Recombinant Expression and Functional Characterization of a G3-family Protein from *Arabidopsis thaliana*

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Dissertation submitted in fulfillment of the requirements for the degree Master of Science in Biology (Plant Biotechnology) at the Mafikeng Campus of the North-West University

Supervisor: Prof. O Ruzvidzo

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DECLARATION

I, Teboho Amelia Tiiti, declare that this work entitled "**Recombinant expression and functional characterization of a G3 Family protein from *Arabidopsis thaliana***" full dissertation submitted to the Department of Biological Sciences at the North-West University, Mafikeng Campus, for the degree of Master of Science in Biology (Plant Biotechnology), has never been submitted for a degree at this university or at any other institution. This is my own work and all the sources used in this document have been duly acknowledged.

SIGNATURES

Signature**:** …… Date**:** ……

TEBOHO AMELIA TIITI **(Student)**

Signature**:** …… Date**:** ……

PROF. O RUZVIDZO **(Supervisor)**

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DEDICATION

This work is dedicated to my family: my father, Petrose Makonye Tiiti, my mother Elizabeth Fumane Tiiti and my sisters, Nthabiseng, Tiisetso, Thato and my niece, Oaratwa.

TO GOD BE THE GLORY

ACKNOWLEDGEMENTS

During my research period for this thesis, there have been many people who have contributed in helping me to get by, and reach the success I have come upon, and to whom I owe my sincere appreciation. First and foremost, I owe the greatest debt of gratitude to God, due to the fact that it is His plan for me to be where I am today. Secondly my gratitude goes to my supervisor, Prof. Oziniel Ruzvidzo, for his advice and knowledge, inspiration and sociable nature that makes working in his laboratory and with him a true pleasure.

Dr Dave Kawadza, for his contribution with this massive work, for his intellectual and good humour, I give my thanks to my never-tiring co-mentor, who always reserved his time and effort to put in a good contribution.

In the laboratory, I convey my appreciation to the "Biotech" group for creating a healthy working environment, with patience and good humour, and I am grateful to everyone else for being fantastic colleagues and workmates – it made all the difference!

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DEFINITION OF TERMS

Adenosine triphosphate: A nucleoside triphosphate used within cells as a coenzyme (ATP) to transport chemical energy for metabolism.

Adenylate cyclases (ACs): Enzymes that catalyze the formation of cyclic 3′,5′-adenosine monophosphate (cAMP) and pyrophosphate from adenosine triphosphate (they are also called adenylyl cyclases).

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

Cloning vector: Genetic elements into which genes can be inserted and replicated.

Cyclic 3′,5′-adenosine monophosphate (cAMP): A second messenger in many biological processes that is derived from ATP and is used for most intracellular signal transduction systems in many different organisms, conveying the cAMP-dependent pathways.

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

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

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

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

LIST OF ABBREVIATIONS

AC: Adenylate cyclase

ATP: Adenosine 5′-triphosphate

bp: Base pairs

G3-FP: G3-family protein

cAMP: Cyclic 3′,5′-adenosine monophosphate

cGMP: Cyclic 3′,5′-guanosine monophosphate

***E. coli*:** *Escherichia coli*

EDTA: Ethylene diamine tetra-acetic acid

IPTG: Isopropyl-β-D-thiogalactopyranoside

LB: Luria-Bertani

OD: Optical density

PBS: Phosphate buffered saline

rpm: Revolutions per minute

RT-PCR: Reverse transcriptase polymerase chain reaction

SDS-PAGE: Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SOC media: Super optimal catabolite repression media

TAIR: The Arabidopsis Information Resource

TBE: Tris-borate EDTA

UV: Ultraviolet

YT: Yeast Tryptone

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ABSTRACT

Adenylate cyclases (ACs) are a diverse group of enzymes, which catalyze the formation of 3′,5′ cyclic adenosine monophosphate (cAMP) from adenosine 5′-triphophate (ATP). Cyclic AMP is a key secondary messenger known to be involved in the mediation of responses to various extracellular stimuli in nearly all living organisms. Recently, a total of 14 candidate AC-encoding proteins in the *Arabidopsis thaliana* genome were identified and proposed through a bioinformatics approach based on the functionally assigned amino acids residues in the catalytic centre of annotated nucleotide cyclases. Among these identified candidates, is a G-3 family protein (G-3FP), which harbours a novel adenylate cyclase catalytic centre and is encoded by the At2g11890 Arabidopsis gene. Incidentally, to date, there are only 4 annotated and experimentally confirmed ACs in plants, which are the *Zea mays* pollen protein (Moutinho *et al.*, 2001), the *Arabidopsis thaliana* pentatricopeptide repeat protein (Ruzvidzo *et al.*, 2013), the *Nicotiana benthamiana* adenylyl cyclase protein (Ito *et al.*, 2014) and the *Hippeastrum hybridum* adenylyl cyclase protein (Świeżawska *et al.*, 2014). Therefore, with a view to attempt and identify other additional higher plant AC candidates, this study was set out to clone, partially express and functionally characterize the annotated G-3FP protein. Findings from this approach unequivocally demonstrated that this putative protein candidate is indeed a *bona fide* functional higher plant AC molecule.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

This research study specifically focused onto those plant molecules which may be involved in signal transduction pathways of stress response and adaptation mechanisms and have since been the main focal point of research in plant biotechnology. The plant molecule which was studied in this research study is an adenylate cyclase (AC), which is an enzyme that catalyzes the formation of 3′,5′-cyclic adenosine monophosphate (cAMP) and a pyrophosphate from adenosine 5′-triphosphate (ATP) as is shown in Figure 1.1 below (Gehring, 2010).

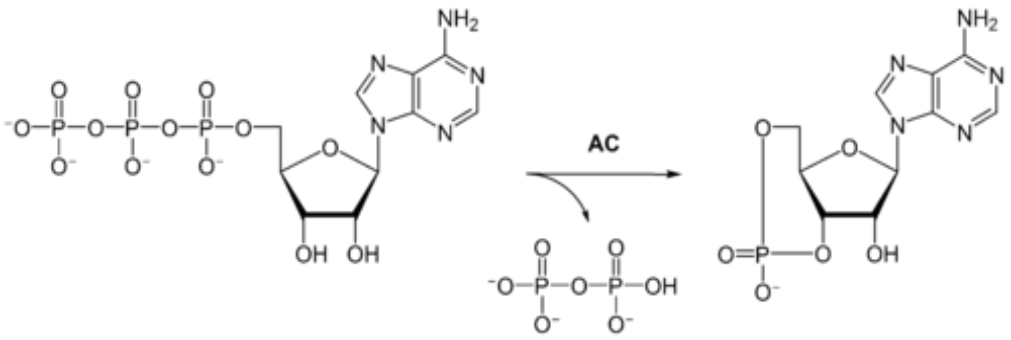

Fig 1.1: The formation of cyclic adenosine monophosphate (cAMP) from ATP (adenosine triphosphate) by action of the enzyme adenylate cyclase (AC) (Gehring, 2010).

While the world population is on the increase, the arable land size has remained constant (Abah *et al.*, 2010). On the other hand, adverse climatic conditions continue to affect agronomic crops, justifying the need to study biological molecules that may bring about stress-resistance in crop plants. To this effect therefore, experiments undertaken on novel ACs will at least give a better understanding of the possible role of the cAMP as a second messenger in plant development and responses to environmental stimuli and/or hormones (Gehring, 2010). In many developing countries, particularly in Africa, crop yields have decreased dramatically and climate change has been considered to have a major impact on food security and sustainable agriculture (Abah *et al.*, 2010). About 70% of the population in Africa is rural and it strongly relies on farming as its main economic and social activity, thus

the issue of food security is of paramount concern (Abah *et al.*, 2010). On another note, the world population is expected to double over the next generation, which, therefore, translates into a critical doubling of food demand in the world (Mccalla, 1999).

Modern technology that is being explored to attempt and facilitate the world to meet its food needs is being driven by the fact that billions of people struggle for a better life in developing countries (Huang *et al.*, 2002). Hence biotechnology is one of the current and modern technologies that can be used as a tool to integrate recombinant DNA techniques into our conventional breeding programs, in order to substantially enhance the efficiency of agricultural research and development. However, agricultural production challenges cannot be solved through the use of biotechnology alone. This is despite evidence that biotechnology has the capacity to increase crop production, the diversification of crops, the enhancement of nutritional values of food, the reduction in environmental impacts on agricultural production and the promotion of market competitiveness (Abah *et al.*, 2010).

By 2010, fourteen candidates from *Arabidopsis thaliana* had been bioinformatically identified using a search motif, which had specificity for ACs (Figure 1.2). These molecules were all shown to contain the putative AC catalytic centre (Gehring, 2010). Among them was the G-3 family protein (G-3FP), which was studied in this reported study (Gehring, 2010). At present, there are only four functionally tested and experimentally confirmed ACs in plants namely; the pollen protein from *Zea mays*, which is responsible for the polarized growth of pollen tubes (Moutinho *et al.*, 2001), the pentatricopeptide repeat protein from *Arabidopsis thaliana*, which is responsible for pathogen responses and gene expressions (Ruzvidzo *et al.*, 2013), the adenylyl cyclase protein from *Nicotiana benthamiana*, which is responsible for the tabtoxinine-β-lactam-induced cell deaths that occur during wildfire diseases (Ito *et al.*, 2014), and the adenylyl cyclase protein from *Hippeastrum hybridum* involved in stress signaling (Świeżawska *et al.*, 2014).

