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Recombinant expression and functional characterization of a fusion F-box protein from *A. thaliana*

by

Grace Humbelani Mabadahanye

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Supervisor : Dr. Oziniel Ruzvidzo

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Declaration

I Mabadahanye Grace Humbelani, declare that the thesis entitled "**Recombinant expression and functional characterization of a fusion F-box protein from *A. thaliana***" submitted to the Faculty of Agriculture Science and Technology, Department of Biological Sciences at University of North West for Msc in Plant Biotechnology has never been submitted at this University or in any other institution elsewhere. This is my own work and all the sources used or quoted have been indicated and acknowledged.

Student: **Mabadahanye G.H**

G.H Mabadahanye ..... **Date:** 19/09/2013.....

Supervisor: **Dr. O. Ruzvidzo**

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

Dedication

I dedicate this research to Wanga, Yoanda, Dziedzi and in memory of my mother Sylvia for the courage and strength they gave me throughout this study. I also dedicate this research to the **Plant Biotechnology Research Group,** friends and all the people who encouraged me in my studies, May God richly bless them.

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Mudzimu a ni shudufhadze

Definitions of Terms

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

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

cAMP: A bio-molecular compound produced from ATP hydrolysis catalyzed by adenyl cyclases at the cell membrane. It is used for intracellular signal transduction, activation of protein kinases, and regulation of the passage of calcium ions through ion channels.

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

F-box proteins: Proteins containing at least one F-box domains.

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

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

PKA: Protein kinase A.

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

Reverse transcription polymerase chain reaction (RT-PCR): A molecular method used to amplify a short RNA segment into a DNA product termed copy DNA (cDNA) using an RNA-dependent DNA polymerase enzyme.

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

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

Abbreviations

AC: Adenylate cyclase

AFBs: Auxin signalling F-Box proteins

ANOVA: A one-way analysis of variance

AtCNGC: *A. thaliana* cyclic nucleotide-gated channel

ATP: 3′, 5′-Adenosine 5′-triphosphate

AUX: Auxin

BLAST: Basic local alignment searching tool

cAMP: Cyclic 3′, 5′-adenosine monophosphate

cGMP: Cyclic 3′, 5′-guanosine monophosphate

cDNA: Copy DNA

CMS: Cytoplasmic male sterility

CCR: Chlororespiratory reduction

EIA: Enzyme immunoassay

ETRs: Ethylene receptors

EIN2: Ethylene signalling protein

GC: Guanylate cyclase

GTP: 3′, 5′-guanosine 5′-triphosphate

HR: Hypersensitive response

IBMX: 3-Isobutyl-1-methyl xanthine

IAA: indole-3-acetic acid

IPTG: Isopropyl-β-D-thiogalactopyranoside

JA: jasmonic acid

kb: Kilo base

kDa: Kilo dalton

LRRs: Leucine-rich repeats

MSMO: Murashige and Skoog basal salt with minimum organics

Ni-NTA: Nickel-nitrilotriacetic acid

OD: Optical density

PBS: Phosphate buffered saline

PBST: Phosphate buffered saline + triton-X 100

PKA: Protein kinase A

RT-PCR: Reverse transcriptase polymerase chain reaction

SCF: F-box containing complex

SDS-PAGE: Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SNK: Student Newman Kuehls

STAND: Signal transduction ATPases with numerous domains

TAE: Tris Acetate +EDTA

TEMED: NNN'N'-Tetramethylethlenediamine

TIR1: Transport inhibitor response

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

UBC: ubiquitin-carrier enzyme

YT: Yeast-tryptone

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Abstract

Adenylate cyclases (ACs) are enzymes that are capable of converting adenine-5'-triphosphate (ATP) to cyclic 3′, 5′-adenosine monophosphate (cAMP). It has been found that cAMP has an important role in cell signalling and as a second messenger in animals, plants and lower eukaryotes. Cyclic 3′, 5′-adenosine monophosphate can affects many different physiological and biochemical processes such as the activities of kinases. However and up to date, the only annotated and experimentally confirmed AC in higher plants is the *Zea mays* pollen capable of generating cAMP and is involved in the growth of polarized pollen tubes. Recently, an F-box protein from *Arabidopsis thaliana* has been bioinformatically annotated as a possible higher plant AC because of its possession of a putative adenylate cyclase catalytic motif in its structural domain. In this study therefore, the main aim was to test and determine if the putative AC containing segment of the F-box gene has any functional AC activity and if so, to further explore if it has any physiological roles in plant cell signaling and transduction systems. Therefore, in order to attempt this aspect, putative AC containing segment of the F-box gene was cloned into a prokaryotic expression vector (pGex-6p2) and expressed in *E. coli* BL21 (DE3) pLysS cells. In order to demonstrate the biological functionality of the F-box's adenylate cyclase catalytic centre, the recombinant protein was tested for its ability to generate cAMP endogenously, in *vitro* and *in vivo*. Results from these three assays all indicated that the recombinant F-box-AC does possess functional adenylate cyclase activity.

Chapter 1: Introduction and Literature Review

1.1 Introduction

Food shortage is a major problem facing the whole world because most of the countries are highly susceptible to droughts and other harsh environmental conditions that render the farming land unproductive. This aspect in turn affects the biomass of cultivated crops yet each and every year there is a decrease in food supplies associated with a rapid increase in the size of the populations. This imbalance in the "demand and supply" dynamics has seen an unprecedented increase in food prices yet and moreover, most inhabitants of the African continent do rely on crops as their main food source in order to sustain themselves. With the present aspect of environmental stresses around Africa, crop farming is somewhat becoming increasingly unsustainable, and this strongly calls for more efficient and cost-effective methods to ensure food security for the current and future generations. 'There is therefore, an urgent need to use rational and integrated approaches to develop crop plants with increased stress tolerance and adaptation mechanisms. This urgent need has, in this regard, has led to an impressive body of work in the areas of plant genetics, plant physiology, plant biochemistry and plant molecular biology, and a realisation that only an integrated and systems-based approach could possibly deliver effective biotechnological solutions (Stuhmer *et al.*, 1989).

1.2 Problem Statement

While previous studies have associated the F-Box protein with plant cellular functions such as signal transduction and regulation of the cell cycle that in turn are linked to the modulation of cellular cAMP levels (Newton *et al.*, 1980; Newton *et al.*, 1986; Chen *et al.*, 2000), no particular study has yet directly characterized this protein as a possible functional plant AC. Besides, considering that the present genome-wide sequence analysis and previous evidence that strongly supports the idea that the complexity of F-box proteins in SCF-mediated ubiquitin proteolytic systems is highly conserved in plants, it also suggests a universal function of these proteins in plants (Dreher *et al.*, 2007). Thus the identification of a novel plant F-box protein with a functional AC activity should facilitate the investigation and further understanding of those signal transduction pathways and other cellular processes regulated by ubiquitin-mediated proteolysis and cAMP-dependent systems in plants and particularly, in the areas of stress response and adaptation mechanisms. Hence in this proposed research, the F-box protein was extensively studied in order to gain a better understanding of its physiological roles in plants.

1.3 Literature Review

1.3.1 *A. thaliana* as a Model Plant for Research

A. thaliana offers important advantages for basic research in genetics and molecular biology. *A. thaliana* is a member of the mustard (*Brassicaceae*) family, which includes cultivated species such as cabbage and radish. Although not of major agronomic significance, Arabidopsis offers important advantages for basic research in genetics and molecular biology (Rédei, 1992). In the laborotary, *A. thaliana* may be grown in petri plates or pots, under fluorescent lights or in a greenhouse (Meinke *et al.,* 1998). Although *A. thaliana* has little direct impact on agriculture, it has many traits that make it a useful model for understanding the genetic, cellular, and molecular biology of flowering plants. Having specialized as a spring ephemeral, it has been used to find several laboratory strains that take about six weeks from germination to maturity. The small size of the plant is convenient for cultivation in a small space and it produces many seeds. Further, the selfing nature of this plant assists genetic experiments. Also, as an individual plant, it can produce plentiful seeds, and each of the above criteria therefore lead to *A. thaliana* being valued as a genetic model organism.

1.3.2. Adenylyl Cyclases in Higher Plants

In early 1970s, the molecule 3'-5'-cyclic adenosine monophosphate (cAMP) had been firmly established as an important signaling chemical and a second messenger in both animals and lower eukaryotes (Goodman *et al.*, 1970; Gerisch *et al.*, 1975; Wiegant, 1978). Adenylate cylases (ACs) are enzyme that synthesizes cyclic adenosine monophosphate or cyclic AMP from adenosine triphosphate (ATP). cAMP is an important signalling molecule in prokaryotes and eukaryotes, but its significance in higher plants has been generally doubted because they have low adenylyl cyclase activity. Cyclic AMP functions as a "second messenger" to relay extracellular signals to intracellular effectors, particularly protein kinase A. A major target of cAMP is protein kinase A (PKA) which is a key player for the activation of several other proteins. PKA and other cAMP-dependent proteins phosphorylate down-stream targets which consequently results in an alteration of metabolism, signal transduction, differentiation, memory or apoptosis (Simpson *et al.*, 1996). Regulation of intracellular concentrations of cyclic AMP is largely as a result in controlled adenylyl cyclases. Given the growing realization of the importance of ACs and cAMP, it is not surprising that plant scientists were also interested in learning if this signaling system was universal and therefore operating in plants too. There are two reasons why the information on AC and cAMP levels in plants are scarce in the literature as opposed to information available for animals and lower eukaryotes. Firstly, the levels of the cAMP detected in plants appear to be very low (< 20 pmol/g fresh weight) (Ashton *et al.*, 1978) compared to those found in animals (> 250 pmol/g wet weight) (Butcher *et al.*, 1968) and secondly, that the vagaries of assays conducted in plants were not conducive to reach firm conclusions (Amrhein *et al.*, 1977). However, the fact that signaling in plants at lower molecular levels is feasible is not uncommon because incidentally, low levels of another cyclic nucleotide, cGMP (< 0.4 pmol/g fresh weight), were also reported in plants where the molecule has a

physiological role in specific responses to virulent pathogens and defense mechanisms (Meier *et al.*, 2009). In addition, the availability of more advanced analytical tools has dramatically enhanced the assaying systems in plants and the affirmation of solid conclusions.

In *A. thaliana*, the functional and structural domains of guanylate cyclases have been experimentally identified and for this reason we may expect that a similar approach might lead to the discovery of novel ACs (Gehring, 2010). To date, the only annotated and experimentally confirmed AC in plants is a *Zea mays* pollen protein capable of generating cAMP, which in turn is a second messenger with a role in polarized pollen tube growth. Cyclic adenosine monophosphate (cAMP) is widely distributed from prokaryotes to eukaryotes as a signal molecule (Moutinho *et al.*, 2001). In most of these organisms, the presence of cAMP can be well-defined in various physiological processes. In animal cells, cAMP functions as a second messenger in cellular sign). The increase of cAMP level in cells promotes the activation of protein kinase A, the phosphorylation of several intracellular enzymes and this phosphorylation results in increased enzymatic activities. In contrast to the well-documented situation in the animal kingdom, the presence of cAMP in higher plants and its physiological role in signal transduction are rare. Only in the last decade new evidence has created new momentum in this field. The existence of cAMP in plants has been established (Newton *et al.*, 1980; Newton *et al.*, 1986) and moreover, its synthesis by adenylyl cyclase in lower plants is widely accepted while the existence of adenylyl cyclase activities in higher plants is growing (Franco *et al.*, 1980; Katayama *et al.*, 1995; Yashiro *et al.*, 1996). Furthermore, the existence of ACs and their activities in higher plants has been reported as was demonstrated in plants such as *Phaseolus vulgaris, Medicago sativa, Risinus communis, Pisum sativum* by several physiological and biochemical experiments (Carricarte *et al.*, 1988; Lusini *et al.*, 1991; Pacini *et al* 1993; Cooke *et al.*, 1994; Roef *et al.*, 1996). Light regulated

adenylyl cyclase have been shown to be present in *Zea mays* and *Sorghum bicolor* (Fedenko *et al.*, 1983; Gasumov *et al.*, 1997) and the genes of light-regulated adenylyl cyclase were isolated and characterised from *Cyanobacteria* (Katayama *et al.*, 1995).

