Functional characterization of the N-terminal kinase containing domain of a Triphosphate Tunnel Metalloenzyme (AtTTM3) protein from *Arabidopsis thaliana*

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DECLARATION

I,NthabisengTshireletso Margaret Mphuthi, declare that the full dissertation submitted to the Department of Biological Sciences at the North-West University, Mafikeng Campus, for the Master of Science in Biology degree has never been submitted at this University or any other institution elsewhere. This is myown work and all the sources used or quoted here have been properly and dulyindicated and acknowledged.

Student: NthabisengTshireletso Margaret Mphuthi

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DEDICATION

This work is dedicated to my parents Mr and Mrs Mphuthi; to my younger sisters,Tlotleho and TsholofeloMphuthi;my extended family; friends; the Plant-Biotechnology Research Group;my mentors(Dr Takundwa,Dr Dikobe); my co-supervisor Prof. O Ruzvidzo and my supervisor Dr Kawadza.

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DEFINITION OF TERMS

AGC GROUP:is a protein kinase group (PKA and PKG, the PKC families).

AUTO-REGULATION:is a process within many biological systems, resulting from an internal adaptive mechanism that works to adjust (or mitigate) that system's response to stimuli.

CALMODULIN: is a multifunctional intermediate calcium-binding messenger protein expressed in alleukaryotic cells.

CELL SIGNALING: is part of a complex system of communication that coordinates cell action and governs basic cellular activities.

DEPHOSPHORYLATION:is the removal of aphosphate(PO_4^{3-}) group from anorganic compoundbyhydrolysis

GEL ELECTROPHORESIS:is a method for separating and analysis of macromolecules (DNA,RNAandproteins) and their fragments, based on their size and charge.

KINASE:is an enzyme affecting phosphate group transfers from a phosphate-rich molecule, such as ATP to another molecule.

METALLOENZYME:is an enzyme that contains metal ion(s) as an integral part of its active structure.

PHOSPHORYLATION:is a process that is frequently applied in nature for enzyme activity regulations and to create recognition areas for the desirable aggregation of multi-protein complexes. These events are utilised in a variety of signal transduction pathways and control systems of cell cycles.

PHOSPHATASE:is an enzyme that uses water to cleave a phosphoric acidmonoesterinto aphosphate ionand analcohol.

PRIMER:is a fragment of short nucleotide sequences capable of forming base pairs with a complementary template RNA/DNA strand and facilitating its specific amplification.

REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR):is a method that converts a short RNA segment into a DNA product termed copy DNA (cDNA), using an RNA-dependent DNA polymerase enzyme.

SECOND MESSENGER:is a biological molecule capable of transmitting external signals intothe cell for the development of appropriate cellular responses through regulated gene expressional and metabolic events.

SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE):is a technique used to separate different protein molecules according to their sizes and migration levels in a polyacrylamide gel system subjected to a strong electrical field.

LIST OF ABBRIVIATIONS

ACs	:	Adenylatecyclases
AGC group	:	(PKA, PKG, and PKC) group
ATP	:	Adenosine5'-triphosphate
BRI	:	Brassonosteroid receptor
cAMP	:	Cyclic adenosine3',5'-monophosphate
cGMP	:	Cyclic guanosine3',5'-monophosphate
DAG	:	Diacylglycerol
DNA	:	Deoxyribonucleic acid
ETR1 gene	:	Ethylene receptor-1 gene
His	:	Histidine
IP3	:	Inositol 1,4,5-trisphosphate
IPTG	:	Isopropyl-β-D-thiogalactopyranoside
LB	:	Luria-Bertani
MAPKs	:	Mitogen Activated Protein Kinase
mRNA	:	Messenger RNA
MS	:	Murashige and Skoog
Ni-NTA	:	Nickel-nitrilotriacetic acid
PI	:	Phosphatidyl inositol
PKA	:	Protein kinase A
PKC	:	Protein kinase C
PKG	:	Protein kinase G
PPPi	:	Tripoly-phosphate

PSK	:	Phytosylfokine
RLKs	:	Receptor-like kinase
RSKs	:	Ribosomal protein S6 kinase beta
RT-PCR	:	Reverse transcriptase polymerase chain reaction
SDS-PAGE	:	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Ser	:	Serine
TTM	:	Triphosphate tunnel metalloenzyme
Tyr	:	Tyrosine
WAKL	:	Wall associated kinase-like
YT	:	Yeast-tryptone

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ABSTRACT

Plants are challenged by many environmental factors such as drought, heat, pest invasions and even field fires, all which may affect crop yield. These factors influence plants to adapt and/or establish means of survival wherever they are situated. In this study,the research model plant *Arabidopsis thaliana*was used to analyse a gene involved in plant responses relating to environmental factors. Thestudy was based on an *A. thaliana*kinase-like protein (At2g11890) which has been annotated as an adenylatecyclase (AC) and kinase, whereby our focus was on its kinase domain. Kinases are enzymes catalysing the transfer of a phosphate groupfrom ATP to another molecule. Plants perform multiple signal transduction processes in response to stresses they encounter whereas during signal transduction G-protein activates AC which converts ATP to the second messengercAMP resulting, in the activation of the protein kinase A and various physiological responses within the cell. The study aimed to determine whether the annotated AtTTM3 has any kinase activity. In this case, the annotated kinase-like gene fragmentwas isolated and ligated into apTrcHis2-TOPO vector. The construct was then used to transformchemically competent *E.cloni* EXPRESS BL21 (DE3) pLysScells for recombinant protein expression. Protein expression was enabled through the addition of IPTG to a portion of the generated culture.The expressed protein was then assessed forits probable kinase activity using anOmnia™ Recombinant system reaching fluorescence signals at 485 nm emission (λ_{em} 485) and a reaction excitation at 360 nm (λ_{ex} 360).The AtTTM3 protein was alsofurther characterised using bioinformatics where a list of co-expressed genes was retrieved from TAIR and few proteins indicated some functional GOs . In this regard, it was shown that the kinase-like (AtTTM3) protein is indeed a bona fide plant kinase that is highly expressedin various plant tissues during expression intensity(peripheral endosperm, chalazal seed coat, chalazal endosperm, testa, general seed coat, micropylar endosperm, suspensor and embryo) and expression profile of the developmental stagesneeded forgrowth and development of the plant.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Biotic and abiotic stress effects on plants

Plants are subjective to biotic and abiotic stresses,leading them to adapt rapidly to the changing environments.These stresses affect plant development andyield. Abiotic stresses are non-living impact factors which affect living organisms in a specific environment.They include light, gravitational forces, wind, temperature, drought, mineral content and ionic radiations (Ho, 2015). Biotic stresses, on the other hand, are living organisms impacting on other living organisms in a specific environment. These include predators, pathogens, pests, herbivores, fungi, other micro-organisms and harmful insects (Ho, 2015).Both the abiotic and biotic factors,individually or in concert, result in inducingmultiplesignal transduction pathways within the plant, in response to damage or against diseases (Ho, 2015). There are adaptation response pathways which plants follow in response to abiotic and biotic stresses, which include various mechanisms such as transduction, transcription and translation that result in an adaptive response (Figure 1.1). In thisFigure, a signal arriving on the surface of a cell will initially be transduced, leading to a specific gene system being transcribed and translated to produce a particular protein, which will bring about the resultant response of the cell to the initial signal.

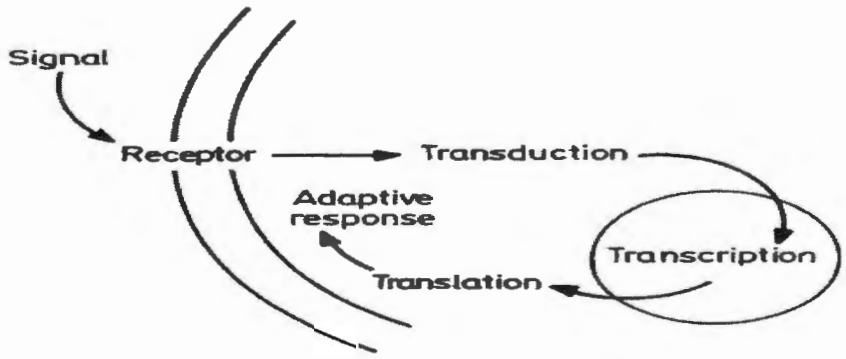


Figure 1.1: Sequence of events diagram representation resulting in an adaptive response. In specific systems, transduction features may openly act on translational events affecting an adaptive response (Gorelick, 1987).