This research study therefore, specifically focused onto the AC-containing domain of the G-3FP protein to assess and determine its possible function, and most importantly, in relation to plant responses and adaptation to the various biotic/abiotic environmental stress factors.

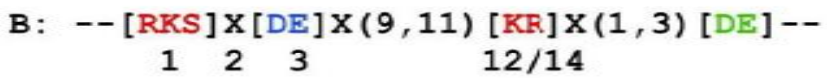

Fig 1.2: The AC catalytic search motif used to identify the G-3FP and other AC protein candidates in *Arabidopsis thaliana* (Gehring, 2010).

1.2 Literature Review

One of the most significant findings in biochemistry was the discovery of the biological molecule, cyclic 3′,5′-adenosine monophosphate (cAMP) by Earl Sutherland in the late 1950's (Rall and Sutherland, 1958). By the mid-1970s, the action of this molecule had been conclusively established as an important signaling chemical and a second messenger in both animals and lower eukaryotes (Goodman *et al.*, 1970; Gerisch *et al.*, 1975). In the past half century, cyclic nucleotides (both cAMP and cGMP) had been implicated in a vast array of biological phenomena in all kingdoms of life (Bridges *et al.*, 2005). Therefore, the discovery of the importance of adenylate cyclases (ACs) and their product cAMP in all other organisms had prompted scientists to also learn this signaling system in plants. However, the levels of cAMP detected in plants were very low compared to those found in animals and lower eukaryotes and for this reason, the acceptance that cyclic nucleotides play a role in plant signaling was rather delayed (Newton and Smith, 2004; Gehring, 2010). The level of cAMP detected in plants was <20 pmol/g fresh weight (Ashton and Polya, 1978) while in animals it was >250 pmol/g fresh weight (Butcher *et al.*, 1968). Furthermore, the unpredictability or erratic nature of assays conducted on plants during that time was also not very conducive for reaching firm conclusions (Amrhein, 1977).

All the same, plants would probably also have a functional cAMP-dependent signal system similar to that in animals and lower eukaryotes still remained alive despite the low, seemingly un-physiological and certainly not animal-like levels of cAMP in plants. This was mainly because of the fact that both the cell permeant 8-Br-cAMP and the stimulation of, *albeit* unknown, ACs with forskolin could elicit concentration and time-dependent biological responses such as increases in Ca^{2+} influx across the plasma membrane (Kurosaki and Nishi, 1993). Furthermore, very low concentrations of yet another cyclic nucleotide, cGMP (<0.4 mol/g fresh weight), were reported in plants whose molecules played a physiological role in specific responses to virulent pathogens and defensive mechanisms, thus demonstrating and proving that signaling in plants at lower molecular levels was very feasible and commonplace (Meier *et al.*, 2009).

To date, various research groups have invested considerable efforts into finding ACs particularly in *Arabidopsis thaliana* because of the important role cAMP plays in cell signaling (Moutinho *et al.*, 2001). Cyclic AMP may have an important role in abiotic stress responses and in particular, responses to NaCl stress since voltage independent channels (VIC) in *Arabidopsis thaliana* roots have been reported to have open probabilities sensitive to

micromolar concentrations of both cAMP or cGMP at the cytoplasmic site (Maathuis and Sanders, 2001). Thus the importance of cyclic nucleotides and their diverse roles in plant signaling systems may be via cyclic nucleotide responsive protein kinases, as well as binding proteins and gated ion-channels (Newton *et al.*, 2004). Given the diverse roles, it is therefore unlikely that a single AC and/or GC could account for all the cAMP- and/or cGMP-dependent processes observed in higher plants (Newton *et al.*, 2004). In line with this hypothesis, a number of *Arabidopsis* molecules with different domain organizations and experimentally confirmed GC activity have also been reported (Ludidi and Gehring, 2003; Meier *et al.*, 2010) and hence it is also most likely that similar findings will hold true for ACs.

Therefore in the year 2010, a fourteen amino acid long search motif deduced from an alignment of conserved and functionally assigned amino acids in the catalytic centre of annotated nucleotide cyclases from lower and higher eukaryotes (Ludidi *et al.*, 2003) was used to search the *Arabidopsis thaliana* genome (Figure 1.3) (Gehring, 2010). The search returned a total of fourteen putative AC candidates of which the G-3FP protein was one among them. In the modified AC search motif, the amino acid residues that confer substrate specificity (position 3 in Figure 1) was substituted with [DE]. Consequently, the core motif within the catalytic centre consists of the functionally assigned residue that does the hydrogen bonding with the adenine (position 1), the amino acid that confers substrate specificity for ATP (position 3) and the amino acid that stabilizes the transition state from ATP to cAMP ([K,R], position 12-14).

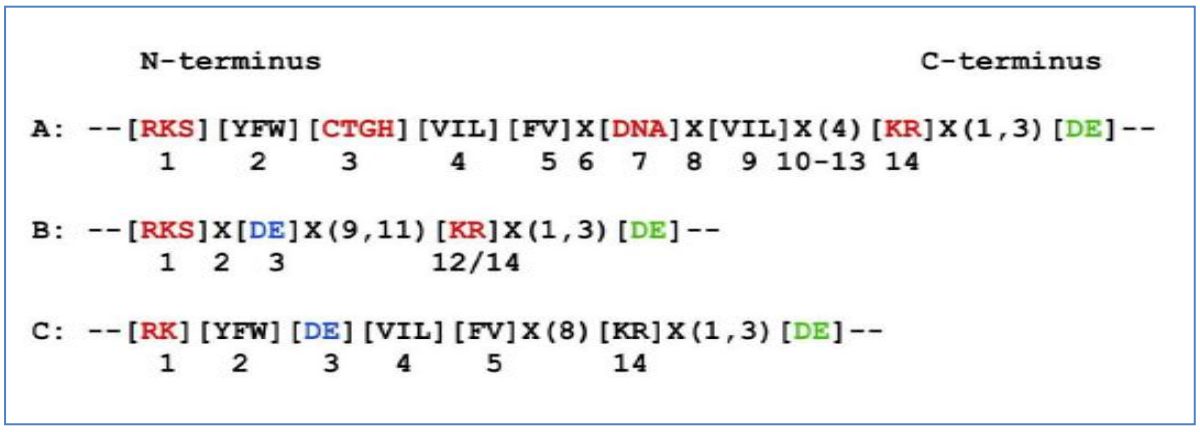

Fig 1.3: Catalytic centre motifs of nucleotide cyclases. (A) Catalytic domain of GCs which have been tested in plants. The hydrogen bonding between hydrogen and guanine are made possible by the residue (red) in position, the amino acid in position 3 confers substrate specificity and the residue in position 14 stabilizes the transition (GTP/cGMP). The Mg^{2+}/Mn^{2+}-binding site is C-terminal (green). In the resultant motifs (B and C) specific for ACs, position 3 (blue) has been substituted to [DE] to allow for ATP binding (Gehring, 2010).

1.2.1 Signal Transduction

Signal transduction underlines the mechanisms through which cells, tissues and whole organisms perceive and process signals (Irving and Gehring, 2013). As such, the process is key to development (Kaplan *et al.*, 2007). Plants are sessile living organisms that are constantly subjected to a number of abiotic and biotic stress factors in their natural habitats, however, plants are forced to acclimatize and respond flexibly to such aggressive conditions by switching their cell metabolism and physiological activities in response to environmental conditions (Lichtenthaler, 1996). In view of the above-given premises, it is outright that plants are sensitive organisms in need of really proactive biological mechanisms that will aid in their survival and also ensuring food security through the tolerance and continued adaptation to various stress factors.

1.2.2 The At2g11890 Gene

The At2g11890 gene encodes for a G-3 family protein (G-3FP). The G-3FP protein functions in ATP binding, ATPase activity and triphosphate activity. It is involved in root development according to the Arabidopsis Information Resource (TAIR) (http://www.arabidopsis.org). The protein is expressed during various growth stages including the 4 leaf senescence stage, C globular stage, D bilateral stage, and expressed in the carpel, cauline leaf, leaf apex, root and seed. The At2g11890 is one of the many bioinformatically annotated higher plant adenylate cyclases which have not been characterized as yet. Hence, its isolation and subsequent functional characterization through the use of various modern molecular biology techniques will entirely provide clear insights into its physiological roles of these special types of molecules, particularly in plant stress response and adaptation mechanisms.

1.3 Problem Statement

The G-3FP protein (At2g11890) in *Arabidopsis thaliana* is one of the few plant proteins that have recently been annotated as possible plant ACs (adenylate cyclases) but no study has yet been undertaken to directly characterized this protein and possibly establish it as a possible functional plant AC (Gehring, 2010). Even though the G-3FP from *A. thaliana* has been annotated as a possible plant AC, its function as a signaling molecule has not yet been

elucidated. A molecular understanding of this biological molecule will undoubtedly advance current scientific knowledge on plant molecules responsible for environmental stress response and adaptation mechanisms. Subsequent to its potential confirmation as a functional adenylate cyclase, it is also very logical that its biological role as a novel protein candidate in cAMP-dependent cell signaling and adaptation mechanisms to various environmental stress factors be further elucidated.