One of the physiological effects of cAMP is its participation in both lower and higher plant signal transduction processes. It was shown that red and far red lights absorbed by phytochromes regulate the activity of cAMP related enzymes in maize seedlings and the red illumination of etiolated seedlings results in increased cAMP levels in cells (Fedenko, 1988; Fedenko *et al.*1983). In a nitrogen-fixing cyanobacterium *Anabaena cylindirica*, an immediate light-to-dark transition caused a 9-fold increase in cAMP concentration within 1 minute (Ohmori 1989). Yashiro *et al.*, (1996) has shown the existence of light regulated membrane and soluble fractions of adenylyl cyclase in *Spirulina platensus*. The report also discusses the presence of membrane and soluble forms of light regulated adenylyl cyclase in sorgum plants. Both fractions were considered to participate in plant photosignal transduction processes that synthesize AMP from ATP.

In addition, Gehring (2010) has also recently reported the identification of nine putative AC genes in the Arabidopsis genome using a search motif consisting of functionally assigned amino acids in the catalytic centre of annotated and/or experimentally tested nucleotide cyclases (Gehring, 2010). Among them is an F-box gene (At3g28223) that encodes for an F-box protein (Table 1.1).

ATG No.	Sequence	Annotation
At1g25240	-KWEIFEDDFCFTCKDIKE-	Epsin N-terminal homology
At1g62590	-KFDVVISLGEKMQR--LE-	Pentatricopeptide (PPR) protein
At1g68110	-KWEIFEDDYRCFDR--KD-	Epsin N-terminal homology
At2g34780	-KFEIVRARNEELKK-EME-	Maternal effect embryo arrest 22
At3g02930	-KFEVVEAGIEAVQR--KE-	Chloroplast protein
At3g04220	-KYDVFPSFRGEDVR--KD-	TIR-NBS-LRR class
At3g18035	-KFDIFQEKVKEIVKVLKD-	Linker histone-like protein-HNO4
*At3g28223	-KWEIVSEISPACIKSGLD-	F-box protein
At4g39756	-KWDVVASSFMIERK--CE-	F-box protein

Table 1.1 The nine bioinformaticall identified AC genes. *A. thaliana* proteins containing the AC search motif [RK][YFW][DE][VIL][FV]X(8)[KR]X(1,3)[DE] (Adapted from [Gehring,2010]).

ATG represents the assigned *A. thaliana* gene bank numbers for the nine genes, followed by nucleotide sequences suspected to be their adenylate cyclase catalytic sites, and the names to which each gene was bioinformatically inferred (annotations).

*The gene for the F-box protein to be functionally characterized in this project.

1.3.3 F-box protein

1.3.3.1 At3g28223

At3g28223 is one of the nine bioinformatically identified plant adenylate cyclases (Gehring, 2010), and this gene belongs to the F-box family protein; and it has not been extensively characterised (the molecular function, biological process and the cellular component of this gene are still unknown). The At3g28223 contains interpro domains: F-box domain, cyclinin-like (interpro: IPR001810), F-box domain, Skp2-Like (Interpro: IPR022364) (http://www.arabidopsis.org).

1.3.3.2 Classification of F-box Proteins

The F-box was firstly observed as a region of homology between the proteins Cdc4, β-TrCP, Met30, Scon2, and MD6, all of which contain WD (Trp-Asp) repeats (Kumar *et al.*, 1995). Another F-box with WD40 repeats, β-TrCP plays a role in both developmental and inflammatory signaling pathways (Maniatis, 1999). The implications of the homology were not appreciated until in 1996 when Bai *et al.*, recognized that the F-box is a widespread motif that is required for protein-protein interaction. The name F-box was identified by Bai *et al.*, 1996 on the basis of the existence of the motif in cyclin F. The F-box motif itself is generally found in the amino-terminal half of proteins and is often coupled with other motifs in the carboxy-terminal part of the protein, the two most common of which in humans are the leucine-rich repeats (LRRs) and the WD repeats.

The nomenclature for human F-box proteins proposed by the Human Genome Organization follows the pattern proposed by Cenciarelli *et al.*, 1999 which denotes FBXL to a protein containing an F-box and LRRs; FBXW to a protein with an F-box and WD repeats; and FBXO to a protein with an F-box and either another or no other motif. F-box proteins are proteins containing a minimum of one F-box motif and its structural domain is of about 50 amino acids that mediate protein-protein interactions. The protein was first known in cyclin F and is one of the three components of the Skp, Cullin, F-box containing complex (SCF complex) (Craig *et al.*, 1999), which mediates ubiquitination of proteins targeted for degradation by the proteasome (Jones-Rhoades *et al.*, 2006). The F-box motif interacts directly with the SCF protein Skp1 and its domains commonly exist in proteins that are in concert with other protein-protein interaction motifs such as the LRR (leucine-rich repeats) and the WD repeats or beta-transducin repeats, which are thought to mediate interactions with the SCF substrates (Kumar *et al.*, 1995).

1.3.3.3 Proteasomal Degradation, Ubiquitination and Function of F-box Protein

Selective proteolysis of proteins has been known as a very important mechanism for regulating many cellular events (Hershko *et al.*, 1998; Zheng *et al.*, 2002). A major pathway for controlled protein destruction is the ubiquitin-mediated proteolysis by the proteasome (Hershko *et al.*, 1998; del Pozo *et al.*, 2000). Ubiquitination of target proteins is carried out over three enzymatic reactions. First, the ubiquitin moiety is activated by the ubiquitin activating enzyme (also known as E1) and forms a thiol-ester bond with the carboxy-terminal glycine of ubiquitin in an ATP-dependent process, then transferred to the ubiquitin conjugating enzyme or ubiquitin-carrier enzyme (UBC, also known as E2), which accepts the ubiquitin from the E1 by a *trans*-thiolation reaction that again involves the glycine at the carboxy-terminus of ubiquitin, and finally, the moiety is bound to substrate proteins by the ubiquitin-protein ligase (E3), that catalyses the transfer of ubiquitin from the E2 enzyme to the amino group of a lysine residue on the substrate (Glickman *et al.*,2002).

Both E1 and E2 are less specific than E3 (Patton *et al.*, 1998). The SKP1, cullin/CDC53, F-box protein (SCF) complexes are the largest and best studied family of E3 ubiquitin-protein ligases and are known to control cell cycle regulation, signal transduction, transcription, and other biological events (Bai *et al.*, 1996; Hershko *et al.*, 1998; Schulman *et al.*, 2000; Zheng *et al.*, 2002). E3 ubiquitin ligases have been shown to play a role in plant growth and development, including phytohormone-signal pathways, of candidates such as Auxin, GA and Ethylene (Moon *et al.*, 2004; Smalle *et al.*, 2004). Among the subunits of the SCF complex, SKP1 acts as an adapter that links cullin to one of the F-box proteins, which are highly variable (Willems *et al.*,1999; Schulman *et al.*, 2000; Zheng *et al.*, 2002). Current studies propose that plants make extensive use of SCF complexes to regulate multiple biological processes (Gagne *et al.*, 2002; Risseeuw *et al* 2003).

Some SCF complexes have been characterized as F-box proteins that contain a conserved domain of about 40 amino acid residues identified firstly in cyclin F at the N-terminal region (F-box domain) and in many cases, they have several protein–protein interaction domains at the downstream of F-box domain that confers the substrate specificity for ubiquitination. Over the past few years, dozens of F-box proteins have been identified in many eukaryotes and many reported F-box proteins have been identified as SCF components. Recently, F-box proteins have been discovered to function in non-SCF complexes or possess enzyme activity. FOG-2, which was a *Caenorhabditis elegans* F-box protein, has been suggested to function as a bridge, bringing GLD-1 that is bound to tra-2mRNA into a multi-protein translational repression complex (Clifford *et al.*, 2000).

In plants, many F-box proteins are represented in gene networks broadly regulated by microRNA-mediated gene silencing via RNA interference RNA (Jones-Rhoades *et al.*, 2006). Many F-box proteins have been found to be involved in plant hormone responses as receptors or important medial components. The proteins have also been associated with cellular functions such as signal transduction and regulation of the cell cycle that in turn is linked to both auxin responses and changes in cellular cAMP content (Ehsan *et al.*, 1998; Leyser, 1998). These findings shed more light on our current understanding of the structure and function of the various F-box proteins, their related plant hormonal signaling pathways and their roles in regulating plant development.

1.3.3.4 Roles of F-box Proteins in Plant Hormone Responses

F-box proteins have been involved in the direct perception or early signal transduction of several phytohormones, including auxin, jasmonic acid and gibberellin (Davies *et al.*, 2004). Therefore, both auxin and JA directly bind to F-box proteins and regulate their ability to bind to their substrates, which are repressors of the respective hormone-induced genes. In the case

of the signaling pathway for the hormone gibberellic acid (GA), the regulation is slightly more complex. GA response also involves transcriptional activation and requires GA-induced degradation of a class of DELLA proteins, which repress GA-activated genes (Esther *et al.*, 2008). For each of these hormones, the F-box protein targets negative regulators of transcription factors that carry out the hormone responses. In the case of auxin, association of the F-box protein TIR1 with an Aux/IAA negative regulator is promoted by auxin-binding in a TIR1 pocket, a non-allosteric interaction in which the ligand acts as "molecular glue". The plant hormone auxin (indole-3-acetic acid or IAA) regulates plant development by inducing rapid cellular responses and changes in gene expression. Auxin promotes the degradation of Aux/IAA transcriptional repressors, thereby allowing auxin response factors (ARFs) to activate the transcription of auxin-responsive genes. Auxin enhances binding of Aux/IAA proteins to the receptor TIR1, which is an F-box protein that is part of the E3 ubiquitin ligase complex SCF^{TIR1}. Binding of Aux/IAA proteins leads to degradation via the 26S proteasome, but evidence for SCF^{TIR1}-mediated poly-ubiquitination of Aux/IAA proteins is lacking.

Other F-box proteins also have been found to control the degradation of key components in other hormonal pathways. In the auxin signalling pathway, a small family of F-box proteins, TIR1 (Transport Inhibitor Response 1) and its paralogs, AFBs (Auxin signalling F-Box proteins), facilitates ubiquitination of key transcription repressors, the AUX/ IAA proteins, in an auxin-dependent manner (Dharmasiri *et al.*, 2005; Kepinski *et al.*,2005; Tan *et al.*, 2007), causing their rapid degradation by the proteasome. This releases the inhibitory effect of AUX/IAA on the auxin response factors (ARFs) to promote the expression of auxin-responsive genes (Leyser *et al.*, 1993; Abel *et al.*, 1994; Gray *et al.*, 2001; Guilfoyle *et al.*, 2007).