The phenotypic and metabolic responses to any changes taking place in an organism are brought about by the nature and types of proteins that are expressed. Once expressed, many proteins may be activated or deactivated through phosphorylation and de-phosphorylation. Phosphorylation may be brought about through a transfer of a phosphate group from a phosphate-rich molecule, such as adenosine triphosphate (ATP), to another molecule with the engagement of kinases. Kinases are involved in multiple cellular responses and plant development.A few examples of cellular responses and regulationsin response to the involvement of protein kinases with regard to adaptive response include self-incompatibility (Klimecka and Muszynska, 2007), initiation of mitosis (Tanenbaum*et al.*, 2015), isoprenoid biosynthesis (Rodríguez-Concepción and Boronat, 2015), MSERK1 activity and phosphoenolpyruvate carboxylase activity (Yan *et al.*, 2015).

The mechanisms involved in these external stresses,whereby intracellular signalling molecules respond to the stressors, are known as signal transduction,and during this process, elements involved in multiple interacting components are required for initial signal recognition, and a network of this orderly signal transduction activates the release of second messengers directly and indirectly in a cell (Ho, 2015).In Figure 1.2, a variety of signal transduction pathways are shown, whereby fat-soluble signals like steroids can easily go across the cell membrane, and their receptors are found in the cytoplasm.Substances, like nitric oxide, may also diffuse into the cell to be recognised by a cytoplasmic receptorinteracts with guanosine triphosphate (GTP), through the action of guanylate cyclase (GC),to produce cyclic guanosine 3',5'-monophosphate (cGMP), which in turn activates a protein kinase G. Hydrophilic ligands or signals will not be able to cross the cell membrane and as such will require a transmembrane receptor.

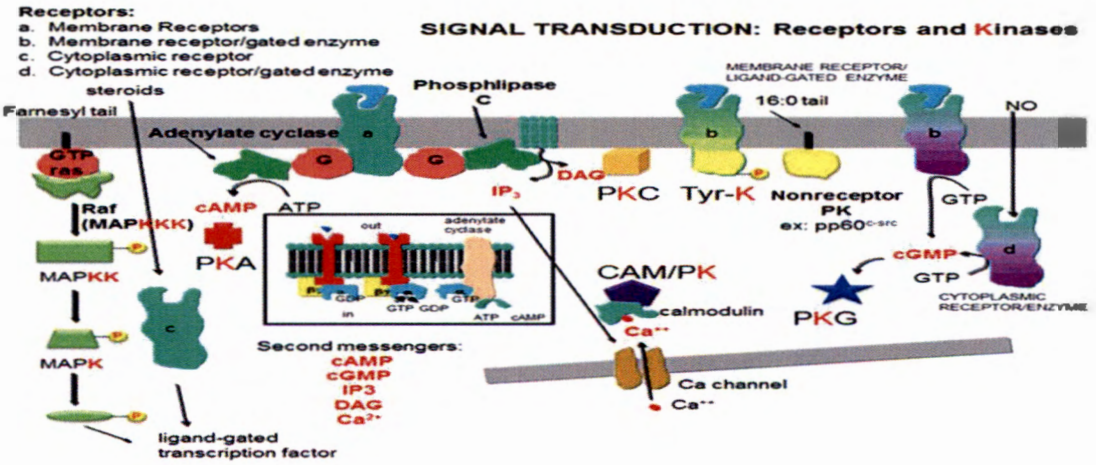


Figure 1.2: The five major protein kinases(http://biochem-vivek.tripod.com/id57.html).

The events of signal transduction result in various mechanisms, which include secondary messengerssuch as cyclic adenosine 3',5'-monophosphate(cAMP)and the phosphatidylinositol(PI) pathway (Figure 1.3) which may involve other secondary messengers such as diacylglycerol(DAG), inositol 1,4,5-trisphosphate(IP3), calciumion (Ca^{2+}) and cyclic guanosine3',5'-monophosphate(cGMP). The interaction between kinases and AC are indicated through asecond messenger mechanism of thecAMP pathway described in Figure 1.2., through the signal transduction pathway.In Figure 1.2, a chemical signal (blue) is picked up by a transmembrane receptor represented by the transmembrane receptor (light green). The transmembrane receptor, which then activates an AC, which in turn activates protein kinase A, the protein kinase A then phosphorylates and influences the actions of the protein that is phosphorylated.

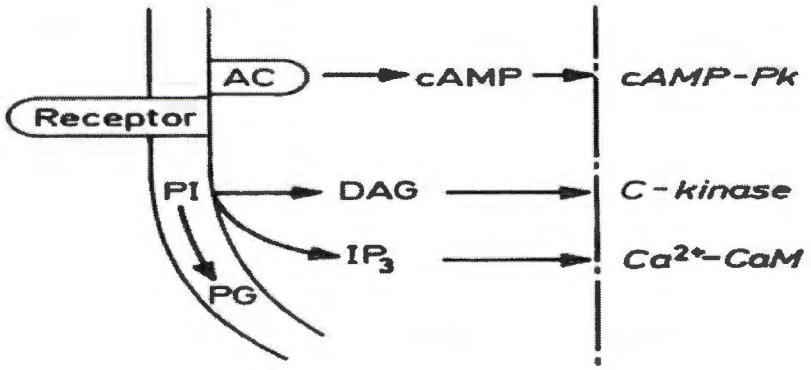


Figure 1.3:Primary effectors summary of the second messenger pathways. Neutral lipid appears to excite merely Protein kinase C (C-kinase); two cAMP-dependent protein kinase types are familiar. In divergence, distinct protein kinase activities can be aroused by the Ca^{2+}/calmodulin system (Gorelick, 1987).

Interaction between kinases and AC

During signal transduction mechanisms, as shown above in Figure 1.2,one of the pathways involves a G-protein (proteins that bind the guanine nucleotides, GDP and GTP),which activates an ACthatconverts ATP to cAMP, which activates protein kinase A that, in turn, phosphorylatesproteins at specific amino acid side chains and influencestheir action (Jakubowski, 1994).ACsare enzymes which have an important regulatory role in all cells.They activate various protein kinases, which are responsible for the various physiological responses within the cell that are involved in plant development and stress responses. Protein kinases are phosphotransferases, which transfer the terminal phosphate

from ATP to substrate proteins (Ho, 2015). There are different types of protein kinases which form part of the AGC group (protein kinase A, G and C).

Kinases which are unique, such as MAPKKK and Try-K (Receptor Tyr kinases) and themediated protein kinases such as the AGC group, are implicated in the earlier events of signal transduction in plants, where we also find ACs converting ATP into cAMP.These signals fall into pathways utilising a more highly conserved universal protein kinase system in animals (Stone and Walker, 1995). A reflection of developmental divergence and environmental signals may be observed in initial signalling steps of transduction pathways to which plants must respond (Stone and Walker, 1995). The relevant second-messenger regulated kinases found in some animals have not been identified in plants, which these second messengers in the cells are significant in signal transduction (Stone and Walker, 1995).

Protein kinase and phosphatase activities

The function of a protein is widely regulated by the covalent addition of a phosphate group to one or more of its amino acid side chains. Protein phosphorylation includes the enzyme-catalysed transmission of the terminal phosphate group of an ATP molecule to the hydroxyl group of serine, threonine or tyrosine side chain of the protein (Albertset *al.*, 2014). Protein phosphatase activity catalyses the reverse reaction of kinases (phosphate removal or dephosphorylation) (Johnson and Hunter, 2005; Hegeduset *al.*, 2016; Korneevaet *al.*, 2016 and Zhu *et al.*, 2016). A protein phosphatase is an enzyme which removes a phosphate group from the phosphorylated amino acid, while a protein kinase is an enzyme that modifies proteins by chemically adding a phosphate to them.

Modification of targeted protein properties by reversible phosphorylation events is one of the most prominent cellular control processes in all organisms (Dissmeyer and Schnittger, 2011). The phosphor status of a protein is dynamically controlled by protein kinases and the counteracting phosphatases. Therefore, kinase monitoring and phosphatase activities, identification of specific phosphorylation sites, and assessment of their functional significance are of crucial importance to understand development and homeostasis (Dissmeyer and Schnittger, 2011) as well as stress response.