1.4 Aim of the Research Study

Currently there are only 4 known plant ACs, the *Zea mays* pollen protein (Moutinho *et al.*, 2001), the *Arabidopsis thaliana* pentatricopeptide repeat protein (Ruzvidzo *et al.*, 2013), the *Nicotiana benthamiana* adenylyl cyclase protein (Ito *et al.*, 2014) and the *Hippeastrum hybridum* adenylyl cyclase protein (Świeżawska *et al.*, 2014). The study also targeted at investigating on whether such a novel biological molecule would be capable of mediating plant responses to different environmental stimuli, mainly biotic and abiotic stress factors. The study aimed at identifying a fifth ever higher plant AC through concerted experimental studies of a putative G-3FP from *A. thaliana*.

1.5 Objectives of the Research

The specific objectives of this study were as follows:

1. To isolate the annotated G-3FP-AC gene fragment from *Arabidopsis thaliana*.
2. To clone the G-3FP-AC gene fragment into a stable and viable heterologous prokaryotic expression system.
3. To optimize the expression regimes and processes of the recombinant G-3FP-AC protein.
4. To determine the adenylate cyclase enzymatic activity of the recombinant G-3FP-AC protein.
5. To bioinformatically determine the functional role of this annotated G-3FP protein in plants.

1.6 Significance of the Research Study

Upon the successful completion of this reported research study, the following significances were set to be accomplished:

1. Another functional adenylate cyclase (AC) in higher plants was going to be established besides the only and currently known four.
2. The project was going to assist future related projects to have a clearer and better understanding on the roles of adenylate cyclases in plants.
3. This work was going to produce some novel outcome(s), which would contribute towards the production of new literature and future scholarships in the broader domain of modern sciences.
4. A better understanding of the functionality aspects of this group of enzymes was going to be vital to both scientists and agriculturists as this could be applied into the improvement of crop yields and the subsequent eradication of hunger and poverty.

CHAPTER TWO

RESEARCH METHODOLOGIES

2.1 Regeneration and Growth of *Arabidopsis thaliana* Plants

2.1.1 Seed Sterilization and Stratification

About 100 *Arabidopsis thaliana* ecotype Columbia seeds were collected in a sterile micro-centrifuge tube and were washed with 70% ethanol through vortexing for 30 seconds and the ethanol subsequently removed. The washing step was performed three times. The seeds were then further washed three times with an aliquot of sterilization buffer (50% (v/v) commercial bleach and 0.1% (v/v) Triton-X) under a sterile laminar flow.. The seeds were rinsed five times with 500 µl of filter-sterilized deionized water with intermittent vortexing for 30 seconds. Thereafter, the seeds were re-suspended in sterile water and stratified for three days at 4°C. The stratification process is a seed treatment process used to overcome some kinds of chemical dormancy and this ensuring a uniform and synchronized germination of the treated seeds (Gupta *et al.*, 2011).

2.1.2 Plant Regeneration and Growth Conditions

Following stratification, the *Arabidopsis thaliana* ecotype Columbia seeds were allowed to germinate and grow on Murashige and Skoog (MS) medium (0.43% w/v of organic salts, 3% sucrose and 0.4% w/v tissue culture grade agar supplemented with 1 ml/l of Gamborg's vitamins) at pH 5.7 for a period of 2 weeks in a growth chamber (Growth chamber system, Lab Companion, Australia). The germinated seedlings were then transplanted into potting soil composed up of a 1:1:1 (w/w) vermiculite, humus and potting mix soil, respectively. The transplanted seedlings were watered with sterile distilled water and then covered with a plastic cling wrap to retain moisture; and were allowed to grow in a growth chamber set at the following growth conditions: 23°C day/night for periods 16 hours day and 8 hours night with the light for the day times set at 10,000 lux. The seedlings were kept in the growth chamber for a further 2-4 weeks.

2.1.3 Extraction of Total mRNA from the *Arabidopsis thaliana* Plants

Approximately 100 mg of Arabidopsis leaves were harvested from the 6-week old *Arabidopsis thaliana* plants and total RNA was then extracted according to the manufacturer's protocol of the Gene Jet Plant RNA Purification Mini Kit, Catalogue # K0801 (Thermo Scientific, Massachusetts, USA). Briefly, the leaf material was placed in a cooled (-80°C) sterile mortar and immediately snap-frozen in liquid nitrogen (Afrox Industrial Gases, Klerksdorp, South Africa), then it was grounded into a fine powder using a cooled (-80°C) sterile pestle. The tissue powder was immediately transferred into a 1.5 ml micro-centrifuge tube containing 500 µl of the Plant RNA Lysis solution. The tube was then vortexed for 10-20 s and mixed thoroughly. The micro-centrifuge tube was centrifuged for 5 minutes at ≥16,300 x *g* (≥14,000 rpm). The supernatant (~500 µl) was thereafter transferred to a clean micro-centrifuge tube. A volume of 250 µl of the 96% ethanol was added and mixed by pipetting. The prepared mixture was then transferred to a purification column inserted into a collection tube and centrifuged for 1 minute at 12,000 x *g* (~11,000 rpm). The flow-through was discarded while the column and its collection tube were re-assembled. The purification column was washed using 700 µl of the Wash buffer 1 and centrifuged for one minute at 12,000 x *g* (~11,000 rpm). The flow-through and the collection tube were discarded. The purification column was then placed into a clean collection tube and washed again with 500 µl of the Wash buffer 2 at 12,000 x *g* (~11,000 rpm) for 1 minute. Finally, the purification column was placed into a clean collection tube and 50 µl of nuclease-free water added directly onto the purification column membrane and centrifuged for 1 minute at 12,000 x *g* (~11,000 rpm) to elute the mRNA. The purification column was discarded and the collection tube with its eluted RNA was stored at -20°C until it was needed.

2.2 Isolation and Amplification of the G-3FP-AC Gene Fragment

2.2.1 Designing and Acquisition of Sequence-specific Primers

The genomic sequence of the G-3FP-AC protein from *Arabidopsis thaliana* was searched for from the *Arabidopsis thaliana* genome using the locus details (*Arabidopsis thaliana* gene accession numbers) as was described by Gehring (2010). The amino acid sequences of the At2g11890 gene fragment encoding the novel G-3FP-AC protein was then retrieved from The Arabidopsis Information Resource (TAIR) (http://www.arabidopsis.org). From the primary protein structure, the forward and reverse primers were then designed with the use of

the Expasy and TAIR programs. Two sequence-specific primers were then manually designed based on the G-3FP-AC gene sequence. The primers were designed in such a way that they would flank and amplify the desired and targeted AC catalytic domain of the G-3FP-AC gene fragment. The designed primers were then sent for chemical synthesis and subsequent supply by the Inqaba Biotechnological Sciences (Pretoria, South Africa). The sequences of the two specifically designed primers are shown in Figure 2.1 below:

***The forward G-3FP-AC primer*:**

5′- gAA gTC gAA gTC AAg CTC CgT CTC CTA ACC -3′

***The reverse G-3FP-AC primer*:**

3′- TTT gCT gTT TTC Cgg TCA ggA AAA CTT CCT -5′

Fig 2.1: The two manually designed sequence-specific G-3FP-AC gene primers. The forward primer is in red while the reverse is in green.

The complete amino acid sequence of the G-3FP-AC protein, indicating its AC catalytic center and its specific priming sites is shown below in Figure 2.2.

```
  1 MEVEVKLRLL TAAAHLRLTT LLTPYHLKTL HQRNTFFDTP KNDLSLRRAV
 51 LRLRFLQNAA VSAASPSPPR CIVSLKAKPT LANGISRVEE DEEEIEYWIC
101 NECVESPAKL SDIGSRVLKR VKEEYGFNDF LGFVCLGGFE NVRNVYEWRG
151 VKLEVDETKY DFGNCYEIEC ETEEPERVKT MIEEFLTEEK IEFSNSDMTK
201 FAVFRSGKLP
```

Fig 2.2: The complete amino acid sequence of the G-3FP-AC protein. The protein consists of a total of 210 amino acids harbouring an adenylate cyclase catalytic motif (red). The underlined and bolded sequences indicate the AC priming sites of this putative protein fragment.

2.2.2 Preparation of the RT-PCR Reaction Mixture

The G-3FP-AC gene fragment was synthesized from the total RNA of the *Arabidopsis thaliana* plants. Essentially, this *A. thaliana* total RNA was used as a template to generate copy DNA (cDNA). The generated cDNA was then simultaneously used together with the acquired sequence-specific primers to amplify the targeted G-3FP-AC gene fragment in a reverse transcriptase-polymerase chain reaction (RT-PCR) system using a Thermo Scientific

Verso 1-Step RT-PCR Reddy Mix kit (Fermentas International Inc., Burlington, Canada), and as is shown in the Table 2 below.

Table 2.1: Reaction components of the 1-step RT-PCR reaction mixture for the specific amplification of the G-3FP-AC gene fragment in a total volume of 50 µl.

COMPONENT	VOLUME	FINAL CONCENTRATION
Verso Enzyme Mix	1 µl	
1-Step PCR Reddy Mix (2X)	25 µl	1X
Forward Primer (10 µM)	1 µl	200 nM
Reverse Primer (10 µM)	1 µl	200 nM
RT Enhancer	2.5 µl	
Water (PCR Grade)	14.5 µl	
Template (mRNA)	5 µl	1 ng
Total Volume	50 µl	

The RT-PCR process was then carried out on a C 1000 thermo-cycling system (Bio-Rad Laboratories Inc, California, USA) and in accordance with the Thermo Scientific Verso ™ 1-Step RT-PCR ReddyMix™ system (Fermentas International Inc., Burlington, Canada), (Table 2.2 below).