1.3.3.5 F-box Protein Regulate Ethylene Signalling in Plants

Ethylene regulates many aspects of plant development and physiology which include seedling development, leaf and flower senescence, floral sex determination, fruit ripening, roots hair development, and abiotic and biotic stress responses (Kendrick *et al.*, 2008; Zhu *et al.*, 2008). In cucumber and other members of the melon family, male and female flowers develop from the same immature flowers with primal of both male and female reproductive organs, the stamens and pistil. Under the influence of ethylene, bisexual immature cucumber flowers undergo programmed cell death in the stamens, resulting in the formation of female flowers with a functional mature pistil, whereas male flowers form in the absence of ethylene (Trebitsh *et al.*, 1997; Hao *et al.*, 2003).

The stability of the ethylene signalling protein EIN2 is modulated by two F-box proteins. In a study that involved an investigation into the stability of the EIN2 protein and also to search for proteins that interact with it using its conserved C-terminal region, and when it is found that there is an EIN2-interacting protein (ETP1) that contains an F-box domain. In addition, it was also shown that a paralog of ETP1, ETP2, also can interact with EIN2. A number of F-box proteins are known to be components of protein ubiquitin ligases called SCF complexes and are important for specifying specific substrates (Qiao *et al.*, 2009). This suggests that ETP1 and ETP2 might facilitate the ubiquitination of EIN2, thereby regulating its degradation by the proteasome. To test this idea, Qiao *et al.* (2009) used plants that had altered the functions of ETP1 and ETP2 and found that EIN2 accumulated to abnormally high levels in plants with reduced ETP1/2 function and was hardly detectable in plants with elevated ETP1/2 function. Moreover, overexpression of ETP1 or ETP2 resulted in a decrease in response to ethylene, whereas reduced ETP1/2 function caused changes in plant morphology and gene expression similar to those induced by ethylene treatment. They also

showed that ethylene could induce a reduction of ETP1 and ETP2 protein levels, but had no detectable effect on ETP1/2 mRNA, suggesting that ETP1/2 themselves are regulated post-transcriptionally.

In short, (Qiao *et al.*, 2009) provided strong genetic and biochemical evidence that the F-box proteins ETP1/2 are significant for modulating ethylene signalling, and that they likely regulate EIN2 protein levels by facilitating its ubiquitination. EIN2 is not the first key mediator of ethylene signalling that is regulated at the level of protein stability and ETP1/2 are not the first F-box proteins found to be important for this process. Previous studies showed that the level of the EIN3 protein, but not EIN3 mRNA, increased following ethylene treatment, indicating that ethylene can stabilize the EIN3 protein. In addition, inhibition of proteasome leads to the accumulation of EIN3, suggesting that EIN3 is degraded by the proteasome. Using the yeast two hybrid methods, two EIN3-binding proteins (EBF1 and EBF2) were identified and found to be F-box proteins. Additionally, EBF1 and EBF2 can cooperate with putative SCF subunits ASKs, suggesting that EBF1 and EBF2 are indeed components of SCF complexes. Mutations in EBF1 and EBF2 lead to accumulation of the EIN3 protein and enhanced ethylene responses (Guo *et al.*, 2003; Potuschak *et al.*, 2003; Gagne *et al.*, 2004).

1.3 Aim and Objectives

1.3.1 Research Aim

1. The main aim is to empirically establish the presence of F-box protein as an AC in higher plants and test for enzymatic activities.
2. To determine if these molecules have any physiological roles in cell signalling, particularly in biotic, abiotic, environmental stress response and adaptation mechanisms.

1.3.2 Objectives

1. To isolate and clone an *A. thaliana* gene (At3g28223) which has been annotated as a F-box gene and contains an putative AC motif.
2. To optimize the expression of the cloned gene and purification of the expressed recombinant F-box protein.
3. To determine the biological/enzymatic activity of the purified recombinant F-box protein and determination of its endogenous AC activity by enzyme-immunoassay.
4. To further functionally characterize the expressed recombinant F-box protein with the intention of establishing its exact physiological roles in plants, particularly in biotic and abiotic environmental stress response and adaptation mechanisms.

1.4 Significance of the Research Project

1. The complete functional characterization of the annotated F-box protein will strongly contribute towards a better understanding of the general mechanisms by which plants respond and adapt to stressful environmental conditions.
2. This understanding would advance our scientific knowledge on plant genes responsible for environmental stress responses and adaptation mechanisms.
3. Further, this understanding also broadens our knowledge of the developments through which environmental stress affects plants.
4. The project may potentially contribute towards the integrated management of both biotic and abiotic stressful conditions of agronomically important crops in South Africa.
5. Upon completion of its functional characterization, the annotated F-box genes may possibly 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: Isolation and Molecular Cloning of F-Box Protein

2.1: Plant Generations and Growth Conditions

2.1.1 Sterilisation of Seeds

A volume of 50 µL equivalence of *A. thaliana* ecotype Columbia seeds were washed with 500 µL of 70% ethanol through vigorous shaking on a vortex mixer for 30 seconds in a sterile 1.5 ml Eppendorf tube. Seeds were then allowed to settle through gravity and the supernatant was discarded as waste. The seeds were sterilised with 500 µL sterilization buffer (0.1% Sodium dodecyl sulfate, 5% commercial bleach) through vigorous shaking on a vortex mixer for 1 minute. After the sterilisation step, the seeds were washed five times in a 1.5 mL Epperndorf tube with 1 mL sterile distilled water by vortexing for 30 seconds and then allowed to settle through gravity before removing the supernatant as waste. Seeds were finally vernalized by storing them at 4°C for three days.

2.1.2 Germination of *A. thaliana*

After vernalization, the seeds were germinated on petri dishes containing Murashige and Skoog (MS) medium (0.4% of organic salts, 3% sucrose and 8% of type 'M' agar, pH of 5.7). The germination medium was always sterilized by autoclaving at 120°C for 20 minutes before plates were poured and solidified for further use. The petri dishes were sealed with parafilm and incubated in an labcon growth chamber (LTGC20, Labex, SA). After 18 days, the seedlings were transplanted to soil composed of 50% peat-based soil and 50% vermiculite, supplemented with the fungicide Gaucho and the seedlings were then allowed to grow for 2-4 weeks under greenhouse conditions. Growth chamber conditions for the germinating seeds were always kept at short and equal day and night lengths of 12 hours each

to promote vegetative growth while all the subsequently generated seedlings were later grown at longer days and short nights of 16 and 8 hours respectively. Temperature was always maintained at 25°C.

2.2 Designing and Acquisition of Sequence-Specific Primers

The *Arabidopsis* Information Resource'' (TAIR) database was used to design F-box gene sequence-specific primers for both the forward and reverse primers based on the F-box gene sequence as shown in figure 2.1 the primers were synthesized and obtained from the DNA oligonucleotide Facility, Department of Molecular and Cell Biology, University of Cape Town. Both primers carried restriction sites complementary and in frame to those of the pGEX-p62 expression vector (Invitrogen, Carlsbad, USA). The forward primers (5′-GGA ATT CCC ATG GCT ACT GGT ACG GAA TCT G-3′) carried an *Eco* RI restriction site while the reverse primer (5′-GAC TCG AGC GTA GCA GCC AAT GCG GGA GAG C-3′) carried a *Xho* I restriction site.

A

MESGTKKKKIDYYTEDLVVNILARLPLKSITAFTLVCKEWKSIV
ESQYLHELFMSHHQDSHPSWSLMCRETHKEVIAHYRCDTWGL
TQSLGSYISSFLTHKFGIHKEKVTVEAYTDVGLILVTDIYKPSGL
ATRIENGVVLGYKVVLMKDTTDTDDISLLIYSSETGLWSFNTVQ
SPYLLKRVAWFNPVSLNGNLYWLCYNNYRDHLVVSHNLYATG
TESDQCRVIEFPHLENDVYFRRAFTTSQGSLMYMKIINEEKDDG
SLGLKLCVWRLKSGKWEIVSEISPACIKSGLDYFPLAINPFDAN
KMYLWSEMHKCLVSTSLLKGKFRRHKKLEYSSDGRIMSFAGD
WSPFEHLFNPCFSRFALPHWLHRIPSSPPTDKLRMRIRLRKRAT

Sequences start

Motif

Sequence ending

B

Forward primer: *EcoRI* restriction site
*GCG CG*GA ATT CCC ATG GCT ACT GGT ACG GAA TCT G
Reverse primer:*Xho I* restriction site'
*GA*C TCG AGC GTA GCA GCC AAT GCG GGA GAG C

Start Codon

Stop Codon

Underlined: Resitriction site

Italic: Protection site

Figure 2.1: Primer Sequence and protein motif information. **(A)** Protein sequence annotated F-box protein retrieved from TAIR. The region highlighted in purple indicates start and blue indicate end of the AC motif respectively whereas the region highlighted in pink is the catalytic center. **(B)** Primer sequences designed to amplify AC catalytic center of F-box gene (At3g28223) where the underlined sequence indicate the restriction sites, and the highlighted green and orange nucleotides indicate the start and stop codons respectively.

2.3 Isolation of Total RNA from *A. thaliana*

At the age of four weeks, total RNA was extracted from fresh leaves of *A. thaliana*. The RNA was then transcribed to cDNA using an RT-PCR RNeasy® plant mini kit (Catalog no 74903, QIAGEN) according to the manufacturer's instructions. Aproximately 100 mg of plant leaf material were harvested and immediately placed in liquid nitrogen and material was then thoroughly ground with a pestle and motar into a fine powder. Throughout the grinding process, the liquid nitrogen was allowed to evaporate but the tissue was not allowed to thaw. After grinding, the tissue was transferred into 2 mL microcentrifuge tube. Buffer RLT (450 µL) was added immediately and vortexed vigorously. The lysate was then transferred to a QIAshredder spin column placed into a 2 mL collection tube and centrifuged for 2 minutes at 18 000x*g*. The supernatant of a flow through was transferred to a new microcentrifuge tube without disturbing the cell debris pellet in the collected tube. Half the supernatant volume of a 96% ethanol was then added to clear lysate and mixed immediately by pipetting. From the sample, 650 µL including any precipitation that could have been formed were transferred to an RNeasy spin column placed into a 2 mL supplied tube. The lid was closed gently and the contents centrifuged for 15 seconds at 8 000×*g* and the flow through discarded. Buffer RW1 (700 µL) were added to the RNeasy spin column followed by a centrifugation for 15 seconds at ≥8 000×*g* and in order to wash the spin column membrane. After centrifugation, the RNeasy spin column was removed avoiding any possible contact with the flow through. Buffer RPE (500 µL) was added to the RNeasy spin column followed by another centrifugation for 15 seconds at ≥8000×*g* in order to further wash the spin column membrane. Buffer RPE (500 µL) was added to the RNeasy spin column and centrifuged for 2 minutes at ≥ 8 000×*g* in order to further and thoroughly wash the spin column membrane. After centrifugation, the RNeasy spin column was carefully removed from collection tube and carefully placed into a new 2 mL collecting tube and centrifuged at full speed for 1 minute in

order to dry the column membrane. The RNeasy spin tube was then placed in a new collection tube and 50 µL RNase-free water was added directly onto the spin column membrane and gently centrifuge for 1 minute at ≥8 000×*g* to elute RNA.

2.4 Reverse Transcription and Polymerase Chain Reaction (RT-PCR)

2.4.1 Preparation of a Reaction Mixture

The isolated RNA from *A. thaliana* was used as a template to amplify the F-box gene using two designed sequence-specific F-box primers in a RT-PCR sustem. The reaction mix is shown in table 2.1 and was perfomed using the Verso™ 1-Step RT-PCR ready to mix™ kit (Thermo Fisher Scientific.Inc, Massachusets) according to the manufacturer's instruction.

Table 2.1: Components of the RT-PCR Reaction Mixture in a Total Volume of 50µL.