Based on various protein kinases and their function our specific protein kinase gene is naturally found in the *A. thaliana* plant. The*A. thaliana* encodes three TTM genes (AtTTM1,

AtTTM2 and AtTTM3) which have been annotated for kinase activity even though their kinase activity has not yetbeen practically demonstrated (Keppetipola*et al.*, 2007; Delvaux et al., 2011; Bettendorff& Wins, 2013; Moeder*et al.*, 2013). The AtTTMs can be either transphosphorylated or autophosphorylated or both (Suzuki *et al.*, 2016). The focus of our studywasspecifically on the AtTTM3 because of its annotation in plant development (http://www.arabidopsis.org).

1.2Literature Review

Sequences of adaptation response pathways

As previous stated, plants face abiotic and biotic stress factors and need an adaptation response to survive these harsh conditions as they are immobile and cannot move away from danger or change places when challenged by the harsh environmental factors. Thisbeing said, plants are a form of food source,and when they cannot survive the challenging condition they face, their yield drops, which affects food security. Plants survive these stressful environmental factors through a responsive mechanism called the adaptation response pathway.Theadaptive response effectorsgenerallyconsist of complex and multilevel cellular networks. It can be estimated that various ligands and their receptors, signal transduction pathways, and transcriptional and translational mechanisms will be recruited by these effectors (Gorelick, 1987).There are two general types of transduction mechanisms which seem to oversee adaptation, the first one appears to act through a modulation of a traditional second messenger pathways, which is acknowledged to be engaged by calcium, lipids, and cyclic nucleotidesand secondly, this system is characterized by highly precise ligand-receptor interactions that seem to activate distinctive transduction pathways. It is predictedthat phosphatase or protein kinase changes activity can mostly signify final mutual pathways for both systems (Gorelick, 1987).

The adaptive response can be a response to cold, heat, or drought or even reproduction as other plants rely on insects or wind to reproduce their kind. Within the adaptative response, in higher plants there are important physiological roles such as in cell survival (Petrov*et al.*, 2015), growth (Hatfield and Prueger, 2015), migration (Pitelka, 2017), proliferation (Ishida *et al.*, 2014), polarity (Tejos*et al.*, 2014), and metabolism (lipid and glucose)may be played by Triphosphorylation including chemical roleresponse in plants through various mechanisms in signal transduction pathway. This pathway contains various receptors, secondary messengers

and protein kinases activated for a specific reaction response for plants to protect themselves or to survive abiotic and biotic factors where they are situated.

Secondary messengers activating various kinases

Kinases are a class of enzymes which use ATP to phosphorylate molecules within the cell, regulating most aspects of cellular functions and the ultimate removal of the phosphate group will be done by phosphatase through hydrolysis. All cellular function aspects are regulated by kinases and phosphatase (Jakubowski, 1994). Below is the interaction of receptors and the second messengers within the signal transduction pathways, within these cascade of events there are various secondary messengers such as thePIpathway,the DAG, the IP3andthe Ca^{2+} shown below in Figure 1.3, the cGMP and cAMP,even though our main focus was on the cAMP pathway, which is described below in Figure 1.2.

A) <u>Protein Kinase A (PKA)</u>

An extracellular biochemical signal binds to a transmembrane receptor without enzymatic activity, leading to a conformational change in the receptor, which transmitsacross the membrane. The intracellular domain of the receptor is bound to an intracellular heterotrimeric G protein (since it binds GDP/GTP) in the cell. The G protein dissociates,and one subunit interacts with and activates an enzyme - KC- which converts ATP into the second messenger -cAMP - in the cell. cAMP activates protein kinase A, which then phosphorylates proteins at specific Ser, Tyr or Thr side chains.

B) <u>Protein kinase C (C-kinase) and calmodulin-dependent kinase (CAM-PK)</u>

A transmembrane receptor without enzyme activity binds an extracellular chemical signal, causing a conformational change in the receptor, which propagates through the membrane. The intracellular domain of the receptor then binds to an intracellular heterotrimer G protein (since it binds GDP/GTP) in the cell. The G protein dissociates,and one subunit interacts with and activates - a phospholipase C - which cleaves the phospho-head group from a membrane PIP_2 into two-second messengers - DAG andIP_3. DAG binds to and activates protein kinase C (PKC). The IP_3 binds to ligand-gated receptor/Ca^{2+} channels on the internal membranes, leading to an influx of calcium ions into the cytoplasm.Calcium ions bind to a calcium modulatory protein, calmodulin, which binds to and activates the calmodulin-dependent

kinase (CAM-PK). The released calcium ions also activate PKC. As in the previous outline, these receptors, which interact with G proteins, are single polypeptide chains which contain sevenmembrane-spanning alpha helices. The cycle of degradation and re-synthesis of PIP2 is called the PI cycle: is activated by the neutral lipid DAG through an increase in calcium affinity even though phorbol esters can be alternatively used for neutral lipids in protein kinase C activation (Gorelick, 1987). The IP3 is the second essential messenger generated from the PI pathway and this soluble product is immediately generated after a ligand-receptor interaction and appears to be liable in large part for cytosolic transient calcium generation seen in multiple systems (Gorelick, 1987).

Resulting from this signal transduction pathway as one of various pathways involved in plant survival during harsh conditions, is a PKA activated by cAMP from AC and PKG activated by cGMP which both fall under the AGC group kinases and also other kinases such as the MAPK and CAM/PK activated by other second messengers in which they all have various roles in the plant as a unit or individually (Agrawal *et al.*, 2002). MAPKs are other types of protein kinases which are signalling units that phosphorylate specific serine/threonine known to regulate numerous cellular functions and activities (Agrawal*et al.*, 2002).

C) Receptor Tyrosine Kinases

A transmembranereceptor with a hormone-dependent enzymatic activity (tyrosine kinase) binds an extracellular chemical signal, causing a conformational change in the receptor, which then propagates through the membrane. The intracellular domain of the receptor becomes an active tyrosine kinase, which can phosphorylate itself (autophosphorylation) or other proteins. Such kinases are usually active in a multimeric state. Typically, binding of two molecules of a ligand or a ligand dimer to individual subunits of the receptor causes the monomers of the receptor to dimerise. In this form, the kinase activity of the receptor is activated. The individual subunits of the multimer are proteins with a single transmembrane helix. Examples are the insulin and epidermal growth factor receptors.

The receptor Tyr kinases autophosphorylate themselves- a process required for their activity. When the receptor is phosphorylated, other proteins can bind to the cytoplasmic domain of the receptor,where they are phosphorylated too. The target substrates phosphorylated by the receptor Tyr kinase are proteins with a typical 100 amino acid domain called the SH for srchomology, based on structural homology to another cytoplasmic protein, Src. Src is an

intracellular Tyr kinase, activated when it binds through SH2 domains to the autophosphorylated receptor Tyr kinase. Specifically, the SH2 domain has been shown to bind phosphorylated peptides. These domains target proteins to the autophosphorylated receptor Tyr kinase.

Second messenger pathways generated from the PI pathway

From the signal transduction mechanisms cascade of the event in Figure 1.2, the PI pathway is further elaborated below in Figure 1.3, indicating the three pathways resulting from it. The PI breakdown intercedes through the action of a sequence of phospholipase C's and kinases, resulting in the generation of the significant three messengers, which includes the DAG, IP3, and PG (Gorelick, 1987). ACsdo not depend on PI for activation; PIactivates DAG,IP3 and the generation of PG, known asicosanoid. DAG, also known as the neutral lipid activates the protein kinase C and IP3 activates Ca^{2+}(calmodulin). The stimulated calcium transient by IP3 is probably responsible for the numerous enzyme activities activated through thecalcium-binding proteins,modulatorycalmodulin effects and amongstthese, are alsothe most essential groups of enzymes,which are protein kinases regulated by calcium/calmodulin (Gorelick, 1987).

The PI breakdown that influences the third messenger system containing generation of icosanoid mediators, depending on arachidonate availability is due to most tissue systems appearing to contain generation of arachidonate ability through phospholipase A2 action on phosphatidylcholine or phosphatidylethanolamine.The contribution of the relative PI breakdown is unclear (Gorelick, 1987). Present investigations propose that arachidonatecan arouse a transient increase in cytosolic calcium by a mechanism that differs from that described for IP3, the transduction pathways acting through neutral lipids, calcium and cyclic nucleotides with an indicated autoregulatory potential (Gorelick, 1987).

Various protein kinases found in plants

It was found that the AGC kinases phosphorylate a massive range of proteins that regulate various cellular procedures, whereby the capability of various AGC kinases to phosphorylate similar substrates has evolved to permit various extracellular stimuli to regulate similar cellular machinery and provoke the same cellular responses (Pearce *et al.*, 2010). Several examples of this are the Akt, RSK, PKA, PKC and S6K isoforms thatphosphorylate

similarSer residues at the N terminus of glycogen synthase kinase (Pearce *et al.*, 2010). Akt isoforms have essential roles in directing metabolism, protein synthesis and proliferation(Laura, 2010).