Table 2.2: The 1-Step RT-PCR thermal cycling program utilized for the specific amplification of the G-3FP-AC gene fragment.

STEP	TEMPERATURE	TIME	NUMBER OF CYCLE
cDNA Synthesis	50ºC	15 min	1 cycle
Thermo-Start Activation	95ºC	15 min	1 cycle
Denaturing	95ºC	20 sec	↕ 45 cycles
Annealing	52ºC	30 sec	
Extension	72ºC	1 min	
Final Extension	72ºC	5 min	1 cycle

2.2.3 Agarose Gel Electrophoresis of the Amplified G-3FP-AC Gene Fragment

The amplified RT-PCR products in section 2.2.2 above were resolved on a 1 % agarose gel supplemented with 0.5 µg/ml ethidium bromide to stain the DNA. The amplified RT-PCR fragments were then resolved against a 100 base-pair Gene-Ruler™ DNA ladder (Fermentas International Inc., Burlington, Canada) and immersed in a 1X TBE buffer (62.5% Tris-HCl, 31.8% boric acid and 5.7% EDTA) at 80 volts and 250 mA current for 50 minutes. Thereafter, the gel was visualized by means of a UV light system on a 2000 UV trans-illuminator system (Bio-Rad Laboratories Inc., California, USA) and the resultant images finally captured with a gel documentation system, Chemi Doc ^{TM} Imaging System, (Bio-Rad Laboratories Inc., California, USA).

2.3 Recombinant Cloning of the Amplified G-3FP-AC Gene Fragment

The amplified G-3FP-AC gene fragment in section 2.2.2 above was ligated into a commercially-acquired pTrcHis2-TOPO® expression vector (Figure 2.3) (Invitrogen, Carlsbad, USA) and as is briefly outlined below. This pTrcHis2-TOPO® expression vector allows for a fast, high level, efficient and well-regulated cloning system of PCR products in various prokaryotic expression systems harbouring the *trc* operon.

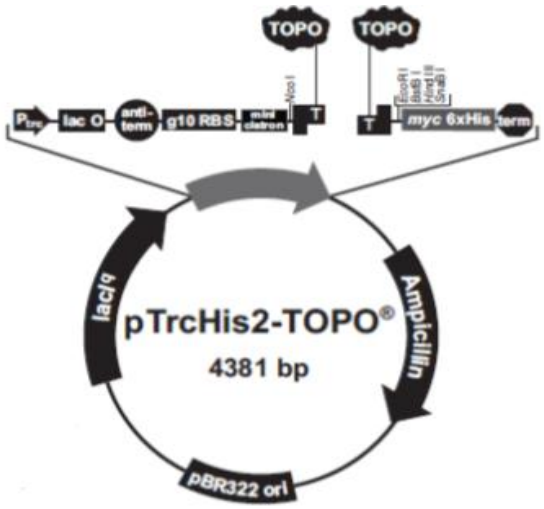

Fig 2.3: The commercially-acquired pTrcHis2-TOPO plasmid expression vector used in this study (Invitrogen, Carlsbad, USA). Overview of the circular map of the pTrcHis2-TOPO® vector showing the position of its 6xHistidine tag promoter, and a point of origin to facilitate replication of the plasmid in bacterial cells such as *E. coli*. In addition, there is an ampicillin resistant gene that allows for the effective screening of all positive recombinants, and other various cloning sites and specific features of the vector. (Adapted from www.lifetechnologies.com).

2.3.1 Addition of the 3'-adenine Overhangs

A volume of 1 µl 1U *Taq* polymerase was added to the 40 µl of the RT-PCR product reaction mixture (the amplified G-3FP-AC gene fragment) and the mixture then incubated at 72ºC for 10 minutes on a C1000 Thermo-cycling System (Bio-Rad Laboratories Inc., California, USA). The resultant reaction mixture was then kept on ice for further use.

2.3.2 Ligation of the Adenylated G-3FP-AC Gene Fragment into the pTrcHis2-TOPO Plasmid Expression Vector

An aliquot of 4 µl was collected from the adenylated G-3FP-AC gene fragment reaction mixture and transferred into a fresh PCR tube before a 1 µl of the pTrcHis2-TOPO expression vector (Invitrogen, Carlsbad, USA) was added. Following this, the ligation mixture was then incubated at room temperature for 5 minutes before its subsequent use for the transformation process of competent *E. coli* One Shot TOP10 competent expression cells (Invitrogen Corp., Carlsbad, USA).

2.3.3 Transformation of Competent One Shot® TOPO 10 *E. coli* Cells with the pTrcHis2-TOPO:G-3FP-AC Fragment Construct

Immediately after the ligation process in section 2.3.2 above, 2 µl of the ligation mixture (pTrcHis2-TOPO:G-3FP-AC Expression Construct) were added into an ice-cold micro-centrifuge tube containing about 100 µl of the TOP10 One Shot chemically competent *E. coli* cells. The reaction mixture was gently mixed by swirling and incubated on ice for 30 minutes. The reaction mixture was thereafter heat-shocked on a dry-bath heating block for 30 seconds at 42ºC before being immediately incubated on ice for 5 minutes. Subsequently, the reaction mixture was supplemented with 250 µl of the Super Optimal broth with Catabolite (SOC) repressor (glucose) medium (2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, and 10 mM $MgCl_2$ and 10 mM Mg_2SO_4, and 20 mM glucose) and incubated in a shaker at 37ºC at 200 rpm for 30 minutes (this incubation step is normally carried out in order to allow cells to produce the β-lactamase enzyme, which would later and during the selection process, detoxify ampicillin). The mixture was afterwards, plated in aliquots of 20 µl and 80 µl, onto two respective Luria Bertani (LB) agar plates (1% (w/v) agar, 1% (w/v) tryptone powder, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl) supplemented

with 100 µg/ml ampicillin and 0.5% glucose. The plates were then incubated overnight at 37ºC.

2.3.4 Growth of the Transformed Cultures

Pre-cultures of the plated cultures above (section 2.3.3) were set-up with double strength yeast-tryptone (2YT) broth (0.8% (w/v) tryptone powder, 0.5% (w/v) yeast extract and 0.25% (w/v) NaCl) supplemented with 100 µg/ml ampicillin and 0.5% of glucose, whereby a single colony of the cells was picked up and inoculated into 5 ml of growth medium. The prepared tubes (falcon) were then incubated overnight at 37ºC with shaking at 200 rpm.

2.3.5 Extraction of the pTrcHis2-TOPO:G-3FP-AC Plasmid Construct from the Transformed Competent *E. coli* One Shot® TOP 10 Cells

The cells from above (2.3.4) were harvested by centrifuging at a speed 6 800×*g* for 5 minutes at room temperature and the supernatant was discarded. Plasmid extraction was then performed at room temperature using the Gene Jet Plasmid Miniprep Kit (Thermo Fisher Scientific Inc., California, USA), and as follows: The pelleted cells were briefly resuspended into 250 µl of the Re-suspension Solution supplemented with RNase through pipetting up and down and/or until no cell clumps had remained, and the mixture was then transferred into a sterile micro-centrifuge tube. This was followed by the addition of 250 µl of Lysis Solution with thorough mixing by inverting the tube 4-6 times or until the solution had become less viscous and slightly clear. Thereafter, 350 µl of the Neutralization Solution was added and mixed immediately and thoroughly by inverting the tube 4-6 times. The mixture was subsequently clarified by centrifuging for 5 minutes at 16 300×*g* and the supernatant transferred into the supplied Gene Jet spin column. The spin column, fitted into a collection tube, was centrifuged for 1 minute at 16 300×*g* and its flow-through discarded while the column was again placed into the same collection tube. Approximately 500 µl of the Wash Solution (diluted with 96% ethanol prior to its first use) was added to the Gene Jet spin column and the column was further centrifuged for 30-60 seconds at the same speed. The flow-through was discarded while the column was placed back into the same collection tube. The washing procedure was repeated twice more before the Gene JET Spin column was centrifuged at 16 300×*g* for an extra 1 minute to remove any residual wash solution. The washed and dried GeneJET spin column was straightaway transferred into a sterile micro-

centrifuge tube where the plasmid DNA was then eluted by adding 50 µl of the Elution Solution to the center of the GeneJET spin column membrane followed by an incubation of 2 minutes at room temperature and centrifugation at 16 300×*g* for 2 minutes. The used column was thereafter discarded while the purified plasmid DNA was stored at -20ºC for further use.

2.3.6 Analysis of Positive Clones

Confirmation of positive clones was carried out by normal and conventional PCR on a C1000 Thermo-cycler system (Bio-Rad Laboratories Inc., California, USA) and in accordance with the Standard MyTaq™ Mix protocol (Bioline, London, UK), to confirm whether the G-3FP-AC gene insert had been successfully cloned into the pTrcHis2-TOPO™ plasmid expression vector and whether such a cloning outcome was in the correct orientation. Successful cloning was confirmed with the amplification of the insert using both of its own primers (forward and reverse primers), and the correct orientation was verified by using the vector's reverse primer and the insert's forward primer. The reaction mixtures for the two stated processes are respectively shown in Tables 2.3 and 2.4 below while the associated thermal cycling conditions for both processes are shown in Table 5 (below).

Table 2.3: Reaction components of a PCR reaction mixture to confirm the successful cloning of the G-3FP-AC gene insert into the pTrcHis2-TOPO™ plasmid expression vector.

