the other outstanding annotated Arabidopsis AC candidates in order to broaden the functional base and understanding of ACs in higher plants.

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