Protein kinase A (PKA) isoforms are in an inactive state before activation by the creation of an R2C2 heterotetrameric complex, containing two catalytic subunits bound to two regulatory subunits, where the regulatory subunits dimerise past an N-terminal coiled-coil motif. At least there are 50 various AKAP members that localise PKA to exact regions in various cell types and a concentration of cAMP that is regulated by the virtual proximities and activities of ACs and phosphodiesterases(Wong, 2004).In divergence to PKA, PKG forms homodimers,and its cGMP-binding domains are situated in similar polypeptides as the catalytic domain. An interface of PKG with cGMP leads to a conformational change in PKG, releasing the inhibitory effect of a pseudosubstrate motif (Lucas, 2000).

A type II calmodulin kinase, which is activated by IP3 is found to be consistent with autonomous autoregulation; this enzyme was established to undergo calcium/calmodulin-stimulatedautophosphorylation soon after it was discovered. The type II calmodulin kinase autophosphorylation results in an enzyme, whose catalytic activityis retained in theabsence of calcium.This kinase autophosphorylation may have a central role in the activation and inactivation of this enzyme. TypeII calmodulin kinase autophosphorylation is an intramolecular event and it is independent of the kinase concentration as it has the ability of occurring in systems containing small amounts of enzyme only. (Gorelick, 1987).

The predicted structural features, which are continually expanding in the family of plant kinases, are made up of the Receptor-Like Kinases (Receptor-like kinases) (Stone and Walker, 1995). Different types of implied common signalling mechanisms are inresponseto the RLKs cloned abundance from plants and their different expression patterns (Walker, 1994).Based on the information regarding protein kinases, it is shown that protein kinases are present within plants and they are also functional. In our study, we investigated if our specific chosen gene (At2g11890) has any probable enzymatic activity and its characterisation as a functional protein kinase.

At2g11890 classified as a triphosphate tunnel metalloenzymes (TTM)

Our gene of interest is found at the locus At2g11890 in *A. thaliana*. It is also known as an ACor TTM, including being known as AtTTM3. A group of enzymes characterised by their ability to hydrolyse a range of tripolyphosphatesis represented by a superfamily named triphosphate tunnel metalloenzymes (TTMs). Three TTM genes; AtTTM1, 2 and 3are encoded by *A. thaliana* (Ung*et al.*, 2017). AtTTM3 has previously been reported to possess apolytriphosphatase activity, which is expressed recombinantly while AtTTM2exhibited apyrophosphatase activity (Ung*et al.*, 2014). The TTM superfamily consists of two groups of enzymes, which are RNA triphosphatases and CYTH phosphatases (CyaBadenylatecyclase, thiamine triphosphatase) thatcontain mutual characteristics within their catalytic sites (Iyer and Aravind, 2002; Gong *et al.*, 2006). The superfamily members canhydrolyse various triphosphate substrates, which result in giving them essential roles in cAMP formation, mRNA capping and secondary metabolism (Iyer and Aravind, 2002; Gallagher *et al.*, 2006; Gong *et al.*, 2006; Song *et al.*, 2008).

Numerous TTMs contain a unique tunnel structure composed of eight antiparallel β strands, establishing a β barrel and characteristic EXEXK motif (whereby X is any amino acid), where the barrel is essential for catalytic activity (Lima *et al.*, 1999; Iyer and Aravind, 2002; Gallagher *et al.*, 2006). Uncommon tunnel domains fold harbouring substrate,and metal co-factor binding sites are contained in TTMs (Ung*et al.*, 2014). There are three TTMs contained in the Arabidopsis genome (AtTTM1-3), with TTM3 beinga small soluble enzyme (Martinez *et al.*, 2015).Regardless of protein kinase involvement, there are interactions between the various hormone-dependent signalling pathways, including plant hormones like ethylene, cytokinin, auxin, gibberellins, abscisic acid and also are other signalling pathways, which include the abiotic stress signal pathways such as those for cold, salt, drought and pathogens attack (Agrawal*et al.*, 2002).

It is confirmed that kinases have an essential role in plants even though the link between plants and animals is not yet clear,but it is shown that just like animals, plants can be protected and able to survive harsh conditions through mechanisms such as signal transduction, hormonal signalling or abiotic stress signal pathways. Literature indicates that kinases produced from such pathways are also involved in the growth and development of these plants even though AtTTM3 kinase activity hasnot yetbeen clearly stated.It is thereforehighly probable that it hasfunctional kinase activity when compared with other kinases involved in various pathways.

1.3 Problem statement

It has been confirmed that the At2g11890 is present within the*A. thaliana* gene pool (Poole, 2007).However, even though the At2g11890gene has been annotatedas aTTM3,its probable function as a kinase has not yet been confirmed.

1.4 Research aim

The study aimed to determine the enzymatic activity and characterisation of AtTTM3 as a functional plant protein kinase.

1.5 Objectives

The following objectives were set:

1- To amplifyand clone the AtTTM3 coding region.

2- To optimise strategies for expression of the recombinant AtTTM3 protein

3- To confirmthe kinase activity of the recombinant AtTTM3 protein*in vitro*.

4- To characterise the kinase activity of the AtTTM3 protein*in silico*.

1.6 Significance of the study

The findings of this study were intended to add more literature to the existing information on plant kinases. This research would add more understanding of the function and role of the AtTTM3 protein in *A. thaliana* and in other related higher plants. Kinases are known to play a significant role in various metabolic processes such as cell signalling, cellular transportation, plant growth and ATP production, hence showing that these proteins (including our own)are essential in plant growth and development.

CHAPTER 2

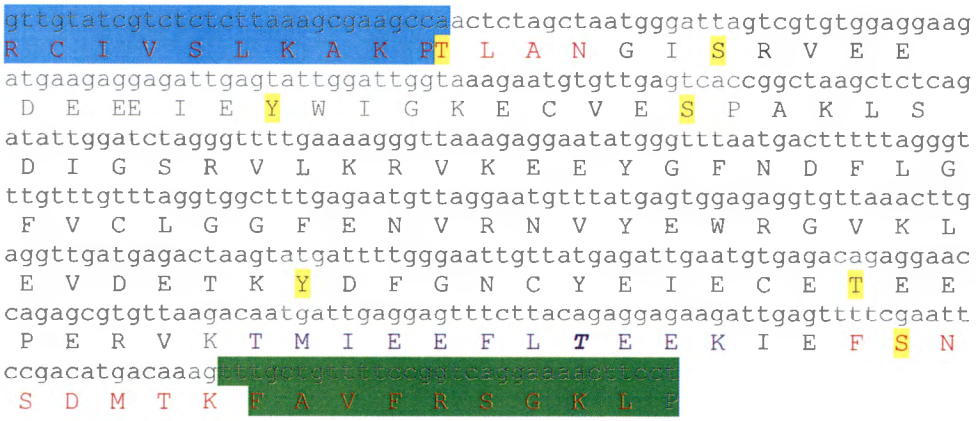
METHODS AND MATERIALS

2.1 Generation of *Arabidopsis thaliana* plants

Arabidopsis seeds (approximately 30) were transferred into an Eppendorf tube and washed threetimes with 500 µL of 70% ethanol. The seeds were then vortexedthreetimesin 500µL sterilisation buffer:(50% (v/v) commercial bleach and 0.1% (v/v) Triton-X)and then washedfive times with 1mlsdH_2Ofivetimes. The washed seeds were then suspended in 200µL sdH_2O to stratify them at 4°Cfor three days. The stratifiedseeds werethen planted into an MS media(Murashig&Skoog media, sucrose, nutrient agar, sdH_2O and pH to 5.7) and grown inside a growth chamber for two weeks to allow seed germination.The seedlings were then transplanted into sterile potting soil (33.3% (w/w)) vermiculite, 33.3% (w/w) Canadian peat humus and 33.3% (w/w) potting mix soil). The set conditions for the growth of the seedlings and the plants were averaged temperatures of 23°C day/night for periods of 16 hours light at 10,000lux and 8 hours darkness at 60% humidity.