Component	Volume
Plasmid (DNA)	2 µl
Insert Primers (20 µM each, 1 µl each)	2 µl
MyTaq Red Mix, (2X)	25 µl
Water (sdH_2O)	21 µl

Table 2.4: Reaction components of a PCR reaction mixture to confirm the correct orientation of the G-3FP-AC gene insert into the pTrcHis2-TOPO™ plasmid expression vector.

Component	Volume
Plasmid (DNA)	2 µl
Insert and Vector Primers (20 µM each, 1 µl each)	2 µl
MyTaq Red Mix, (2x)	25 µl
Water (sdH_2O)	21µl

Table 2.5: The reaction thermal cycling conditions for the step by step assessment episode of the successful cloning and correct orientation of the G-3FP-AC gene insert into the pTrcHis2-TOPO™ expression vector.

Step	Temperature	Time	Cycles
Initial Denaturation	95ºC	1 minute	1
Denaturation	95ºC	15 seconds	25-35
Annealing	52ºC	15 seconds	
Extension	72ºC	10 seconds	

2.3.7 Agarose Gel Electrophoresis of the Correctly Cloned G-3FP-AC Gene Fragment

The PCR products from both Tables 2.3 and 2.4 were resolved on a 1% agarose gel stained with ethidium bromide and immersed in a 1X TBE buffer at a voltage of 80 volts, 250 mA for 50 minutes. The samples were resolved against a 1 kilo-base Thermo Scientific GeneRuler™ DNA ladder and visualization was undertaken under UV light using a 2000 UV trans-illuminator system (Bio-Rad Laboratories Inc., California, USA). The Chemi Doc Imaging System (Bio-Rad Laboratories Inc., California, USA) was subsequently used to capture the resultant images. Double amplification of the targeted G-3FP-AC gene insert in both reaction samples (Tables 2.3 and 2.4 respectively) was to correspondingly confirm the successful and positive cloning of the G-3FP-AC gene insert into the pTrcHis2-TOPO™ plasmid expression vector.

2.4 Partial Expression of the Recombinant G-3FP-AC Protein

2.4.1 Transformation of the *E. cloni*® EXPRESS Chemically Competent BL21 (DE3) pLysS DUOs with the G-3FP-AC Fusion Expression Construct

Immediately after the cloning process of the G-3FP-AC gene insert into the pTrcHis2-TOPO™ plasmid expression vector had been positively confirmed, its resultant expression construct (pTrcHis2-TOPO:G-3FP-AC) was then used to transform some chemically competent *E. cloni* EXPRESS BL21 (DE3) pLysS DUOs cells. The transformation was carried out in a sterile micro-centrifuge tube and in accordance with the pTrcHis2-TOPO™ TA Expression protocol and instructions (Lucigen, Wisconsin, USA), whereby a 1 µl of the pTrcHis2-TOPO:G-3FP-AC fusion construct was aseptically added to 40 µl of the ice-cold chemically competent *E. cloni* EXPRESS BL21 (DE3) pLysS DUOs cells. The mixture was then incubated on ice for 30 minutes before being heat-shocked for 45 seconds at 42ºC and immediately placed on ice for 2 minutes. The reaction mixture was then supplemented with 250 µl of the Expression Recovery Media (Lucigen Inc, Wisconsin, USA) and incubated at 37℃ in an orbital shaker at 250 rpm for 60 minutes. This step allows cells to start expressing the antibiotic-resistance enzyme (β-lactamase), which later was used for the selection criteria of the transformed and recombinant cells. The reaction mixture was thereafter plated onto 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 34 µg/ml chloramphenicol and the plates incubated overnight at 37ºC.

2.4.2 Confirmatory PCR for the Recombinant pTrcHis2-TOPO®:G-3FP-AC

The confirmatory PCR was conducted in accordance with the Standard MyTaq™ Mix protocol (Bioline, London, UK), to ascertain that the expression host cells indeed were carrying the recombinant pTrcHis2-TOPO®:G-3FP-AC. In this regard, the reaction mix was prepared as is in Table 2.3 but replacing the plamsd DNA with an intact transformed colony (section 2.4) as a template and the thermal cycling program was again then run as is shown in Table 2.5 above. Following the PCR reaction, a 1% agarose gel was then run and the gel was subsequently visualized by means of UV light using a 2000 UV trans-illuminator system (Bio-Rad Laboratories Inc., California, USA) and the associated image captured with a Chemi Doc™ Gel Documentation Imaging System (Bio-Rad Laboratories Inc., California, USA).

2.4.3 Recombinant Protein Expression

The transformed chemically competent *E. cloni* EXPRESS BL21 (DE3) pLysS DUOs colonies harbouring the pTrcHis2-TOPO:G-3FP-AC expression construct were used to inoculate 20 ml of the 2YT media supplemented with 0.2% glucose, 34 µg/ml chloramphenicol and 100 µg/ml ampicillin in a 50 ml falcon tube. The falcon tubes were then incubated overnight at 37ºC with shaking at 200 rpm. On the subsequent day, 200 µl of the overnight transformation culture were used to inoculate 20 ml of fresh 2YT media containing 34 µg/ml chloramphenicol and 100 µg/ml ampicillin and 0.2% glucose. The culture was then incubated at 37ºC, shaking at 200 rpm and until an OD_{600} of 0.6 was reached (which was measured by the Helios spectrophotometer (Merck, Gauteng, RSA)). Immediately, the culture was split into two 15 ml falcon tubes, each containing 5 ml. One culture was induced to express the intended G-3FP-AC recombinant protein by adding 1 mM of the isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma-Aldrich Corp., Missouri, USA) while the other culture remained uninduced (control). The split cultures were then shaken in an incubator (200 rpm) at 37ºC for 3 hours. After the 3 hours, 500 µl samples of each culture were transferred into micro-centrifuge tubes and centrifuged at 8 000×*g* for 5 minutes to pellet the cells. The supernatant was discarded while the pelleted cells were then analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.5 Activity Assaying

2.5.1 Determination of the Endogenous Activity of the Recombinant G-3FP-AC Protein

An overnight culture of cells confirmed to be harbouring the recombinant pTrcHis2-TOPO:G-3FP-AC expression construct were prepared using 200 µl of its glycerol stock to inoculate 20 ml of fresh 2YT media supplemented with 100 µg/ml of ampicillin and 34 µg/ml of chloramphenicol. The culture was grown overnight at 37ºC in a shaking incubator at 200 rpm. The following day, a fresh 100 ml 2YT media containing 100 µg/ml ampicillin and 34 µg/ml chloramphenicol was inoculated with 1 ml of the overnight culture and incubated at 37ºC in a shaker at 200 rpm and up until its OD_{600} has reached 0.6. The culture was thereafter, immediately placed onto ice and split into four separate but equal portions of 3 ml each. Protein expression was induced by the addition of 1 mM IPTG into three cultures while one tube was left un-induced (control). From two of the three induced cultures, one

culture was supplemented with 100 µM forskolin (Sigma-Aldrich Corp., Missouri, USA) while the other culture supplemented with 100 µM 2',5'-dideoxyadenosine (Sigma-Aldrich Corp., Missouri, USA). All four cell cultures were then incubated at 37ºC for a further 2 hours before the cells were harvested by centrifugation at 9 200×*g* for 10 minutes.

The harvested cells were lysed in 1 ml lysis buffer 1 (Amersham Healthcare, California, USA) supplemented with 2 mM 3-isobutyl-1-methylxanthine (IBMX) (Sigma-Aldrich Corp., Missouri, USA) to inhibit phosphodiesterases. The samples were essentially shaken at 100 rpm, at 37ºC for 30 minutes on an orbital shaker to intensify the cell lysis process. The samples were then centrifuged at 9 200×*g* for 5 minutes and their lysates transferred into a fresh micro-centrifuge tubes where 200 µl of the lysis buffer 2 (Amersham Healthcare, California, USA) was added and mixed. Thereafter, 220 µl of the mixture was transferred into a fresh micro-centrifuge where 11 µl of the acetylating reagent (Sigma-Aldrich Corp., Missouri, USA) was added before the mixture was gently mixed by pulsing. The individual endogenous cAMP contents from each of the cell lysates were then measured by a cAMP-linked enzyme immunoassay kit (Catalog # CA201; Sigma-Aldrich Corp., Missouri, USA) following the acetylation version of its protocol and as was described in the manufacturer's manual. The measurements and/or readings were taken using a Microplate Reader (Labtech International Limited, East Sussex, UK) at 405 nm and the obtained results were then subjected to the statistical analysis of variance (ANOVA) in triplicate formats.

2.5.2 Complementation Testing of the Recombinant G-3FP-AC Protein

Determination of the *in vivo* AC activity of the recombinant G-3FP protein was carried out using the complementation test. This test was performed in order to check for the biological functionality of the AC gene segment of this recombinant protein. This was done by dividing some competent *E. coli cya*A cells into two portions. The first portion was transformed with the pTrcHis2-TOPO:G-3FP-AC expression construct while the second portion was left un-transformed (control). A MacConkey agar plate supplemented with 15 µg/ml kanamycin and 0.1 mM IPTG (Sigma-Aldrich Corp., Missouri, USA) was prepared and then sub-divided into 3 segments using a permanent marker. The first segment was left unstreaked (no *cya*A cells), the second segment was streaked with the non-transformed *cya*A mutant cells while the last segment was streaked with the *cya*A mutant cells transformed with the pTrcHis2-TOPO:G-3FP-AC expression construct. The plate was thereafter, inverted and incubated at 37ºC for

40 hours. After the incubation period, all segments were then visually inspected for various phenotypic characteristics. A reddish or deep purple color on the transformed *cya*A mutant cells would mean a positive AC activity for the cloned and expressed G-3FP-AC recombinant protein, and in essence signifying a functional typical lactose-fermenting phenotype.