	Volume	Final Concentration
Verso Enzyme Mix	1 µL	
1-Step PCR ReddyMix (2x)[a]	25 µL	1x
Forward primer (10 µl)[b]	1 µL	200 nM
Reverse primer (10 µM)[b]	1 µL	200 nM
RT Enhancer	2.5 µL	
Water (PCR grade)[c]	19.5 µL	
Template (RNA)[d]	1 µL	1 ng
Total volume	50 µL	

2.4.2 Polymerase Chain Reaction

The F-box gene was amplified on a Peltier Thermo Cycler (DNA Engine DYAD Model PTC-220, MJ research Machiner USA) according to the Verso™ Reverse Transcriptase kit (Thermo scientific) under the following conditions as shown in table2.2 below. The PCR amplified AT3g28223 gene was resolved on a 1% agarose gel and visualised under UV (Gene genius Bio imaging system) and the image captured using a 6.000.022 software version of the same machine.

Table 2.2: Conditions of a 1-Step RT-PCR Thermal Cycling Program for Amplification of the F-box Gene.

	Temperature	Time	Number of cycle
cDNA Synthesis[d]	50°C	15 minutes	1 cycle
Thermo-Start activation	95°C	15 minutes	1 cycle
Denaturation	95°C	20 seconds	45 cycles
Annealing	65°C	30 seconds	
Extension	72°C	1 minutes	
Final extension	72°C	5 minutes	1 cycle

2.5 Purification of PCR Product from Reaction Mixture

The amplified F-box gene amplicon was purified from the PCR solution using the DNA Clean and Concentrator™-5 kit (Catalog number D4003, Zymo Research Corporation, California, USA) according to the manufacturer's instructions. Volumes (5)of DNA binding buffer were added to one volume of amplified DNA in a 1.5 mL microfuge tube and mixed by vortexing. The DNA was bound by transferring the mixture to a Zymo-Spin™ column. The column was centrifuged (Corning® LSE™ High Speed Microcentrifuge, Corning Inc., Amsterdam, The Netherlands) at 9200 x *g* for 30 seconds and the flow through was

discarded. The DNA bound onto the column was then washed by adding 200 µL of wash buffer and centrifuging at 9200 x *g* for 30 seconds. The flow through was discarded as waste and this step was repeated for a second time. The amplicon was eluted from the column by adding 10 µL of nuclease-free water directly onto the column matrix followed by a 2-minute incubation at room temperature. The eluent was collected into a sterile 1.5 mL microcentrifuge tube by centrifuging the column at 9200 x *g* for 30 seconds. The concentration of the DNA was then quantified using a Nanodrop spectrophotometer (Nanodrop 2000, Thermo Scientific, USA).

2.6. Restriction Double Digestion of the PCR Gene Product

The cleaned F-box (PCR) gene product was double-digested with *Xho* I and *Eco* RI in a 50 µL reaction mixture as follows: 10 units *Xho* I, 10 units of *Eco* RI, 2x Tango buffer (Thermo scientific) and 10 µL of insert. The mixture was incubated for 4 hours at 37°C. Thereafter, the restriction enzymes were inactivated by incubation at 80°C for 20 minutes.

2.7. Purification of Digested PCR Product

The digested PCR product was purified using the DNA Clean and Concentrator™-5 kit (Catalog number D4003, Zymo Research Corporation, California, USA) and according to the manufacturer's instructions. The clean amplicon was then resolved on a 0.8% agarose gel at 80 V and quantified using a nanodrop spectrophotometer (Nanodrop 2000, Thermo Scientific, USA).

2.8. Plasmid DNA Isolation and Purification

Escherichia coli MC106 cells harbouring the pGex-p62 plasmid vector (commercially obtained) were supplied by the Department of Biotechnology, University of the Western Cape. Single colonies were selected from the plates, sub-cultured on to fresh LB agar plates containing 100 µg/mL of ampicillin and incubated at 37°C for 24 hours. An aliquot of 10 mL 2YT expression media (1% yeast extract, 1.25% tryptone powder, 0.5% sodium chloride, 0.4% of glucose) supplemented with 100 µg/mL ampicillin was inoculated with cells from a single colony and the culture was incubated overnight at 37°C at 200 rpm.

The alkaline lysis method was then used to isolate plasmid vector DNA. Cells were collected through centrifugation at 12 750 x *g* for 5 minutes. The supernatant was discarded as waste and the pellet was resuspended in 400 µL ice cold GTE (50 mM glucose, 25 mM Tris HCl,100 mM EDTA, pH 8.0) and kept on ice for 5 minutes. An aliquot of 200 µL of the cell suspension was transferred into a clean 1.5 mL Eppendorf tube. An aliquot 400 µL of 0.2 M NaOH/1% SDS were added to the tube and mixed by gentle inversion before being placed on ice for 5 minutes. An aliquot of 300 µL 3 M KAc: (pH 5.5) was added to the tube and mixed by gentle inversion before being placed on ice for 5 minutes. Cellular debris were separated out through centrifugation at 12 750 x *g* for 5 minutes. The supernatant was transferred into a clean 1.5 mL Eppendorf tube without disturbing the pellet. The KAC salts were added as 1/10 final volume of the supernatant and isopropanol was added as 0.6X the volume of the supernatant. The plasmid vector DNA was precipitated at -20°C for 1 hour. After precipitation, the solution was centrifuged at 12 750 x *g* for 5 minutes and the supernatant discarded as waste. The pellet was washed twice with 500 µL of 70% ice-cold ethanol. The pellet was then dried at room temperature by inverting the tube on a paper towel and gently

tapping to get rid of the excess ethanol. After drying out the pellet, 50 μL of sterile distilled water was added to resuspend the pellet.

To the 50 μL of plasmid DNA, 200 μL of sterile distilled water was added to make a final volume of 250 μL and treated with 10 μg/mL of RNAse. The mixture was incubated 37°C for an hour. Working with a ratio of 1:1 plasmid:phenol, the DNA was extracted by mixing vigorously on a vortex with this mixture. After centrifugation at 12 750 x *g* for 5 minutes, the top aqueous fraction was carefully removed and transferred to a clean sterile 1.5 mL Eppendorf tube. The supernatant and chloroform were mixed in a ratio of 1:1 and vortexed vigorously. The samples were then centrifuged at 12 750 x *g* for 5 minutes and the supernatant was transferred into a clean 1.5 mL Eppendorf tube. DNA was precipitated for an hour with 70% ethanol and then harvested by centrifugation for 10 minutes at 12 750 x *g*. The supernatant was discarded. The pellet was resuspended in 500 μL of 70% ethanol and the suspension was then centrifuged for 5 minutes at 12 750 x *g*. The supernatant was discarded. The wash step was repeated. The pellet was allowed to dry at room temperature and then resuspended in 50 μL of sterile distilled water. The extracted plasmid DNA was resolved on a 0.8% agarose gel at 80 V for 2 hours.

2.8.1 Purification of Plasmid DNA from Agarose Gel

The plasmid DNA was purified from the agarose gel using the Zymo Clean Large Fragment DNA Recovery Kit (Zymo Research, USA) and according to the manufacturer's instructions. The DNA fragments from the agarose gel were excised using a razor blade and transferred into a 1.5 mL Epperndorf tube. Three volumes of ADB buffer were added to each gram of the excised agarose and the mixture was then incubated at 55°C for 10 minutes and until the gel slice was completely dissolved. The melted agarose solution was transferred to Zymo-spin™ 1C-KL column in a collection tube, centrifuged at 16 000 x *g* for 1 minute and the flow through discarded. An aliquot of 200 µL wash buffer was added to the column, centrifuged for 30 seconds at 16 000 x *g* and the flow through discarded. The wash step was repeated one more time. An aliquot of 30 µL sterile distilled water were added directly to the column matrix and incubated for 1 minute. The column was placed into a 1.5 mL Eppendorf tube and centrifuged for 30 seconds to elute the DNA. The eluted plasmid DNA was quantified on a nanodrop spectrophotometer (Nanodrop 2000, Thermo Scientific, USA) and then stored at -20°C.

2.9 Restriction Double Digestion of the Plasmid Vector

The purified pGEX-6p2 plasmid vector was double-digested with *Xho* I and *Eco* RI (Thermo scientific) in a 50 µL reaction mixture as follows: 1x Tango Buffer, 20 units of *Xho* I, 20 units of *Eco* RI and 15 µL of pGEX-6p2 vector. The reaction was incubated for 4 hours at 37°C. The enzymes were then inactivated at 80°C for 20 minutes. The digested vector was resolved on a 0.8% agarose gel and and the concentration of the digested pGex-6p2 (Invitrogen) was determined using a Nanodrop spectrophotometer.

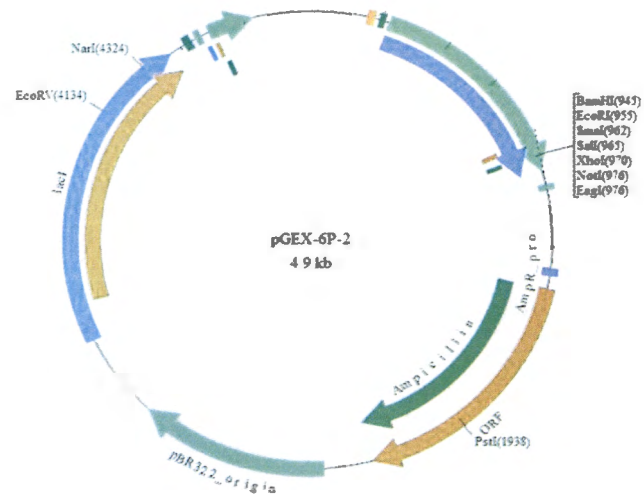

Figure 2.2: Commercial prokaryotic expression vector (pGex-p62) map used to ligate the generated F-box amplicon. The map is showing all of its cloning features including the Xho1 and Eco RI restriction sites (adapted from BvTech).

2.10 Purification of the Digested pGex Vector

The digested pGex-6p2 was purified using the DNA Clean and Concentrator™-5 kit (Catalog number D4003, Zymo Research Corporation, California, USA) and according to the manufacturer's instructions. The clean plasmid was resolved on an agarose gel of 0.8% at 80 V and quantified using a nanodrop spectrophotometer (Nanodrop 2000, Thermo Scientific, USA).

2.11 Ligation of the Gene Product into the Plasmid Vector

The ligation of the F-box gene insert into the pGEX-6p2 vector was carried out using a volumetric ratio of 1:3 vector: insert in a 40 µL reaction mixture and as follows: 1 µL of pGEX-p62 vector, 3 µL of F-box insert, 10x of T4 DNA ligase buffer and 1x T4 DNA ligase (catalog# EL0014, Fermentas International Inc., Burlington, Canada). The reaction mixture was incubated at 22°C for 60 minutes before being incubated at 4°C overnight and in order to maintain the stability of ATP. The ligated mixture was then stored at -20°C until use.

2.12 Preparation of Competent *Escherichia coli* BL21 (DE3) p*LysS* Cells

A trace of *E. coli* BL21 (pLysS) (*DE3*) cells was removed from the storage vial using a sterile toothpick and inoculated onto Luria Bertani (LB) agar plate containing 34 µg/mL chloramphenicol using the streak plate method. The plates were incubated at 37°C overnight. Thereafter, 10 mL of fresh Luria Bertani (LB) broth containing 34 µg/mL chloramphenicol was inoculated with cells from a select colony using a wire loop and then incubated at 37°C overnight in a shaking incubator at 220 rpm. The overnight culture of *E. coli* BL21 (pLysS) (*DE3*) was subcultured by adding 1 mL of the overnight culture to 100 mL fresh pre-warmed LB broth containing 34 µg/mL chloramphenicol antibiotic, and incubated in an incubating shaker at 37 °C until an OD_{600} of 0.5 was reached. The culture was cooled on ice for 5 minutes and transferred to two sterile 50 mL falcon tubes, and then centrifuged at 4 000 x *g*, at 4 °C for 5 minutes. The supernatant was discarded while cells were kept on ice then the cells were resuspended in 30 mL of ice-cold TFB1 (Transformation buffer 1) [30 mM KAc, 50 mM $Mncl_2$, 100 mM RbCl, 10 mM $CaCl_2$, 15% glycerol, pH 5.8 with KOH] and the suspension was kept on ice for 90 minutes. After that, the cells were collected by centrifuging at 4 000 x *g* for 5 minutes at 4°C.