2.2 Specific primer sequence design

The At2g11890 gene fragmentsequenceand AtTTM3 amino acid sequenceswere obtained from TAIR (The Arabidopsis Information Resource) (http://www.arabidopsis.org). Both the forward and reverse primers based on the AtTTM3 gene were manually designed and sent off to Inqaba Biotechnologies (Pretoria, South Africa) for chemical synthesis. During the primer designing stage, the first nucleotide was made to start with the guanine base as adenine bases cannot be successfully cloned when using the pTRCHIS2-TOPO expression vector.



Reverse and forward primer sequence

Forward: 5′-GGT TGT ATC GTC TCT CTT AAA GCG AAG CCA-3′

Reverse: 5′-GGG AAG TTT TCC TGA CCG GAA AAC AGC AAA-3′

Figure 2.1: The DNA and protein sequences for the truncated At2g11890 (Adapted from TAIR, Expasy websites). The blue highlight indicates the forward priming site and green highlight indicates the reverse priming site. The letters in red indicate phosphorylation hotspot of the truncated At2g11890 while letters in yellow indicate predicted phosphorylation sites. The bold-italic T letter indicates the phosphorylation site found in experiments. The manually designed sequence-specific primers are shown below the DNA and protein sequences.

2.3 RNA isolation and amplification of the AtTTM3 coding region

After six weeks in the growth chamber, the *A. thaliana* plantswere removed from the soil and the soilwashed off its roots with sdH_2O. Using aseptic conditions, the plantswere placed in a frozen mortar, where liquid nitrogen was added to snap freezethem before being ground intofine powder with a chilled pestle. Using aBioline Isolate II RNA Plant Kit protocol(Bioline,London,UK), the ground plant tissue powder was transferred into an Eppendorf tube containing 500µL of Plant RNA Lysis Solution, vortexed for 20seconds and then incubated for 3minutes at 56°C. Using an LSE High-SpeedMicrocentrifuge (Corning Inc., Amsterdam, Netherlands), the tube was centrifuged for 5minutes at 12,000×g.The supernatant was collected and then transferred into aclean Eppendorf tubebefore 250µL of 96% ethanol was added and mixed by pipetting. The prepared mixture was then transferred to a purification column inserted into a collection tube. The column was then centrifuged for 1minute at 12,000×g. The flow-through solution was discarded,and the column placed back into a collection tube. Some 700µL of Wash Buffer WB 1was added to the purification column (after ensuring that ethanol was added to the Wash Buffer WB 1) and then centrifuged for 1minute at 12,000×g. The flow-through was discardedand the purification

column placed into a clean 2mL collection tube. About 500µL of Wash Buffer 2 was added to the purification column (ensuring that ethanol was added to the Wash Buffer 2) and then centrifuged for one minute at 12,000×g.The flow-through was discardedwhilethe purification column was reassembled into thesame collection tube (the washing step with the Wash Buffer 2 step was then repeatedthrough centrifugation at 20,000×g). The collection tube containing the flow-through was discarded and the purification column transferred to an RNase-free 1.5mL collection tube. To elute RNA, 50µL of sd$H_2O$was added to the centre of the purification column membrane then centrifuged for one minute at 12,000×g.The purification column was then discarded while the purified RNA was stored at -20°C. The concentration of the obtained RNA was measured using a Nanodrop-2000 Spectrophotometer (Thermo ScientificInc, Massachusetts, USA).

The extracted RNA was used as a template for generating cDNA. The sequence-specificprimers, sdH_2O and the Thermo ScientificVerso 1-Step RT-PCR Ready MixRT-PCR kit (Thermo Scientific Inc., Massachusetts, USA),wereused as per the manufacture's instructions, to generate the construct. The RT-PCR reaction conditions in Table1.2 were used on the RT-PCR reaction mixture shown in Table 1.1.

Table 2.1: Components of an RT-PCR reaction mixture in a final reaction volume of 50 µl.

Composition	**Volume**	**Final Concentration**
Verso Enzyme Mix	1 µl	
1-Step PCR Master Mix	25 µl	1X
Forward Primer (10 µM)	2 µl	200 nM
Reverse Primer(10 µM)	2 µl	200 nM
RT Enhancer	2.5 µl	
Water (PCR Grade)	15.5 µl	
Template (RNA)	2 µl	1 ng
Total Volume	50 µl	

Table 2.2: The 1-step RT-PCR thermal cycling conditions used for amplification of the kinase fragment gene.

Step	Temperature	Time	Cycles
cDNA Synthesis	50°C	15 minutes	1
Thermo-start Activation	95°C	15 minutes	1
Denaturing	95°C	20 seconds	45
Annealing	65°C	30 seconds	
Extension	72°C	1 minute	
Final Extension	72°C	30 minutes	1

2.3.1 Gel electrophoresis

A 1% agarose gel was prepared according to the standard protocol. The mixture was left to cool and (10µg/ml) ethidium bromidewas added to the cool gel. The mixture was poured onto a gel electrophoresis tray with combs before setting. A 10µLaliquot of the100bp Gene-Ruler DNA ladder was loaded into a gel. After that, mixtures of a 3µL loading dye (6Xloading dye) and 7µL RT-PCRsamplewere loaded into other wells. The tray containing the agarose gel and TBEwas then connected to the power supply set at 80volts and 250mA current for 45minutes. The agarose gel was viewed under UV light using the UV trans-illuminator 2000 system (Bio-Rad Laboratories Inc., California, USA) to visualise the amplified bands. Thegeneratedimages were then captured by a Chemi DocImaging System (Bio-Rad Laboratories Incorporated, California, USA).

2.4 Preparation of the pTrcHis2-TOPO–TTM3 construct

2.4.1 The pTRCHIS2-TOPO expression vector.

The vector used for cloning in this study is the pTrcHis2-TOPO expression vector because it has convenient features. Itallows forfast, efficient cloning and expression systems of eukaryotic proteins in *E. coli*, and high level, regulated expression from the *trc*. The vector also has a 6xHis tag for detection and purification of the recombinant protein through animmobilised metal affinity chromatography.

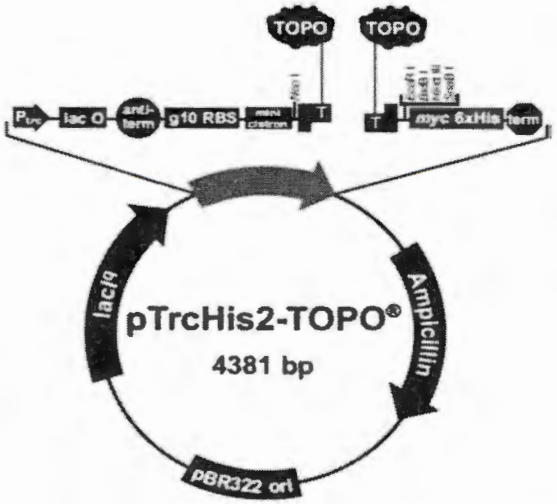


Figure 2.2: Map of pTrcHis2-TOPO vector. Overview of the circular map of the pTrcHis2-TOPO® vector showing the position of its 6xHistidine tag,promoter, and a point of origin to facilitate replication of the plasmid in bacterial cells such as *E. coli*. Thereis also an ampicillin resistant gene that 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).

The vector has the *lacO* sequence for binding the Lac repressor encoded by the *lacI*^{q} gene. In the absence of IPTG, the Lac repressor binds to the *lacO* sequence, repressing transcription. The expressionis inducedupon the addition of IPTG.The vector also has the Amp^{R} gene (ampicillin resistant gene) which is a good selection marker during transformations, in which case, cells with the plasmid can be differentiated from those without the plasmid by growing them on growth media supplemented with ampicillin. Only those cells that have anAmp^{R} gene will grow while those without are inhibited.

2.4.2 Addition of the 3'-adenines overhangs

A volume of 1µL *Taq* was added to 10µL of the amplified RT-PCR product and incubated at 72°C for 10minutes, using a C1000 Thermo-cycler System (Bio-Rad Laboratories Inc., California, USA).

2.4.3 Ligation of the kinase gene insert into the pTrcHis2-TOPO vector

An aliquot of 4µL product was mixed with 1µL of the pTrcHis2-TOPO expression vector (Invitrogen, Carlsbad, USA) into a fresh PCR tube and mixed with a tip. The mixture was incubated at room temperature for 5minutes.