2.5.3 Bioinformatic Expressional Analysis of the *G-3FP-AC* Gene

2.5.3.1 Co-expressional Analysis

In order to establish the co-expressional profile of the *G-3FP-AC* gene with the other related *Arabidopsis thaliana* genes, the co-expression tool (http://www.arabidopsis.leeds.ac.uk) (Hruz *et al.*, 2008) was used. The tool analysis was performed across all the available microarray experiments using the At2g11890 (*G-3FP-AC*) as the driver gene and leaving the gene list limit blank to obtain a full correlation list. This tool utilizes hybridization signal intensities from microarray experiments to calculate a Pearson correlation co-efficient (r-value), which is a scale-invariant measure of expression similarity that expresses the strength and direction of the linear relationship between the reference gene (GOI, *G-3FP-AC* in this case) and all other Arabidopsis genes represented on the selected chip. The tool calculates and returns both negative and positive correlations (ranging from –1 to +1), associated probability (p), and expectation (e) values, which are a measure of the statistical significance.

2.5.3.2 Stimulus-specific Microarray Expressional Analysis

After retrieving a *G-3FP-AC* co-expressional group of 25 genes (*ECGG25*), the expression profiles of the *G-3FP-AC:ECGG25* were then initially screened over all of the available ATH1:22K array Affymetrix public microarray data in the Genevestigator V3 version (https://www.genevestigator.com) using the stimulus/perturbations tool (Zimmermann *et al.*, 2004). In order to obtain greater resolution of gene expression profiles, the normalized microarray data were subsequently downloaded and analyzed for experiments that were found to induce differential expression of the genes. The data were downloaded from the following repository sites: GEO (NCBI) http://www.ncbi.nlm.nih.gov/geo/), (NASCArrays (http://affymetrix.arabidopsis.info/narrays/experimentbrowse.pl) and TAIR-ATGenExpress (http://www.ebi.ac.uk/microarray-as/ac/). The array data were then analyzed and fold-change (log2) values calculated for each experiment. Expression values were then generated using

the Multiple Array Viewer program from the Multi-Experiment Viewer (MeV) software package (vesion 4.2.01) created by The Institute for Genomic Research (TIGR).

CHAPTER THREE

RESULTS

3.1 Results

3.1.1 Generation of Arabidopsis Plants

Wild-type *Arabidopsis thaliana* ecotype Columbia seeds were surface sterilized, cold-stratified and germinated on solidified Murashige and Skoog basal medium in a growth chamber. The germinated seedlings were then transplanted into planting potting mix before their leaf material was subsequently harvested for the isolation of total RNA.

(A) **(B)**

Fig 3.1: Generation of the *Arabidopsis thaliana* plants. (A) A depiction of the newly germinated Arabidopsis seedlings on Murashige and Skoog growth medium under the growth chamber conditions. (B) Six week-old *Arabidopsis thaliana* ecotype Columbia plants on potting soil from which the leaves for total RNA extraction were obtained.

3.1.2 Amplification of the G-3 Family Protein Gene Fragment

Total RNA was extracted from the 6-week old *Arabidopsis thaliana* leaf tissue material. Copy DNA (cDNA) for the G-3 family protein gene was then synthesized in a 1-Step RT-PCR system and together with the sequence-specific primers, the targeted G-3FP-AC gene fragment was eventually isolated and amplified.

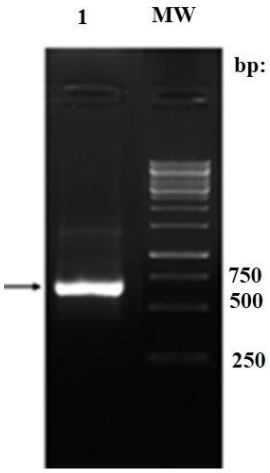

Fig 3.2: Isolation and amplification of the G-3FP-AC gene fragment. An agarose gel resolution of a 627 bp G-3FP-AC gene fragment amplified from the total RNA extracted from the Arabidopsis plants in a 1-Step RT-PCR system. The arrow indicates the amplified G-3FP-AC fragment in lane 1 while MW represents the molecular weight marker (1 kb DNA ladder).

3.1.3 Confirmation of the Cloning Success of the G-3FP-AC Gene Fragment

The successfully amplified G-3FP-AC gene fragment was ligated into the pTrcHis2-TOPO® expression vector to create a pTrcHis2-TOPO®:G-3FP-AC construct, which was further analyzed in two settings. Firstly, to confirm cloning and secondly, to confirm correct orientation of the insert amplicon in the expression vector.

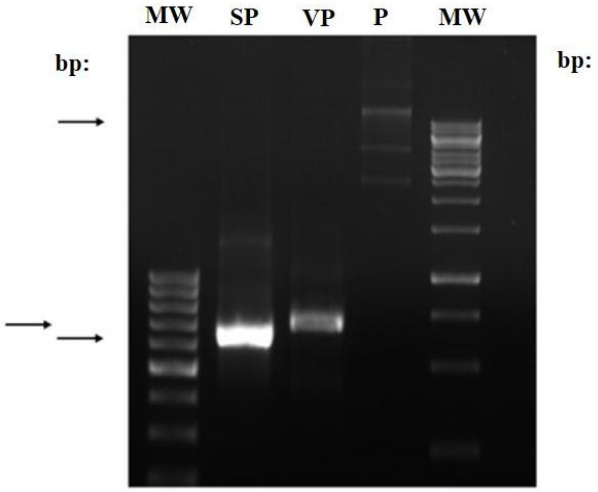

Fig 3.3: Confirmation of the cloning success of the G-3FP-AC gene fragment. A 1% agarose gel resolution for screening of the correct orientation of the G-3FP-AC gene fragment in the pTrcHis2-TOPO® expression vector using the MyTaq™ Mix protocol. MW (from left to right) represents the molecular weight markers (100 bp (left) and 1 kb (right) DNA ladder, SP represents the ligation confirmatory outcome (ran with insert self forward and reverse primers), VP represents the orientation confirmatory outcome (ran with vector reverse and insert self-forward primers) and P representing the pTrcHis2-TOPO®:G-3FP-AC gene construct. The arrows represent the respective bands.

3.1.4 Confirmatory PCR for the Recombinant pTrcHis2-TOPO®:G-3FP-AC

The confirmatory PCR was conducted in accordance with the Standard MyTaq™ Mix protocol (Bioline, London, UK), to ascertain whether the expression host cells were carrying the correct recombinant pTrcHis2-TOPO®:G-3FP-AC fusion construct.

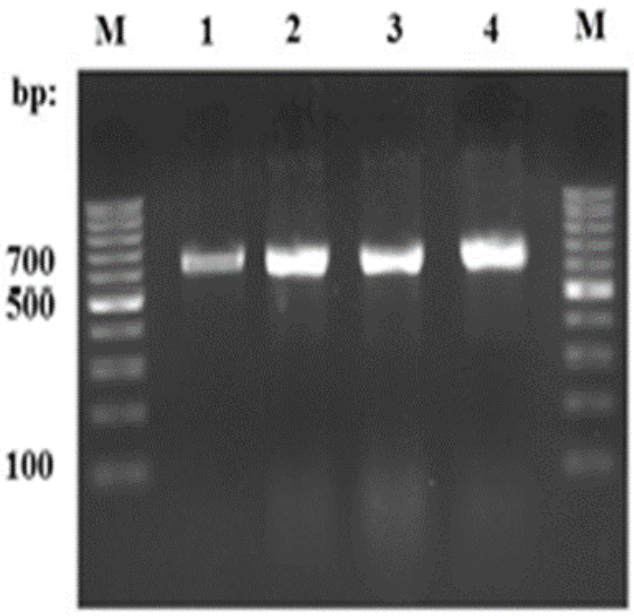

Fig 3.4: Confirmatory PCR. An agarose gel resolution of the competent *E. coli* BL21 (DE3) pLySs DUOs cells harbouring a pTrcHis2-TOPO®:G-3FP-AC expression construct, where (M) represents the DNA marker and the bands in lanes 1 to 4 represent the re-amplified and expected G-3FP-AC gene fragment.

3.1.5 Partial Expression of the Recombinant G-3 Family Protein

The competent *E. coli* BL21 (DE3) pLySs DUOs cells harbouring the pTrcHis2-TOPO®:G-3FP-AC expression construct were induced with 1 mM IPTG in order to partially express the targeted and desired G-3FP-AC recombinant protein.