The cells were resuspended in 4 mL ice cold TFB2 (transformation buffer 2) [10 mM MOPS, 75 mM $CaCl_2$, 10 mM RbCl, 15%. pH 6.8 with KOH]. Aliquots of 100-200 µL were then prepared in 1.5 mL Eppendorf tubes, snap-frozen in liquid nitrogen and stored at -80°C until use.

2.13 Transformation of the Competent Cells with the Vector-gene Construct

10 µL of the ligation mix (pGex-p62:F-box) were transferred into an ice-cold, sterile 1.5 mL microcentrifuge tube and kept on ice. The competent *E. coli* BL2 (DE3) pLysS cells (Invitrogen) were allowed to thaw on ice and gently resuspended. 100 µL of cells were carefully mixed with the ligation mix in a microcentrifuge tube and kept on ice for 20 minutes. Thereafter, the tube was incubated at 42°C for 90 seconds. After that, 500 µL LB broth were added to the tube and incubated for 90 minutes at 37°C at 200 rpm. After incubation, the tube was centrifuged for 5 minutes at 9 200 x *g* and the supernatant was discarded. Aliquots of 100 µL were spread-plated onto LB agar plates supplemented with 100 µg/mL ampicillin and 34 µg/mL chloramphenicol, and incubated at 37°C an overnight.

2.14 Determination of the Cloning and Transformation Success

After transformation, selected colonies were inoculated into 10 mL 2YT media containing 100 µg/mL ampicillin and 34 µg/mL chloramphenicol and incubated at 200 rpm overnight. In the morning, 400 µL of the overnight cell culture was sub-cultured into 20 mL of fresh 2YT broth containing 100 µg/mL ampicillin and 34 µg/mL chloramphenicol and incubated at 37°C for 3 hrs at 200rpm. When the optical density at 600 nm had reached 0.5, half of the culture was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma, USA) and incubated under the same conditions for a further 8 hours and alongside the equivalent

non-induced culture. After the incubation, the samples were transferred into 1.5 mL Eppendorf and centrifuged for 5 minutes at 9 200 x *g*. The supernatant was discarded and the pellet stored at -20°C and until use.

2.14.1 Analysis of Protein by SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis)

Both induced and uninduced pellets were resuspended in 200 µL of sterile distilled water and solubilised by vortexing for an hour. 40 µL of each samples were separately mixed with 10 µL of a 5X loading buffer. The tubes were boiled for 5 minutes to denature the proteins and then analysed by SDS-PAGE on a 12% polyacrylamide gel at 80 V for 50 minutes. The percentage of acrylamide used was based on the molecular weight range of the F-box protein (12% acrylamide for 22 kDa – 200 kDa). To facilitate visualization, the gel was stained with 30% Commassie staining solution (10% absolute ethanol, 10% absolute methanol, 10% absolute acetic acid, and 0.5% Commassie stain) for 15 minutes. The stained gel was then de-stained using a de-staining solution (10% absolute ethanol, 10% methanol and 10% absolute acetic acid) for 30 minutes. The expressed recombinant protein was then determined by visually analysing the resultant protein banding pattern.

Table 2.3: Components of the SDS-PAGE Separating and Stacking Gels.

	12% Separation gel	5% Stacking gel
30% Acrylamide	4000 µL	800 µL
0.8% SDS	1250 µL	625 µL
3M Trs HCL	1250 µL	_
1M Tris HCL	_	625 µL
10% APS	50 µL	25 µL
Sterilised distilled Water	3430 µL	2905 µL
TEMED	20 µL	20 µL

2.15 Extraction of Recombinant pGex-6p2/F-box

The extraction of the recombinant pGex-6p2/F-box vector was conducted using the Zvppy™ Plasmid Minprep kit (Zymo Research, USA) according to the manufacturer's instructions. 100 µL of recombinant cells glycerol stock was inoculated into 10 mL 2YT medium containing 100 µg/mL ampicillin and 34 µg/mL chloramphenicol and incubated overnight 37°C and 200 rpm. 1.5 mL of the overnight culture was centrifuged for 30 seconds at maximum speed and the supernatant was discarded. 100 µL of Lysis Buffer (Blue) and mixed with the cell pellet by inverting 6 times. 350 µL of cold Neutralization Buffer (Yellow) was added and mixed thoroughly by inversion and complete neutralisation was ensured by inverting for an additional 2-3 times. The sample was centrifuged at 1600 x *g* for 4 minutes. The supernatant of approximately 900 µL was transferred to the Zymo-Spin™ IIN column. The column was placed into the collection tube and centrifuged for 15 seconds. The flow-through was discarded and the column was placed back into the same collection tube. 200 µL of Endo-Wash Buffer was added into the column and centrifuged for 30 seconds. 400 µL of Zyppy™ Wash Buffer was added to the column and centrifuged for 1

minute. The column was transferred into a new 1.5 mL microcentrifuge tube and 30 µL of Zyppy^{Tm} Elution Buffer was added directly to the column matrix, incubated for 1 minute at room temperature and centrifuged for 30 seconds to elute the plasmid DNA.

2.15.1 Confirmatory PCR for the Recombinant pGex-6p2/F-box Vector

The confirmatory PCR was conducted using a *Taq* DNA polymerase Recombinant Kit (Fermentas International Inc, Burlington,, Canada) to ascertain that the expression host cells carried the recombinant pGex-6p2/F-box vector. The reaction mix was prepared and the thermal cycling program was run respectively as shown in Tables 4.3.2.1 and 4.3.2.2 below. The confirmatory PCR product was resolved on a 1 % agarose gel for 60 minutes at 100 V. The gel was visualized on the UV transilluminator light to verify the presence of a DNA fragment corresponding to the size of the AtF-box insert. The Gene Genius Bio Imaging System (Syngrene, Synoptics, UK) was used to capture the gel using Gene Snap software (Version 6.000.022).

Table 2.4: Components of Confirmatory PCR for Recombinant Vector.

	Volume	Final Concentration
Taq buffer	1 µL	10 X
dNTP mix	1 µL	200 µL
Forward primer (10 µl)[b]	2.5 µL	0.5 µM
Reverse primer (10 µM)[b]	2.5 µL	0.5 µM
$Mgcl_2$	4 µL	25 mM
Water (Nuclease-free)[c]	12 µL	
Template (Vector Expressed)	2 µL	1 µg
Total volume	25 µL	

Table 2.5: Conditions of a *Taq* DNA Polymerase Thermal Cycling Program for Confirmation of the Recombinant pGex-6p2/F-box Vector

Steps	Temperature	Time	Number of cycles
Initial Denaturation	95°C	3 minutes	1 cycles
Denaturation	95°C	30 seconds	30 seconds
Annealing	65°C	30 seconds	30 seconds
Extension	72°C	1 minute	30 seconds
Final extension	72°C	5 minutes	1 cycle

2.16 Expression and Purification of the Recombinant F-Box Protein

2.16.1 Optimization of Expression Conditions for the Production of Soluble F-box Recombinant Protein

2.16.1.1 Large Scale Expression

A volume of 100 µL glycerol stock from the pilot expression was inoculated into 50 mL 2YT media containing 100 µg/mL ampicillin and 34 µg/mL chloramphenicol and incubated overnight at 37°C at 200 rpm. The overnight cell culture was subcultured by adding 6 mL to 300 mL of fresh 2YT media containing 100 µg/mL ampicillin and 34 µg/mL chloramphenicol, and incubated for 3 hours at 37°C. When optical density of the culture had reached 0.5 at 600 nm, an aliquot of uninduced culture was removed and the rest of the culture induced with 0.5 mM IPTG. Both the induced and non-induced cultures were left to grow for a further 8 hours at 30°C. After incubation, the samples were then transferred into 1.5 mL Eppendorf tubes and centrifuged for 5 minutes at 15600 x *g*. The supernatant was discarded and the pellets were stored at -20°C or until use.

2.16.1.2 Determination of the Solubility State of the Recombinant F-Box Protein by Lysozyme Extraction

The frozen *E. coli* cells (pellets) harbouring the F-box gene from Section 3.1.1 were resuspended in 1 mL PBS buffer [140 mM NaCl, 3 mM KCl, 4 mM Na_2HPO_4:H_2O and 1.5 mM KH_2PO_4], containing 1 µg/mL of lysozyme. The cell suspension was incubated on ice for 30 minutes and vortexed for 2 minutes with 30 second intervals of incubation on ice. After vortexing, the resuspended cells were centrifuged at 9 200 x *g* for 5 minutes. The

supernatant was transferred into a clean 1. 5mL Epperndorf tube. The pellet was resuspended with 1mL PBS. The solubility of the recombinant F-box protein was determined by assessing both the soluble and insoluble fractions using SDS-PAGE.

2.16.2 Purification of the Recombinant F-box Protein Under Native Non-denaturing Conditions

About 20 µL of Glutathione Agarose (Sigma-Aldrich, USA) was left to swell in 500 µL of filter sterilised distilled water for an hour at room temperature. The supernatant was removed and 500 µL of PBS-T buffer (PBS supplemented with 1% Triton-X) was used to equilibrate the beads by gently shaking the tube on a rotary shaker. Thereafter, the beads were washed using the same buffer and the wash step was repeated twice. After washing, the PBS-T buffer was discarded. The supernatant from the lysozyme extraction containing the soluble recombinant F-box protein was then added to the bead slurry and allowed to bind for 1 hour on a rotary shaker at 4°C. After binding, the flow-through was collected and the beads were washed three times with the 1 mL 1X PBS-T buffer.

The purified protein was eluted using PBS buffer supplemented with 0.01 mM PMSF and 200 mM reduced glutathione, whereby 500 µL of the beads (carrying the bound purified protein) were transferred into a new Eppendorf tube and 1 000 µL of the elution buffer added to resuspend the beads. The mixture was incubated at 4°C on a rotary mixture for 20 minutes. After incubating, the beads were sedimented using a minifuge (Labnetspectrafuge mini centrifuge model C1301B Labnet International, New Jersey USA) and the eluent was transferred into a new Eppendorf tube. The purified protein fraction was then analyzed by SDS-PAGE.

2.16.3 Concentrating and Desalting of the Recombinant Protein

A volume 1 mL of eluent of the purified protein fraction was transferred into a concentrator (Spin-X UF Concentrator, Corning, USA). The concentrator was then centrifuged at 4°C at 2540 x *g* (Hermle 2300k, Hermle Labortechnik, Germany). The protein volume was monitored for 30 minutes and 0.2 mL was obtained. The concentrated protein was removed from the concentrator and transferred into a new Epperndorf tube. Protein concentration was then determined using a Nanodrop spectrophotometer (Nanodrop 2000, Thermo Scientific, USA).