2.4.4Transformation of competent *E. coli* One Shot TOPO 10 competent cells with the pTrcHis2-TOPO:AtTTM3 gene expression construct

A 2µl TOPO cloning reaction (pTrcHis2-TOPO:AtTTM3 Expression Construct) was added into a chilledEppendorf,containing about 100µl of the One-shot TOPO 10 *E.coli*cells.The mixture was mixed gently with a tip. The mixture was then incubated on ice for 30minutes before being heat-shocked on a heating block for 30seconds at 42°C,followed by incubation on ice for 5minutes. Afterthis, 250µLof room temperature SOC media (2%(w/v) tryptone, 0.5%(w/v) yeast extract, 10mMNaCl, 2.5mMKCl, and 10mM $MgCl_2$ and 10mM Mg_2SO_4 and 20 mM glucose) was added to the mixture and then incubated in a shaking incubator at 200rpm,set at 37°C for 30minutes (incubation is for cells to express the β-lactamase enzyme for the subsequent detoxification of ampicillin). Thereafter, the mixture was centrifuged for 1minute to concentrate the cells and 10µl, 20µl and 50µl on to Luria Bertani(LB) agarplates (1%(w/v) agar, 1%(w/v) tryptone powder, 0.5%(w/v) yeast extract, 0.5%(w/v) NaCl)supplemented with 250µL ampicillin and 5ml glucose. The plates were incubated overnight at 37°C.

2.4.5Extraction of the pTrcHis2-TOPO:AtTTM3 construct from the One-Shot10competent cells

Single colonies which grew on the LB Agar plates were picked and used to prepare overnight cultures. The colonies were inoculated into 5ml of double strength yeast-tryptone (2YT) media (0.8%(w/v) tryptone powder, 0.5%(w/v) yeast extract and 0.25%(w/v) NaCl)supplementedwith of 50µg/ml ampicillin and 0.5% of glucose in 15 ml falcon tubes. The falcon tubes were incubated overnight at 37°C in a shakingincubator at 200rpm. In the morning,the tubes were centrifuged at 11,000×*g* for 5minutes, discarding the supernatant. The GeneJet plasmid miniprep kit was then used. The resuspended pellet was mixed with 250µL of a resuspensionsolution the mixture was transferred into an Eppendorftube and vortexed to re-suspendthe cell clumps. A volume of 250µL lysis buffer was added and mixed by inverting until the solution had become slightly clear. Avolume of 350µL neutralisation solution was also added and inverted to mix. The mixture was then centrifuged for 5minutes and the supernatant transferred into the supplied GeneJet spin column. The supernatant in the spin column was centrifuged for 1minute, flow-through discardedwhilethe column was

placedback into its collection tube. A volume of 500µL wash solution was added into the spin column and centrifuged for 1 minute(this step was repeated). The GeneJet spin column was then placed into an Eppendorftube and incubated at room temperature for 2 minutesbefore 50µLof sd$H_2O$was used to elute (centrifuged the column at maximum speed then collected the flow-through) the plasmid. The column was discardedwhilethe plasmidstored at-20°C.

2.4.6 Confirmation of positive clones

The plasmid (construct) was checked to determine whetherthe insert was ligated successfully into the pTrcHis2-TOPOexpression vector using reaction components in Table 2.3and confirmation of positive orientation was also checked using reaction components in Table 2.4. A PCR was performed for both settings as is presented in Table 2.5

Table 2.3: PCR reaction mixture for confirmation of successful ligation of the kinase-like gene fragment into the pTrcHis2-TOPO expression vector.

Component	**Volume**
Template (10 ng DNA)	3 µl
Forward Insert Primers (10 µM)	1 µl
Reverse Insert Primers (10 µM)	1µl
PCR Mix (2X)	25 µl
Water (sdH_2O)	20 µl

Table 2.4: PCR reaction mixture for confirmation of the correct orientation of the kinase-like gene insert in the pTrcHis2-TOPO expression vector.

Component	**Volume**
Template (10 ng DNA)	2 µl
Forward Vector Primers (10 µM)	1 µl
Reverse Insert Primer (10µM)	1µl
PCR Mix (2x)	12,5 µl
Water (sdH_2O)	8,5 µl

Table 2.5: The thermal cycling reaction program for the step by step conditions of the successful ligation and orientation of the kinase-like gene fragment into the pTrcHis2-TOPO expression vector.

Step	Temperature	Time	Cycles
Initial Denaturation	95°C	1 minute	1
Denaturing	95°C	15 seconds	25-35
Annealing	50°C	15 seconds	
Extension	72°C	10 seconds	

Amplification of the insert with the components listed in Table 2.3 indicate a successful ligation of the insert into the vector, while amplification of the insert with the components listed in Table 2.4 would indicatea correct orientation of the ligated insert into the same vector. This approach has been designed as a quick, reliable and user-friendly way of confirming cloning in this kind of a vector.

2.4.6.1 Gel electrophoresis

A 1% agarose gel was prepared. The mixture was shaken to mix the powder with 1XTBE and heated in a microwave until it was clear. The mixture was left to cool,and 10µL per 100ml of ethidium bromide was added to the cooled gel. The mixture was poured onto a gel electrophoresis tray with combs before setting. A 10µL of 100bp Gene-Ruler DNA ladder was loaded into thefirst well. After that, mixtures of a 3µL loading dye (6X loading dye) and 7µL PCR sampleswere loaded into the next wells. The tray containing the agarose gel and TBEwas connected to a power source providing 80volts and 250mA current for 45minutes. The agarose gel was then viewed under UV light using the UV trans-illuminator 2000 system (Bio-Rad Laboratories Inc., California, USA) and band images captured by a Chemi DocImaging System (Bio-Rad Laboratories Incorporated, California, USA).

2.4.7 Transformation of chemically competent *E.cloni*EXPRESS BL21 (DE3) pLysS DUOs Cells and optimisation of protein expression

After confirmation of a successful, correct ligation and orientationof the insert into the TOPO vector, its resultant expression construct (pTrcHis2-TOPO:AtTTM3) was used to transform chemically competent *E.cloni* EXPRESS BL21 (DE3) pLysS expression cells. According to the manufacturer's protocol and instructions (Lucigen, Wisconsin, USA), a 2ml tube

containingthe BL21competent *E.coli* cells were thawed on ice for 10minutes. An aliquot of 5µL plasmid DNA was added to the cells and mixed by flicking the tube. The mixture was incubated on ice for 30minutes without shaking,and heat shocked at 42°C for 10seconds. The mixture was further incubated on ice for 5minutes. A room temperature conditioned SOC medium was added and incubated at 37°C for 60minutes in a shaker shaking at 250rpm. The components were mixed with a pipette tip and aliquots of 10µL,20µl and 50µL plated on prepared LB agarcontaining100 µg/ml ampicillin and 0,5% glucose. The plates were then incubated at 37°C overnight.

2.5 Expression of the recombinant AtTTM3protein

A transformed *E.coli* BL 21 (DE3) pLys cell colony,containing thepTrcHis2-TOPO:AtTTM3 expression construct, was used to inoculate 5ml of 2YT media containing 50% glucose and 100 µg/ml ampicillin in a 15ml falcon tube. The Falcon tube was incubated overnight to shake at 250rpm at 37°C and in the morning, subcultures were prepared by transferring 1ml of the overnight culture into 30ml 2YT media containing 100µg/ml ampicillin and 50% glucose. The culture was incubated to shake at 250rpm in a shaker at 37°C and up until an OD_{600} of 0.5 was reached, as measured by a Helios spectrophotometer; (Merck, Gauteng, RSA). The culture was divided intotwo equal parts.One culture wasthen induced to express the intended AtTTM3recombinantproteinby adding 1mM of isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma-Aldrich Corp, Missouri,) while the other culture was left un-induced (control). The divided cultures were incubated to shake at 37°C at a speed of 250 for 3 hours incubation. After incubation, portions of both the induced and uninduced cultures were then analysed by SDS-PAGE while the rest of the culturewasstored for further assays.

2.6 Determination of the kinase activity of the recombinant AtTTM3 protein

The generated crude protein extracts from above wereused to confirmthe predictedkinase activity of the recombinantAtTTM3 protein. This assessment was carried out *in vitro*, whereby the AtTTM3's ability to direct the phosphorylation of particular substrate peptides as is described by the Omnia™ Recombinant system (Catalog # KNZ1241; Life Technologies, Carlsbad, USA) was ascertained. Briefly, 100 µl reaction systems containing 20 µL of the generated crude protein extract (induced or non-induced), 1X reaction buffer,

1mM of ATP and, 0.2 mM DTT, and 25 µM of the Ser/Thr peptide were prepared. The prepared samples were then carefully transferred into a black FluoroNuncMaxisorp 96-well plate (AEC Amersham, Little Chalfont, UK) in triplicate forms (30 µl apiece) and incubated at 30°C for 5 minutes. The plate was then mounted onto a pre-equilibrated (30°C) Fluoroskan Ascent FL fluorometer (AEC Amersham, Little Chalfont, UK), followed by an immediate measurement of the phosphorylation activity in form of fluorescence signals at 485 nm emission (λ_{em} 485) and after a reaction excitation at 360 nm (λ_{ex} 360). Reaction activity readings were then recorded as relative fluorescence units (RFUs) after every minute for 5 minutes.