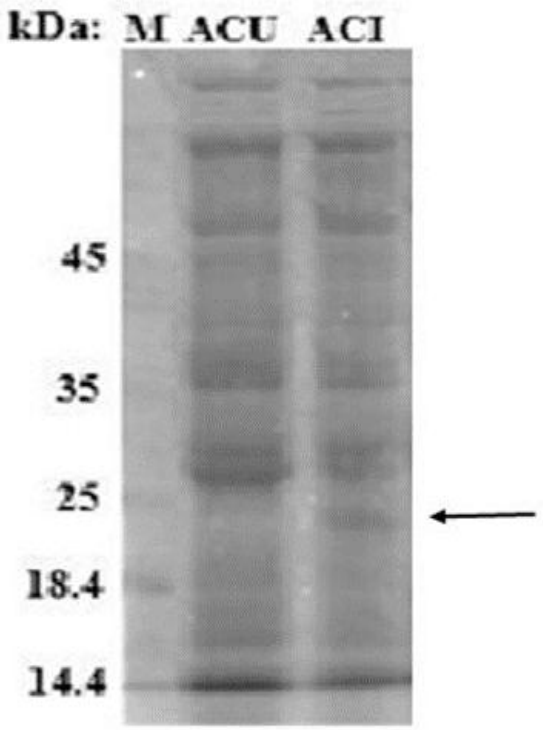

Fig 3.5: Partial Expression of the recombinant G-3FP-AC protein. An SDS-PAGE of protein fractions expressed in chemically competent *E. cloni* EXPRESS BL21 (DE3) pLysS cells transformed with the pTrcHis2TOPO:AC-like fusion construct, where lane 1 (M) is a low molecular weight marker (Fermenters Int., Burlington, Canada), while lane 2 (ACU**)** represents the un-induced control cell culture and lane 3 (ACI) representing the bacterial cell culture treated with 1 mM IPTG to induce the partial expression of the targeted and desired recombinant AC-like protein. The arrow marks the partially expressed recombinant G-3FP-AC protein.

3.1.6 Determination of the Endogenous AC Activity of the Recombinant G-3FP-AC Protein

In order to determine the levels of cAMP generated by the recombinant G-3FP-AC protein, the BL21 (DE3) pLysS *E. coli* cells harbouring the G-3FP-AC gene fragment were checked through enzyme immunoassaying and under different growth conditions.

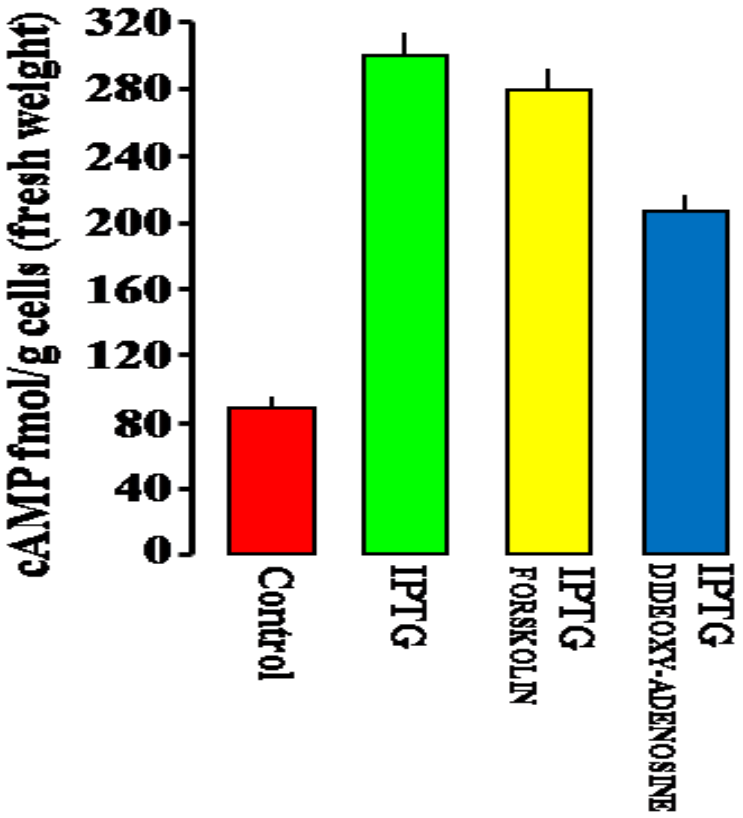

Fig 3.6: Determination of the endogenous adenylate cyclase activity of the recombinant G-3FP-AC protein. Cyclic AMP levels generated by the un-induced (control) and induced (IPTG) *E. cloni* EXPRESS BL21 (DE3) pLysS DUOs cells harbouring the G-3FP-AC gene fragment, and also from induced cell cultures in the presence of either forskolin or dideoxyadenosine. All cAMP levels were determined by the cAMP-linked enzyme immunoassaying system based on its acetylation protocol (Catalogue # CA201; Sigma-Aldrich, Missouri, USA), where error bars represent the standard errors of the mean readings (n = 3).

3.1.7 Determination of the *In Vivo* AC Activity of the Recombinant G-3FP-AC Protein

In order to determine the *in vivo* adenylate cyclase activity of the G-3FP-AC protein, its ability to convert the non-lactose fermenting mutant *E. coli cyaA* SP80 cells to the lactose fermenting wild type cells was tested and examined through a complementation system approach.

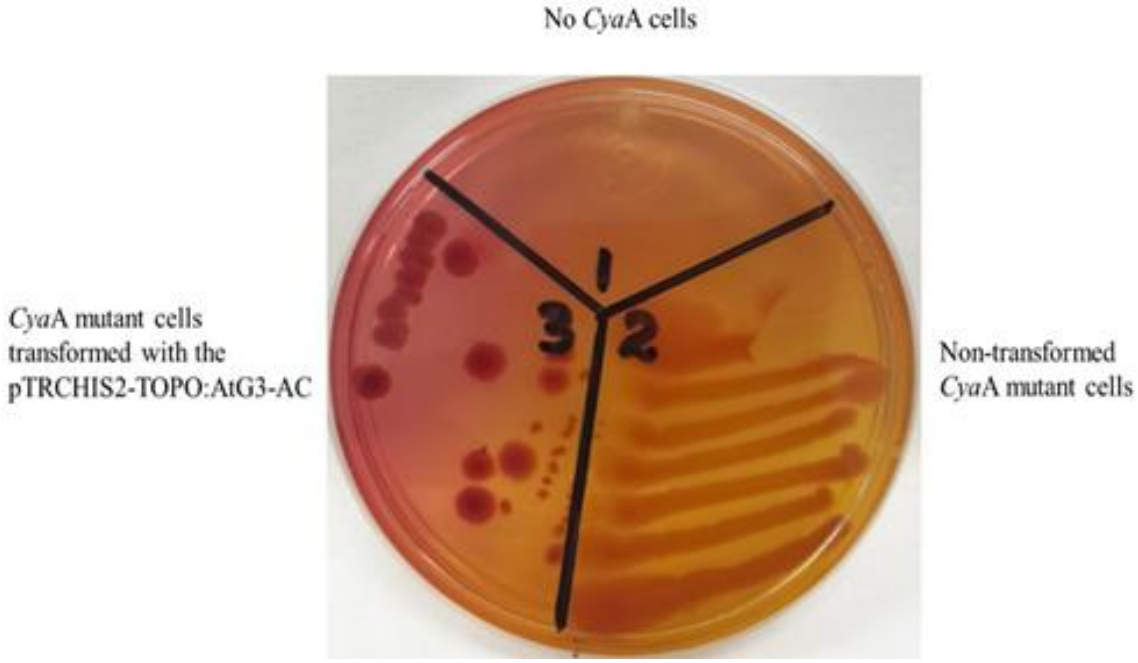

Fig 3.7: Functional complementation testing of the recombinant G-3FP-AC protein. Quadrant 1 of the plate contains no cells, quadrant 2 contains the non-transformed *cyaA* mutant cells and quadrant 3 contains the mutant cells transformed with the pTrcHis2-TOPO®:G-3FP-AC fusion construct. Cells in quadrant 2 are non-lactose fermenters and therefore, produce white or yellow colonies. Cells in quadrant 3 have a deep-purple phenotype signifying a lactose-fermenting phenotype.

3.1.8 Co-expressional Analysis of the *G-3FP-AC* Gene in *Arabidopsis thaliana*

In order to understand the expressional profile of the *G-3FP-AC* gene in *Arabidopsis thaliana*, a desktop analysis of the publicly available microarray data was performed. Out of the 322 diverse transcriptome experiments, the *G-3FP-AC* gene was found to be highly co-expressed with numerous other genes in the Arabidopsis genome, with the top 25 genes having a Pearson's correlation coefficient (r-value) of between 0.815 and 0.894 (Table 3.1 below). These 25 top most co-expressed genes (hereafter referred to as the G-3FP-AC:Expression Correlated Gene Group (*G-3FP-AC: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: List of the top 25 genes that are co-expressed with the *G-3FP-AC* gene (At2g11890).