2.17 Activity Assaying and Functional Characterization of the Recombinant F-Box Protein

2.17.1 Determination of the Endogenous Adenylate Cyclase Activity

200 µL of the glycerol stock of cells containing the pGex-6p2:F-box gene were inoculated into 10 mL 2YT media (1% of yeast extract, 1.25% of tryptone powder, 0.5% of sodium chloride and 2 g of glucose) containing 100 µg/mL ampicillin and 34 µg/mL chloramphenicol and incubated at 37°C at 200 rpm overnight. The following day, 2 mL of the overnight cell culture was inoculated into 100 mL of fresh 2YT media containing 100 µg/mL ampicillin and 34 µg/mL chloramphenicol and was incubated at 37°C up until the optical density at 600 nm had reached 0.5. The culture was placed on ice and sub-divided into four equal portions of 10 mL in falcon tubes. Tube 1 was used as a control lacking IPTG, tube 2 was supplemented with 1 mM IPTG (Sigma, USA) tube 3 was supplemented with 1 mM IPTG and 100 µM foskorlin and tube 4 was supplemented with 1 mM IPTG and

100 μM dideoxyadenosine. All four tubes were then incubated for 3 hours at 37°C and then centrifuged at 10000 x *g* (Hermle 2300k, Hermle Labortechnik, Germany) for five minutes.

The supernatants were removed as waste and to each pellet, 1 mL of lysis buffer (Amersham, Healthcare USA) and 20 mM 3-isobutyl-1-methyl xanthine (IBMX) were added and the tubes swirled for 30 minutes at 200 rpm using AGITATOR Orbital Shaker (Comecta, S.A. optic ivymen system). The samples were then centrifuged at 16300 x *g* for 5 minutes (Corning LSE High Speed Microcentrifuge 6766-HS) and 200 μL of each supernatant was transferred into a new Eppendorf tube and 200 μL of lysis buffer 2 (Amersham, Healthcare USA) was also added and thoroughly mixed. An aliquot (11 μL) of the acetylation reagent Sigma Aldrich Corp, Missouri) was added to 220 μL of the sample and the sample was thoroughly mixed by vortexing. The cAMP content of each sample was then measured by cAMP-specific enzyme immunoassay assay kit (cAMP Enzyme Immunoassay Kit, Catalog Number CA201, Sigma, USA) and in accordance with the acetylation protocol of the manufacturer's instructions. Samples were measured in triplicates using Microplate Reader (Labtech International Limited, East Sussex,UK) at 405 nm and the results were subjected to a biological statistical analysis of variance (ANOVA) (Super-Anova, Stats graphics Version 7, 1993, Stats graphics Corporation, USA).

2.17.2 Determination of the *In Vitro* Enzymatic Activities

The *in vitro* enzymatic activity of the purified recombinant F-box protein was determined by assessing its ability to convert ATP to cAMP. Reaction mixes were prepared as shown in Table 4.1 below.

Table 2.6: Molecular Characterization of the Recombinant F-box Protein.

	Tube 1 (Control)	Tube 2	Tube 3	Tube 4	Tube 5	Tube 6	Tube 7	Tube 8
Tris	50 mM	50 mM	50 mM	50 mM	50 mM	50 mM	50 mM	50 mM
IBMX	2 mM	2 mM	2 mM	2 mM	2 mM	2 mM	2 mM	2 mM
$MgCl_2$	5 mM	5 mM	-	5 mM	5 mM	5 mM	5 mM	5 mM
Protein	-	25 µg	25 µg	25 µg	25 µg	25 µg	25 µg	25 µg
ATP	1 mM	1 mM	1 mM	1 mM	1 mM	1 mM	-	1 mM
$MnCl_2$	-	-	5 mM	-	-	-	-	-
$CaCl_2$	-	-	-	100µM	-	-	-	-
GTP	-	-	-	-	-	1 mM	1 mM	-
$NaHCO_3$	-	-	-	-	1 mM	-	-	-

Each reaction mix was incubated at room temperature for 20 minutes then inactivated by adding 1 mM EDTA to bind all the divalent cations, and was then stopped through boiling for 5 minutes to denature the protein. The samples were then centrifuged at 16,300 x *g* for 5 minutes to spin down any precipitate. The cAMP in the supernatant was then acetylated by adding 11 µL of acetylating reagent (triethylamine: acetic anhydride 1:0.5 v/v) to every 220 µL of the supernatant and the mixture was briefly pulsed. The cAMP content was measured by a cAMP-specific enzyme immunoassay assay kit (cAMP Enzyme Immunoassay Kit, Catalog Number CA201, Sigma USA) and according to the manufacturer's instructions. The samples were measured in triplicates using a Microplate Reader (Labtech,International Limited East Sussex,UK) at 405 nm and all results were subjected to a statistical analysis of variance (ANOVA) (Super-Anova, Stats graphics Version 7, 1993, Stats graphics Corporation, USA).

2.17.3 Determination of the *In vivo* Adenylate Cyclase Activity

Some *E. coli cya*A SP850 cells from Coli Genetic Stock Center, (Yale University, Connecticut, USA) were prepared to become chemically competent as is described in Section 2.13 but replacing 34 ug/mL chloramphenicol with 15 µg/mL kanamycin. The chemically competent *cya*A cells were sub-divided into three equal portions. The first portion was transformed with the pGex-6p2:F-box expression construct as is described in section 2.14, while the second portion was transformed with an empty pGex-6p2 vector. The third portion was left un-transformed. One MacConkey agar plate was prepared and supplemented with 15 µg/mL kanamycin and 0.1 mM IPTG and sub-divided into four equal sections using a permanent ink marker. Section 1 of the plate was left un-streaked, section 2 was streaked with the *cya*A SP850 only, section 3 was streaked with mutant cells carrying the, empty vector pGex-6p2, and section 4 was streaked with the mutant cells carrying the pGex-6p2:F-box expression construct. The plate was then inverted and incubated at 37°C for 40 hours and some visual observations were then made on the various phenotypic natures of the grown cells.

Chapter 3: Results

3.1 Cloning of the F-box Gene, Expression and Purification of the Recombinant F-box Protein

Total RNA was extracted from *A. thaliana* and used as a template for the amplification of the F-box gene using the gene specific primers (Figure 3.1A). The amplified gene was cloned into pGEX-6p2 as a tagged fusion (Figure 3.1C) and expressed in *E.coli* BL21 (plysS) *(DE3)* cells. Subsequently the expressed recombinant protein was isolated and purified via a reduced glutathione column (Figure 3.1D). The cloned gene insert was first confirmed by colony PCR of the transformed *E.coli* BL21 plysS *(DE3)* cells using the sequence specific primers (Figure 3.1B)

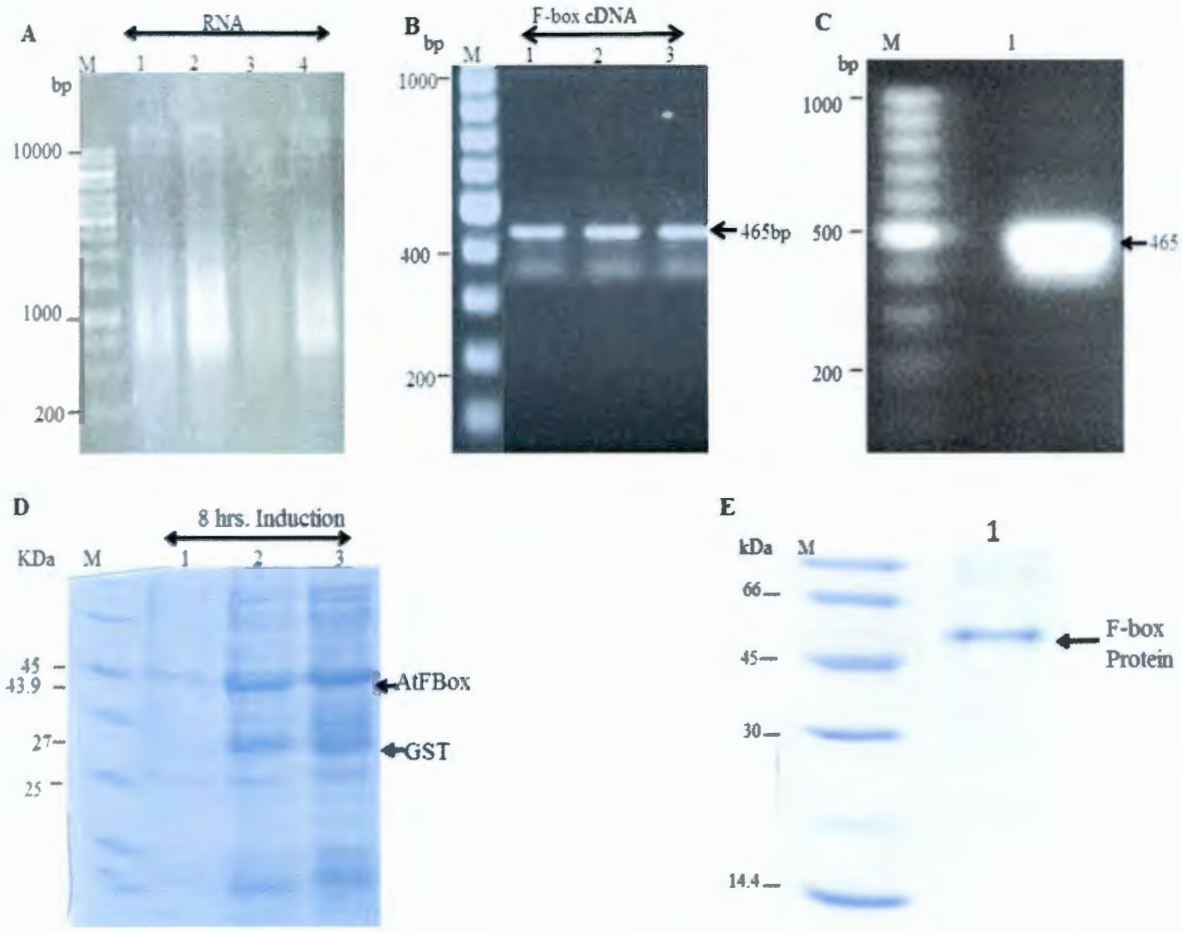

Figure 3. 1: Cloning of the F-box gene, Expression and Purification of the Recombinant F-box Protein. **(A)** Agarose gel showing RNA extracted from *A. thaliana*. Lane M-1 Kb molecular weight marker (Fermentas Life Science); lanes 1-4 are RNA extracted. **(B)** Agarose gel showing the *A. thaliana* F-box cDNA (At3g28223) amplified via RT-PCR. Lane M- 100 bp MW marker (Catalog# SM1143-Fermentas International Inc., Burlington, Canada); lane 1-3 shows amplified F-box cDNA. **(C)** Agarose gel showing the colony PCR product of the *A. thaliana* F-box gene (At3g28223) after cloning into *E.coli* BL21 plysS (DE3). Lane M-100bp MW marker (Fermantas); lane 1- *A. thaliana* F-box gene. **(D)** SDS-PAGE of protein fractions expressed in BL21 (plysS) *(DE3)* cells transformed with the pGex-p62:F-box fusion construct, M-unstained molecular weight marker (Catalog# SM1143-Fermentas International Inc., Burlington, Canada); lane 1-protein fractions from the uninduced culture; lanes 2-3 shows protein fractions from induced cultures. **(E)** SDS-PAGE of the purified F-box. M-low molecular weight marker (Thermo scientific); lane-purified recombinant protein.

3.2. Determination of cAMP Levels and AC Activity Assay

3.2.1 Determination of the Endogenous Activity of the Recombinant F-Box Protein

To determine the levels of cAMP generated by the recombinant *E. coli* BL21 (DE3) pLysS carrying the F-box gene under different growth conditions, the cells were cultured up until their OD_{600} was 0.5. The cells were divided into four portions, whereby the first portion was used as a control without the IPTG, the second portion was treated with 1 mM IPTG only (Figure 5.2A), the third 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 (Figure 5.2B). The generated cAMP was then extracted from the cells and measured by a cAMP-specific enzyme immunoassay kit (Catalogue Number CA201, Sigma, Missouri, USA) based on the acetylation protocol.