2.6.1 Statistical analysis of the *in vitro* kinaseactivityassays

Data obtained from the above studies was subjected to an analysis of variance (ANOVA) Super-ANOVA, Stats graphics Version 7; 1993 (Stats graphics Corp., Missouri, USA). Wherever ANOVA revealed significant variations between treatments, the affected means (*n=3*) were then separated with a post-hoc Student Newman Kuehls (SNK), multiple range test ($p \leq 0.05$).

2.7Bioinformatic expressional analysis of the AtTTM3 gene

Bioinformatic analysis wasused tocomplementthe biochemical work undertaken on the AtTTM3 protein. A wide range of web-based software and computer programs were used toelucidatethe probable physiological roles of theAtTTM3 protein in *A. thaliana*and other relating higher land plants.

2.7.1Anatomical expression analysisof the AtTTM3 gene

The AtTTM3 gene expression levels in various tissues of the *A thaliana* were acquired through a microarray database,using the expression-data analysis tool, GENEVESTIGATOR version V3 (www.genevestigator.com/gv/) (Zimmermann *et al.*,2004; Grennan, 2006). The tool operates through anAffymetrix Arabidopsis genome array platform of the 260011_At probe in the provision of information regarding the genomic transcriptome information of the specifically selected gene. Regarding this case, the AtTTM3 gene fragment was used as a

probe term ahead of the arbitrary values of the expression concentrations of the AtTTM3 in various acquired Arabidopsis tissues.

2.7.2 Developmental expression analysis of the AtTTM3 gene

For determination of the expressional levels of the AtTTM3 in the *A. thaliana* plant at various developmental stages, usage of the AtGeneExpress visualization and developmental tool (www.arabidopsis.org) was instituted, whereby the AtTTM3 gene fragment was probed against 10 various developmental stages of the plant (Zimmermann *et al.*,2004; Grennan, 2006). These developmental stages were set to be from the radicle emerging from the seed coat up to the release of mature seeds from the pod.

2.7.3 Co-expressional analysis of the AtTTM3 gene

For determination of the co-expressional profile of the AtTTM3 gene with the other related genes in the Arabidopsisplant, usage of the Arabidopsis co-expression tool (ACT) (http://genevestigator.com) was effected. The analysis was applied across all microarray experiments with the usage ofthe AtTTM3 gene fragmentsas the search gene, leaving a blank on the gene list limit in order to acquire a full correction list. Signal intensity from microarray experiments is used in the tool to acquire the Pearson correlation coefficient (r-value), indicating various expression of the linear associations among a reference gene AtTTM3 gene fragmentand all other Arabidopsis genes represented on the selection chip. Both the positive and negative correlation (ranges from -1 to +1) were then calculated by the tool, whereby values of statistical significance, expressed as a probability (P) and expectation (E) weremeasured and generated.

2.7.4)Stimuli specific analysis of the AtTTM3 gene

As the stimulus tool was used, the screening of the expression profiles of the AtTTM3 and the top 50 most co-expressed genes (ECGG-50) (the AtTTM3:ECGG-50) was carried out over the ATH1:22K array Affymetrix public microarray data in the GENEVESTIGATOR version V3 (http://genevestigator.com). The normalised microarray data were then downloaded,andanalysationwas done by the GEO (NCBI) (www.ncbi.nlm.gov/geo), TAIR GeneExpress (www.ebi.ac.uk/microarrays-as/ac) andNASC Arrays

(www.affymetrix.arabidopsis.info/narrays/experimentbrowse.pl) for experiments that induced differential expression of the co-expressed genes. Measurements were then taken for each fold change (log2) value of an experiment that induced expression and consequently, providing expression values through a Multiple Array Viewer program of the Multiple Viewer (MeV) software package (version 4.2.01) (The Institute for Genomic Research (TIGR)).

CHAPTER 3

RESULTS

3.1 Generation ofthe *A. thaliana* plants

A. thaliana seeds were planted on MS media to generate seedlings. After growing for two weeks, the seedlings were then transplanted onto sterile potting soil for further growth of another four weeks under greenhouse condition using a growth chamber grown at 25° C within a growth chamber set at 16/8 hour day/night cycle with humidity of 60. After six weeks, the whole plantswere used for extraction of total RNA. As seen below plants grew and ready to be used for RNA extraction.

A) B)

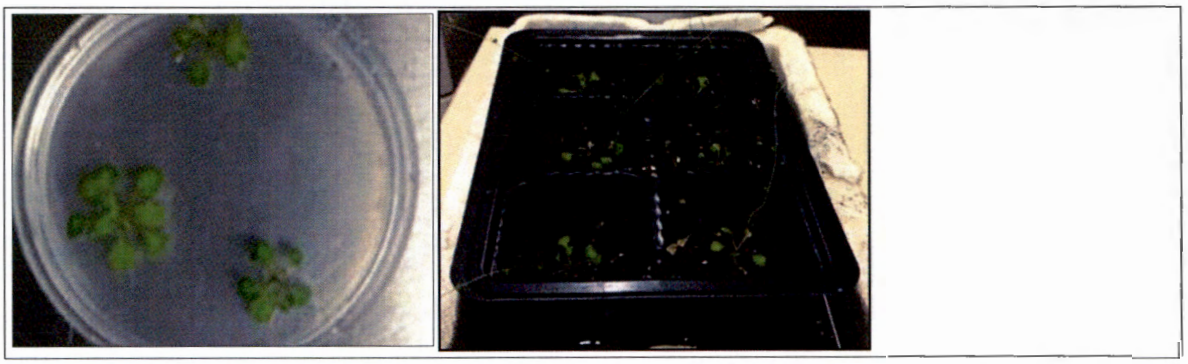


Figure 3.1: Generation of the *A. thaliana* plants. A) Planted *A. thaliana* seeds planted on the MS media. **B)** The transplanted seedlings after six weeks in the growth chamber that were then used for total RNA extraction.

3.2 Isolation and cloning of the AtTTM3 gene fragment

The coding region of the AtTTM3 gene (At22g11890) was amplified from the*A. thaliana* total RNA, using RT-PCR and the synthesised sequence-specific primers. Theampliconwas then ligatedinto a pTrcHis2-TOPO vector forming the pTrcHis2-TOPO:AtTTM3construct.Successfulligation of the AtTTM3 coding region into the pTrcHis2-TOPO vector wasverified usingconventionalPCR and self-primers. Below results are indicated to show positive results as the bands were observed after using gel agarose electrophoresis.

A) B)C)

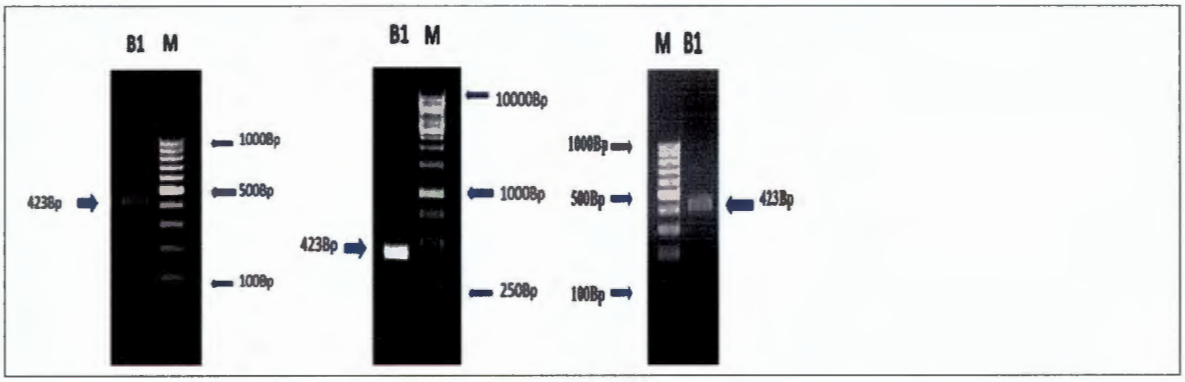


Figure 3.2: Isolation and cloning of the AtTTM3 gene fragment. A 1% agarose gel showing **A)** the AtTTM3 coding region amplified using RT-PCR; **B)** confirmation of a successful ligation of the AtTTM3 coding region into the pTrcHis2-TOPO expression vector using the PCR with the coding region specific primers; **C)** confirmation of the successful orientation of the AtTTM3 coding region insert in the pTrcHis2-TOPO expression vector using the PCR witha vector specific primer (forward) anda coding region specific primer (reverse). M represents the DNA ladder (Stipulate which DNA ladder was used).