Locus	GO terms	*r* value	Annotation
AT2G11890		1.000	G3 AC family protein
AT4G11170	DR	0.894	Disease Resistance Protein (TIR-NBS-LRR class)
AT1G57650	DR	0.869	Disease Resistance Protein (NBS-LRR class)
AT2G35980	DR, RBS, ROO	0.868	NHL10 (NDR1/HIN1-LIKE-10)
AT1G15520	RBS, PM	0.866	Pleiotropic Drug Resistance (PDR) transporter
AT1G19180		0.862	Jasmonate-Zim-Domain protein 1 (JAZ1)
AT1G66090	DR	0.843	Disease Resistance Protein (TIR-NBS class)
AT1G71100		0.842	Ribose 5-phosphate isomerase
AT1G74360		0.841	LRR-TM PK (BRI1-LIKE 2)
AT4G39030	DR, RBS, ROO	0.841	SID-1/ EDS5
AT1G29690		0.841	CAD1, neg. reg. SA-med. pathway
AT4G11370		0.837	Zinc finger (C_3HC_4-type RING) family protein
AT1G26420		0.834	FAD-binding domain-containing protein
AT2G18690		0.834	Expressed protein
AT1G22400		0.829	UDP-glucoronosyl/UDP-glucosyltransferase
AT2G15390		0.828	Xyloglucanfucosyltransferase, putative (FUT4)
AT1G57630	DR	0.825	Disease Resistance Protein (TIR class)
AT5G26920		0.825	Calmodulin-Binding Protein 60g (CBP60g)
AT5G38710		0.824	Prolineoxidase, osmotic stress-responsive e
AT5G12340		0.823	Expressed protein;
AT3G45060		0.821	High-affinity nitrate transporter 2.6 (NRT2.6)
AT4G18170		0.820	WRKY28 transcription factor
AT2G32140	DR	0.819	Disease Resistance Protein (TIR class)
AT5G44990		0.819	Similar to intracellular chloride channel
AT4G34390		0.816	Extra-Large GTP 2 binding protein (XLG2)
AT3G09010		0.815	PK family protein

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

3.1.9 Stimulus-specific Microarray Expressional Profile of the *G-3FP-AC:ECGG25*

In order to determine the biological processes in which the *G-3FP-AC:ECGG25* is involved in *Arabidopsis thaliana*, this gene set (*G-3FP-AC:ECGG25*) was subjected to an *in silico* global expressional analysis of the specific experimental conditions that were able to induce a differential expression of all the genes contained in this set. The histogram generated from

this microarray expression analysis revealed that the transcriptional processes of the *G-3FP-AC* and its associated *ECGG25* complement are generally and collectively induced in response to a variety of pathogenic fungal stress factors (Figure 3.8 below).

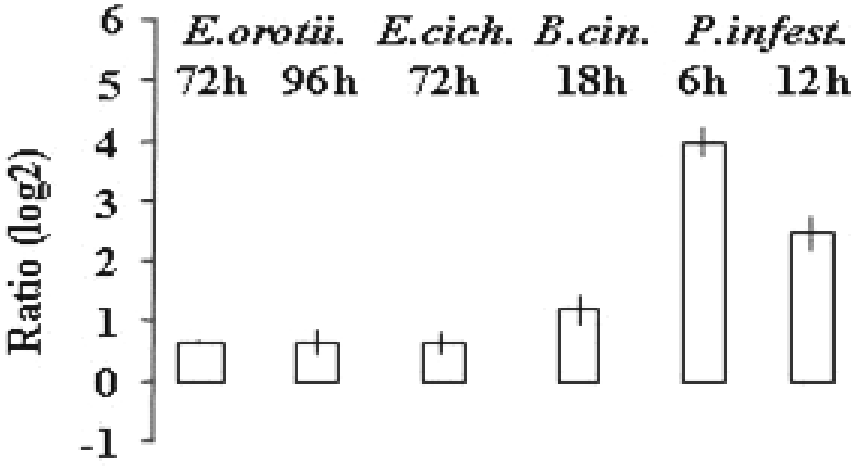

Fig 3.8: Expression profiles of the *G-3FP-AC* gene in response to plant infection with various fungal pathogens. The gene was strongly induced by both the biotrophic and necrotrophic pathogenic moulds. *Erysiphe orontii* is a virulent obligate biotrophic fungal pathogen, *Erysiphe cichoracearum* is a biotrophic parasitic fungus and *Phytophthora infestans* is a biotrophic pathogenic fungus and their infection induces an SA-dependent defense mechanism in plants (Ward *et al.*, 1991; Glazebrook, 2005). *Botrytis cinerea* is a necrotrophic fungal pathogen whose infection induces the JA/ET-dependent defense mechanisms in plants (Penninckx *et al.*, 1998; Glazebrook, 2005).

CHAPTER FOUR

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

4.1 Discussion

The G-3FP-AC protein, which is encoded by the At2g11890 gene, is one of the putative candidate molecules that contain an adenylate cyclase catalytic core motif as has been mentioned in Chapter 1. This protein has never been demonstrated to be able to generate cAMP by itself despite the fact that it has recently been shown to harbour an adenylate cyclase catalytic motif (Gehring, 2010). Hence in this study, the G-3FP-AC protein was targeted and cloned, and then partially expressed for functional characterization. In order to achieve the set objectives for the study, some gene specific primers were manually designed, by the help of the amino acid sequence retrieval using the TAIR (The Arabidopsis Information Resource) to amplify the targeted gene site and were sent for synthesis and subsequent supply by the Inqaba Biotechnological Sciences (Pretoria, RSA Specifically, the assessment was undertaken in non-induced cells, in induced cells as well as in induced cells supplemented with some commonly known AC modulators; 100 µM forskolin or 100 µM dideoxyadenosine (Ehsan *et al*., 1998, Volotovski *et al*., 1998). As is shown in Figure 3.6 , cells induced with 1 mM IPTG had a cAMP generation increase of up to 3.5-fold as compared to the un-induced cells while notably, the treatment of induced cells with 100 µM forskolin had no apparent increase in the levels of the generated cAMP. On the other hand, the treatment of induced cells with 100 µM dideoxy-adenosine did significantly reduce the levels of cAMP by a factor of more than 1.5. Conceivably and in relation to one previous study, where the PSiP coding region of a pollen-specific putative AC from *Agapanthus umbellatus* (*Liliaceae*) was cloned into bacterial cells, and treatment of such transformed cells with 1 mM IPTG did increase the cAMP levels by a factor of 3.0 while treatment of induced cells with 100 µM of the potent AC inhibitor - dideoxyadenosine to the growing pollen tubes, did transiently cause a temporary growth arrest that was accompanied by a reduction of the cAMP concentration by a factor of 1.8 (Moutinho *et al*., 2001). All these previous findings are directly and consistently in line with the findings in this study. At this point and in brief summary, it could be positively postulated that the partially expressed G-3FP-AC recombinant protein was either a *bona fide* AC molecule capable of directly converting ATP to cAMP or else it was simply another functional plant molecule capable of

stimulating the functional activities of other resident ACs (*E. coli* ACs in this case) to produce cAMP. Additionally and by virtue of it being metabolically insensitive to forskolin (Zippin *et al.*, 2004) but being biochemically sensitive to dideoxyadenosine (Moutinho *et al.*, 2001), this further proposes this recombinant protein as a soluble AC type (sAC) (Kamenetsky *et al.*, 2006).

Then, in order to confirm that the cloned and partially expressed G-3FP-AC recombinant protein was indeed a functional AC, an *in vivo* assaying of this protein's activity was further undertaken through a functional complementation test. This test makes use of crystal violet-free, neutral red bile-supplemented lactose agar, also known as MacConkey agar (Macconkey, 1905), which is normally used for the presumptive identification of coliforms, including *E. coli*. The complementation test was performed using an SP850 mutant *cya*A strain that is deficient in endogenous adenylate cyclase activity and therefore cannot ferment lactose (Moutinho *et al.*, 2001). When this mutant *cya*A strain is grown onto the crystal violet-free neutral red bile-supplemented agar, it produces white/yellowish colonies as compared to the magenta deep purple colonies produced by the wild-type counterpart (Moutinho *et al.*, 2001). Therefore, and in order to determine whether the G-3FP_AC had a functional adenylate cyclase activity, the SP850 mutant cells were then transformed with the pTrcHis2-TOPO®:G-3FP-AC expression construct followed by the assessment of the colony phenotypes on MacConkey agar supplemented with 0.1 mM IPTG and 15 µg/ml kanamycin. As is shown in Figure 3.7, the transformed cells stained magenta deep purple, signifying a conversion of the mutant cells into wild types and therefore, unequivocally validating the G-3FP-AC as a functional adenylate cyclase.

Furthermore, outcomes from the bioinformatic expressional analysis of the *G-3FP-AC* gene in the *Arabidopsis thaliana* firmly showed that this annotated gene is mainly expressed in the nucleolic region of the root tip tissues of the Arabidopsis plant and alongside other genes that are principally involved in defense response, response to biotic stimuli, response to other organism and the plasma membrane processes (Table 3.1). In addition, a stimulus-specific microarray expressional profiling of the *G-3FP-AC* gene essentially indicated that this putative gene is generally and specifically induced by a variety of pathogenic fungal stress factors (Figure 3.8).

Therefore, by summing up all the findings in this study, it is clear that the studied G-3FP-AC protein is indeed an important higher plant signalling molecule with a central role in responses to a variety of pathogenic fungal stress factors (Figure 3.8), and whose mechanism

of action is principally mediated by the second messenger, cAMP. Thus this present study, therefore, has experimentally confirmed the G-3FP-AC protein as a *bona fide* higher plant adenylate cyclase besides the four currently known and confirmed, *Zea mays* pollen protein (Moutinho *et al.*, 2001), *Arabidopsis thaliana* pentatricopeptide repeat protein (Ruzvidzo *et al.*, 2013), *Nicotiana benthamiana* adenylyl cyclase protein (Ito *et al.*, 2014) and *Hippeastrum hybridum* adenylyl cyclase protein (Świeżawska *et al.*, 2014).

4.2 Conclusion

This work established the *Arabidopsis thaliana* G-3FP-AC protein as a *bona fide* higher plant adenylate cyclase.

4.3 Recommendations

- Firstly, all the other bioinformatically identified AC proteins should also be practically tested as possible higher plant ACs following confirmation of the G-3FP-AC protein as a *bona fide* higher plant AC.
- Secondly, since the G-3FP-AC protein has been confirmed as a functional AC, it is imperative that its exact physiological roles in cell communication and signal transduction systems are further investigated so that its exact mode of action, specifically in plant stress response and adaptation mechanisms, is also established.

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