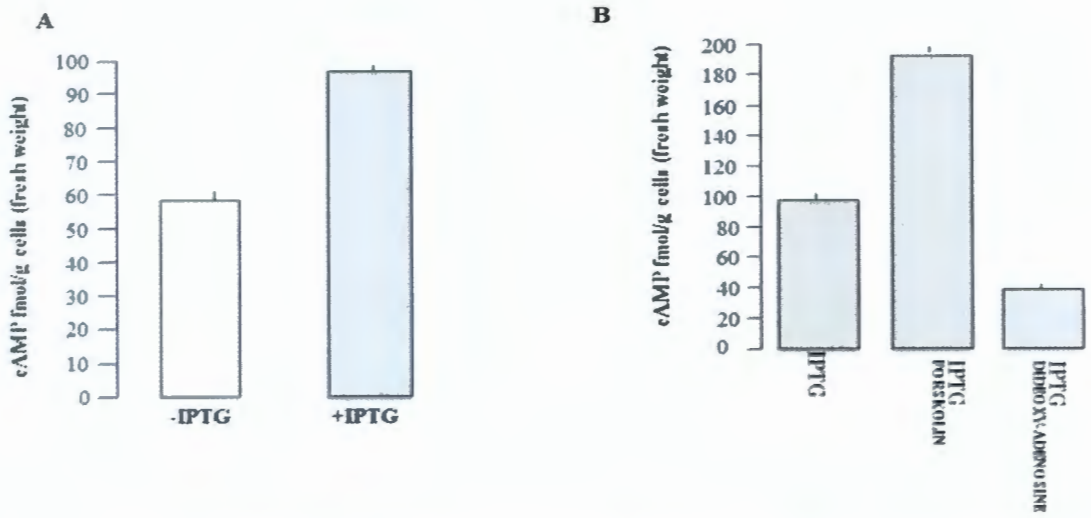

Figure 3.2: Determination of the endogenous adenylate cyclase activity of the recombinant F-box protein. (A) cAMP levels generated by uninduced (-IPTG) and induced (+IPTG) *E. coli* BL21 (DE3) pLysS cells harbouring the F-box gene. **(B)** cAMP levels generated by induced cell cultures in the presence of either forskolin or dideoxyadenosine. 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).

3.2.2 Determination of *In vitro* Adenylate Cyclase Activity of F-box Protein

The expressed recombinant F-box protein was subsequently purified as a GST-tagged fusion product on a reduced glutathione affinity column. The purified recombinant protein was synthesized and tested for *in vitro* activity with the AC catalytic centre conforming to the F-box gene. To test for AC activity Enzyme immunoassay was used to determine the quantitative level of cAMP formed from the recombinant F-box protein. The generated cAMP was then measured by a cAMP-specific enzyme immunoassay kit (Catalogue Number CA201, Sigma, Missouri, USA) based on the acetylation protocol. The effects of other reaction additives like 5 mM Mn^{2+}, 100 μM Ca^{2+}, 5 mM GTP and 50 mM CO_3^{2-} were also assessed and determined with the same assaying system (Figure 5.3). The recombinant F-box protein has the substrate preference for ATP than GTP. Activity was evaluated for both of substrates combined together, as there was a competition for binding between these two substrates (GTP, ATP), where a trivial increase in the generation of cAMP level was detected (Figure 3.3 D).

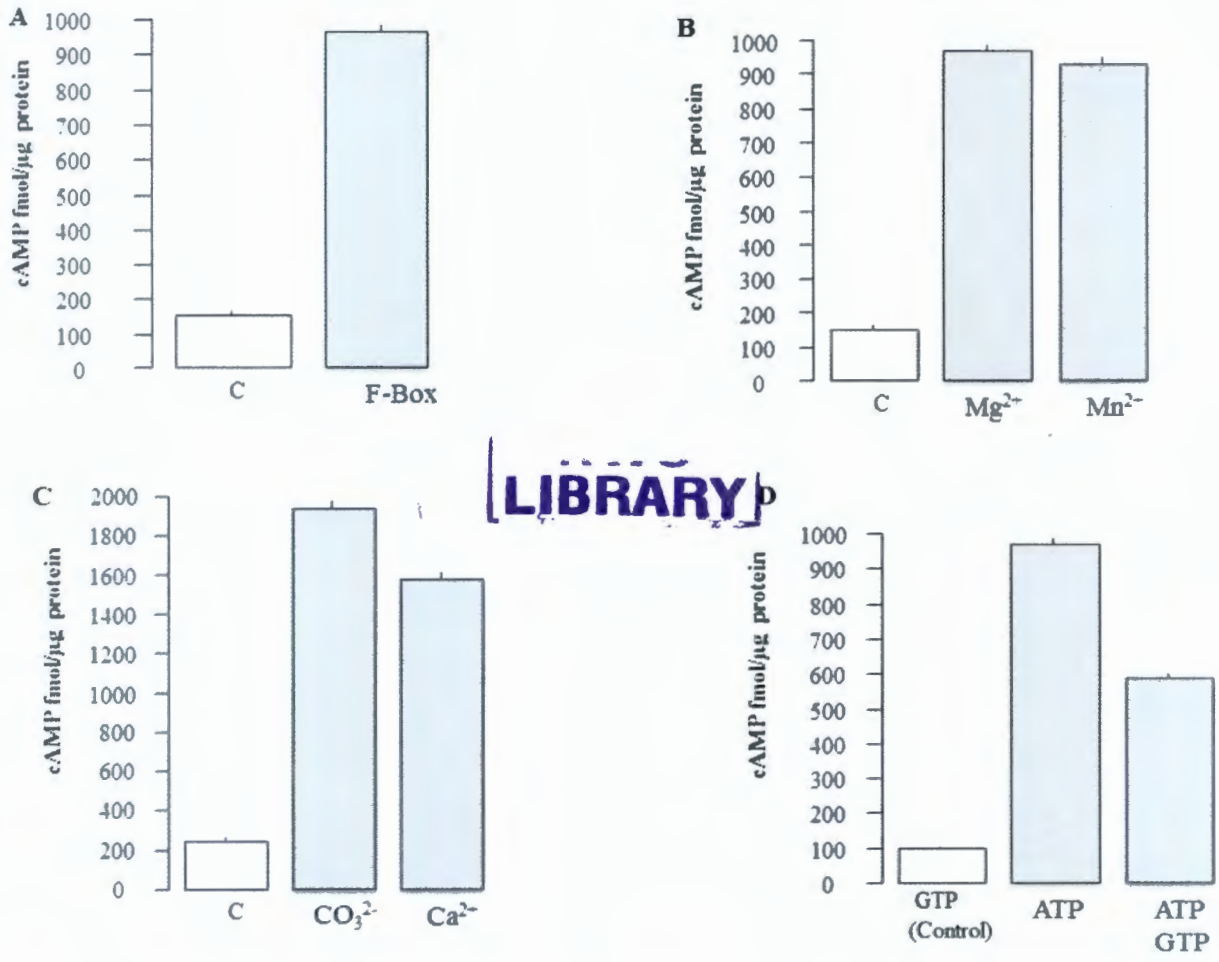

Figure 3.3: Determination of *in vitro* adenylate cyclase activity of the recombinant F-box protein. A reaction mixture containing of 10 µg the purified recombinant F-box protein, 50 mM Tris HCl pH 8.0, 2 mM IBMX, 5 mM Mg^{2+}, and 1 mM ATP and/or in the presence of other additives was incubated at room temperature for 20 minutes. The generated cAMP was then measured by a cAMP-specific enzyme immunoassay system based on the acetylation protocol. **(A)** cAMP levels generated in the absence and presence of the protein (F-box). **(B)** The empty control bar contains the reaction mixture without the recombinant protein; other bars contained protein with 1mM ATP in the presence of 5 mM Mg^{2+} and 5 mM Mn^{2+}. **(C)** AC assay represented by empty control which contains no recombinant protein and other bars contains protein in the presence of CO_3^{2-} and Ca^{2+}. **(D)** AC activity of At3g28223 represented by the first bar (GTP) a control with no recombinant protein, second bar contains recombinant protein with 1mM ATP; the third bar contains both ATP+GTP as the substrates. All error bars represent the standard errors of the means from triplicate assays (n = 3).

3.2.3 Determination of the *In vivo* Adenylate Cyclase Activity of the Recombinant F-box Protein

The adenylate cyclase activity of the F-box-protein was tested by examining its ability to convert the non-lactose fermenting mutant *E. coli cya*A SP80 cells to lactose fermenting wild type cells. In this case, the mutant cells were then transformed by the pGex-6p2: F-box construct and their response assessed phenotypically on MacConkey lactose agar (Figure 3.4). When an empty vector was transformed with *CyaA* showed no activity since this *E. coli* strain lacking cAMP were unable to ferment lactose and produced a white colour this is due to the fact that the mutant cannot ferment lactose as a results of the gene being silenced thus it only requires cAMP to ferment lactose. This test showed a positive response on functional analysis of this recombinant pGEX-6p2: AtF-box-AC expression construct with a mutant *CyaA* and confirmed that it has an adenylate cyclase activity.

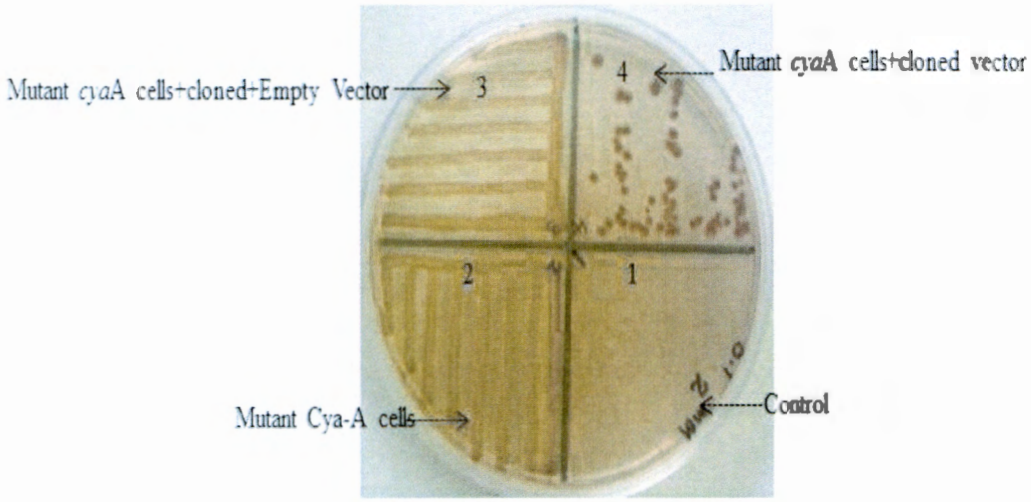

Figure 3.4: Determination of the *In Vivo* Adenylate Cyclase Activity of the recombinant F-box protein by complementation. Three different *cya*A cells were plated onto MacConkey agar supplemented with 0.1 M IPTG and incubated for 40 hours at 37°C. Quarter 1 of the plate contains no cells, quarter 2 contains non-transformed *cya*A mutant cells, and quarter 3 contains *cya*A mutant cells transformed with the empty pGEX-p62 vector while quarter 4 contains *cya*-A mutant cells transformed with the pGEX-p62-AtF-box expression construct. Cells in sections 2 and 3 are both non-lactose fermenters and produce white or yellowish colonies. Cells in section 4 have picked a deep purple phenotype a characteristic signifying the ability to ferment lactose.

Chapter 4: Discussion and Conclusion

4.1. Discussion

F-box proteins are interchangeable substrate receptors and are known to contain protein-interacting domains, such as LRR domains, at their C-terminal. To date, the biological function of only 20 F-box proteins has been explained, and it has been reported that these proteins are involved in a wide range of physiological processes such as cell cycle control, circadian rhythms, floral development, as well as different phytohormone responses. In general, F-box proteins contain two regions; the N-terminal region and the C-terminal region. The F-box motif in the N-terminus interacts with Skp1 proteins while the C-terminal region determines the substrate specificity. One or more protein–protein interacting domains are often found in the C-terminal regions. The F-box protein family has also been associated with cellular functions such as signal transduction and regulation of the cell cycle that, in turn, is linked to auxin responses and changes in cellular cAMP content (Newton *et al.*, 1980; Newton *et al.*, 1986; Chen *et al.*, 2000). Some F-Box proteins have been characterized as components of SCF ubiquitin ligase complexes while others have been shown to be involved in plant hormonal response as receptors or medial components (Haichuan *et al.*, 2007).

However, despite the fact that the F-box protein has been extensively implicated in various cell signalling systems that are mediated by cyclic nucleotides (cAMP and/or cAMP), the protein has never been demonstrated to be able to generate cAMP by itself although a specific *A. thaliana* gene has recently been shown to habour an AC catalytic domain (Gehring, 2010). Therefore in this study the F-box segment containing the AC catalytic domain was targeted and cloned for functional characterization. In order to target the catalytic AC centre in the F-box sequence, we first retrieved the At3g28223 gene sequence

from TAIR and then determined its cDNA sequence for subsequent cloning and expression. the predicted open reading frame was then translated into a recombinant protein product that was flanked 50 amino acids on both the amino and carboxyl-termini sites of the sequence contained the motif (KWEIVSEISPACIKSGLD), and as is shown in Figure 2.1A. The two gene specific primers were manually designed to amplify this targeted gene site (Figure 2.1B), and the primers carrying restriction enzyme sites (*Xho* I and *Eco* R1) complementary to those of the pGex-6p2 expression vector (Figure 2.2). After designing the F-box sequence specific primers, total RNA from *A. thaliana* leaves was isolated (Figure 3.1A) and then reverse transcribed by an RNA dependent DNA polymerase to synthesize cDNA. Sequence specific primers were then used to amplify and isolate the targeted AtF-box gene fragment. When resolved on a 1% agarose gel, the expected fragment size of 487 bp was obtained (Figure 3.1B)

The amplified AtF-box gene fragment and the pGex-6p2 vector were both double-digested successfully using the restriction endonucleases, *Xho* I and *Eco* R1 that created sticky ends for directional cloning between the two DNA molecules. After digestion, the two DNA molecules (*AtF-box* insert and pGex-6p2 vector) were ligated together to form a pGex-6p2 expression construct. The formed expression construct was then used to transform some competent BL21 (DE3) STAR pLysS cells and confirmation of the cloning of a correct expression construct verified by colony PCR (Figure 3.1C). Maximal expression of the AtF-box recombinant protein was then undertaken by inducing the cell cultures at OD_{600} of 0.5 with 0.5 mM IPTG and the expressed recombinant protein resolved by SDS-PAGE. As expected, a recombinant fusion product of approximately 43.87 kDa was obtained (Figure 3.1 D).

To assess if the adenylate cyclase catalytic centre in the AtF-box protein had any enzymatic activity, an endogenous assaying of the cAMP levels generated without cell induction, with cell induction as well as with cell induction in the presence of 100 μm forskolin or 100 μM dideoxy-adenosine was undertaken. As is shown in Figure 3.2A, cells induced with 1 mM IPTG had their cAMP generation increased by a 1.58 fold factor as compared to the un-induced cells. Additionally, the treatment of induced cells with 100 μM forskolin further increased the level of cAMP generation by a factor of 1.95 (Figure 3.2B). On the other hand, the treatment of induced cells with 100 μM dideoxy-adenosine significantly reduced the levels of cAMP by a factor of 2.5 (Figure 3.2B). In a related previous study where a PSiP coding region of a pollen-specific putative AC from *Agapanthus umbellatus* (*Liliaceae*) was cloned into bacterial cells, treatment of cells with I mM IPTG increased the cAMP levels by a factor of 3.0 while treatment of induced cells with 100 μM of the AC inducer forskolin, increased the levels of cAMP generation by a factor of 1.83 (Moutinho *et al.*, 2001). Forskolin elicits cellular responses which have been suggested to be dependent on cyclic AMP as a second messenger (Seamon *et al.*, 1981). Therefore forskolin, provides an important tool for the search of the role of cyclic AMP in physiological responses to hormones, both through it direct activation of adenylate cyclase and through its ability to potentiate hormonal activation of adenylate cyclase (Seamon *et al.*, 1981). Furthermore, the application of 100 μM of the AC inhibitor – dideoxy-adenosine to the growing pollen tubes transiently caused a temporary growth arrest accompanied by a reduction of cAMP concentration by a factor of 1.8 (Moutinho *et al.*, 2001). These findings are similar to our study.

These results suggest that *A. thaliana* F-box gene is probably either an actual adenylate cyclase capable of directly converting ATP to cAMP or else it is a functional plant molecule capable of stimulating other resident bacterial adenylate cyclases to produce cAMP.

In order to verify if the recombinant AtF-box had any specific functional adenylate activity, the protein was purified via reduced gluthathione affinity matrix (Figure 3.1E) followed by its assessment for *in vitro* AC activity (Figure 3.3). From the results obtained, (Figure 3.3A) there is an increase of cAMP (5.4 fold) in the presence of recombinant F-box protein, a relatively high level of AC activity for the AtF-box was revealed with no specific biased preference for either Mg^{2+} or Mn^{2+} as cofactor ions for its activity (Figure 3.3B). As previously reported (Tesmer *et al.*, 2000; Geng *et al.*, 2005), some adenylate cyclases may exhibit no inherent preference of either Mg^{2+} or Mn^{2+} metal ions as cofactors for enzymatic activity. In the presence of either of these metal ions, the adenylate cyclase activity does increase in a dose-dependent manner (Tesmer *et al.*, 2000; Geng *et al.*, 2005). Another study (Kwezi *et al,* 2011) showed that AtPSKR1-KD2 also exhibited GC activity that showed no preference in metal ion selectivity between Mn^{2+} or Mg^{2+}, Signaling molecules have been documented to require and be responsive to cofactors e.g Mn^{2+} or Mg^{2} ions; however the presence of one or the other metal ions was essential to observe activity. Therefore the speculation of F-box-AC is that, Mg^{2+} and Mn^{2+} may possibly act as a feedback to adenylate cyclase or cAMP can directly control a feed back to Mg^{2+} and Mn^{2+}.

In addition, the results obtained in Figure 3.3C indicate that the recombinant F-box protein can cyclize ATP into cAMP in the presence of both Ca^{2+} and CO_3^{2-} ions and as its functional modulators. The increased activity (1.6 fold) as a result of the presence of calcium ions might possibly be due to the responsive activities of the cyclic nucleotide-gated ion channels

(CNGC$_S$), which are crucial for plant development (Talke *et al.*, 2003). This trend is common since in previous studies Ca^{2+} had been shown to be required for the development of freezing tolerance in *A. thaliana* (Knight *et al*, 1996). The same study further established that intracellular Ca^{2+} levels increase briefly in response to cold shock in *Arabidopsis* (Knight *et al*, 1996; Lewis *et al*, 1997; Polisensky *et al*, 1996). In the present of calcium the protein also promoted cAMP, in other studies the other known signalling molecules known to function and activated with calcium. homomeric channels formed by CNGC2 polypeptides conduct Ca^{2+} and are activated by cAMP as well as cGMP. However, it is still unclear (Lemtiri-Chlieh *et al*, 2004) whether channels formed by any translation product of the 20 Arabidopsis CNGC genes and specifically,CNGC2 form cGMP-, as opposed to cAMP-, activated channels in native plant cell membranes. Ca^{2+} is a universal second messenger that acts as a mediator of stimulus response coupling in eukaryotes. In plants, various abiotic and biotic stimuli, including phytohormones, mechanical and oxidative stresses, and microbial elicitors, trigger changes in intracellular Ca^{2+} concentration (Ma *et al* 2007, McAinsh *et al*, 2009) (figure 3.3C). In figure 3.3 the control shows high level of cAMP generated because the presence protein. Possibly in the presence of calcium, F-box molecule is activated because the signal molecule is affected by calcium, this is not surprising because AC's have a binding domain. Hypothetically and in the presence of calcium, the F-box-AC molecule is probably activated and further modulated by cAMP whose activity can be regulated by the binding of a number of various co-factors such as magnesium or manganese ions.

The recombinant F-box protein had also shown an elevation (1.9 fold) in cAMP generation in the presence of carbonate ions and thus suggesting the positive stimulatory effects of these ions on the enzymatic activity of protein. Once more, this finding since carbonate is known

to increase cAMP generation in a pH-independent manner, which is noted to directly modulate the enzyme (Yanqiu *et al.*, 2000).

Substrate specificity for the recombinant AtF-box protein between ATP and GTP as possible substrates was also assessed by testing its ability to generate cAMP from either of the two substrates (Figure 3.3D). As is shown in the figure, the generated cAMP levels from ATP were more than 9.5 times the levels generated from GTP and thus indicating the strict specificity of the AtF-box for ATP. GTP Furthermore, when these two substrates were provided to the protein in equimolar concentrations, the overall activity was somewhat reduced by a factor of 1.5, probably as a result of competitive binding to the enzyme between these two structural analogues. Notably, in all class III ACs, the specific amino acids involved in the binding of the adenine moiety are Lys and Asp and it results in a very high prediction which enable the enzymatic ATP specificity over GTP (Sunahara *et al.*, 1998).

From these *in vitro* findings, it can be speculated that the AtF-box is indeed a functional AC. Nevertheless and in order to further validate this outcome, an *in vivo* assessment of the AtF-box activity was further undertaken through a functional complementation test. This test was performed using an SP850 mutant *cya*A strain that is deficient in endogenous adenylate cyclase and therefore cannot ferment lactose (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 activity of the AtF-box could rescue the mutant cells, these cells were transformed with the pGex-6p2-AtF-box expression construct followed the assessment of the colony phenotypes on MacConkey agar supplemented with 0.1 mM IPTG (Figure3.4). As is shown in the figure, the transformed cells stained magenta deep-purple,

signifying the rescuing of the mutant cells into wild types and thus validating AtF-box as a functional AC.

4.2. Conclusions

Findings from this study firmly ascertains the F-box domain that harbours the annotated AC motif as functional higher plant adenylate cyclase and thereby becoming the second ever identified higher plant adenylate cyclase after the only *Zea mays* pollen protein last identified in 2001 (Moutinho *et al.*, 2001). These findings therefore provide a novel and elegant outcome with huge and massive significance to both scientific literature and academic scholarship.

4.3 Recommendations

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

- Firstly, since this molecule (F-box) was bioinformatically identified together with other eight putative molecules (Gehring, 2010) and in this study, it has been confirmed to be a higher plant adenylate cyclase, it is therefore suggested that the other eight putative molecules also be tested as possible higher plant adenylate cyclases.
- Secondly, since the AtF-box-AC has been firmly confirmed as a functional adenylate cyclase, it is impeartive that its exact physiological role in cell signal transduction and particularly, in plant stress response and adaptatiom mechanisms be further investigatedl.

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