3.3 Expression of the recombinant AtTTM3 protein

The recombinant plasmid construct carrying the correctly-oriented AtTTM3-Kcoding region was used to transform chemically competent *Ecloni* EXPRESS BL21 (DE3) pLysS cells for recombinant protein expression.Protein expression was then induced through the addition of 1 mM IPTG to the transformed cells while a portion of the same culture was left un-induced (acting as a control). After using SDS-PAGE results below were indicated that our recombinant AtTTM3 protein indicated a size of 15.51 kDa.

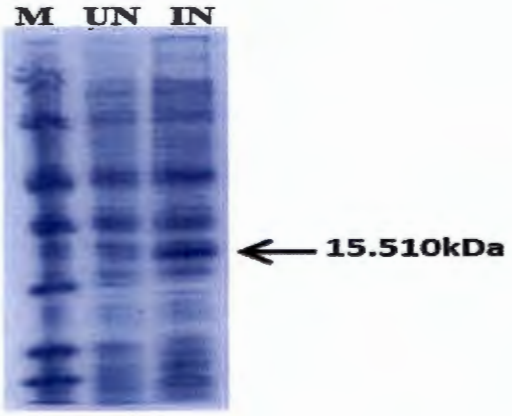


Figure 3.3:Expression of the recombinant AtTTM3 protein. SDS-PAGE showing the level of AtTTM3 expression in the un-induced (UN) and induced (IN) cell cultures. M represents the unstained low molecular weight marker (catalog#SM0431 Fermentas International Inc., Burlington, Canada), while the arrow is marking the expressed recombinant AtTTM3 protein.

3.4Determination of the kinase activity of the recombinant AtTTM3 protein

After expression, the generated crude protein was assessed forpossiblekinase activity of the recombinant AtTTM3 protein. The assessment was carried out *in vitro*measuring phosphorylation activity in the form of fluorescence signal at 485 nm emission (λ_{em} 485) and after a reaction excitation at 360 nm (λ_{ex} 360) usingthe Omnia^{TM} Recombinant system.

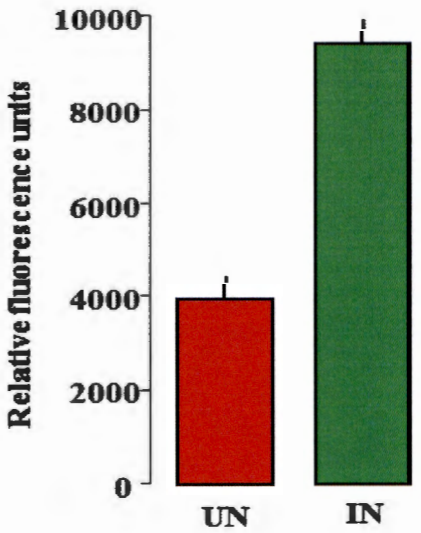


Figure 3.4:Determination of the kinase activity of the recombinant AtTTM3 protein. Phosphorylation activity level of the un-induced (UN) and induced (IN) cell cultures of the recombinant AtTTM3 protein.

3.5Determination of theanatomical expression of the AtTTM3 gene

Ananalysis of At2g11890 usingGenevestigator showed that the AtTTM3 protein is transiently expressed in various tissues of the*A. thaliana* plant, which include the cell embryo, stamen, seedling, the leaf, the root, the cotyledon, the meristem, the root and the collective flower carpel (www.arabidopsis.org). Moreover, the analysis indicated that the AtTTM3 protein is highly expressed in the peripheral endosperm,chalazal seed coat, chalazal endosperm, testa, general seed coat,micropylar endosperm, suspensor and embryo (Figure 3.5 below).

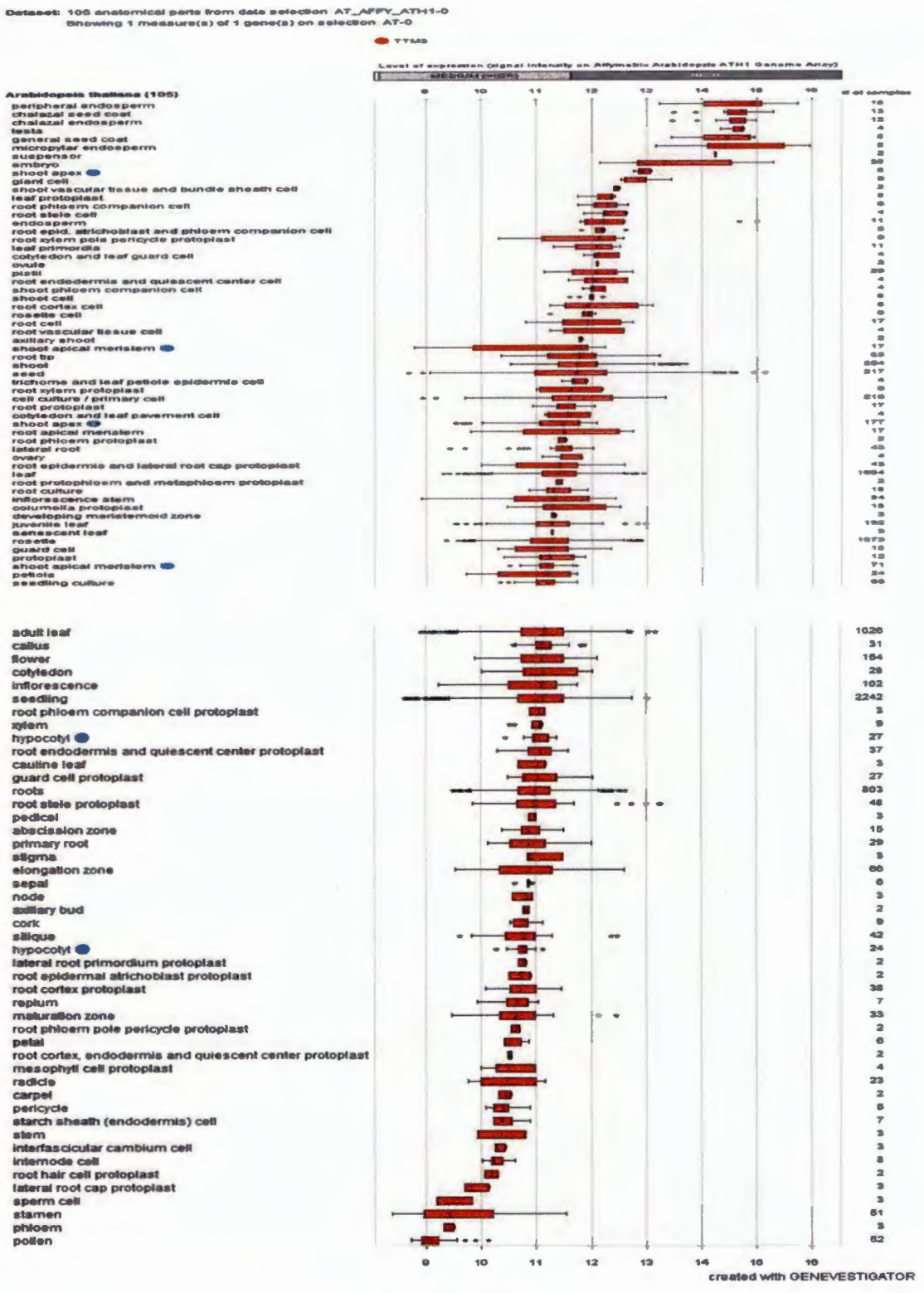


Figure 3.5:Levels of expression intensity of the AtTTM3 protein in the various tissues of the *A.thaliana*. The Figure shows that the AtTTM3 protein is highly expressed in the peripheral endosperm, chalazal seed coat, chalazal endosperm, testa, general seed coat, micropylar endosperm, suspensor and embryo and lowly expressed in the sperm cell, stamen, phloem and pollen (Retrieved from the Genevestigator anatomy tool).

3.6Determination of the developmental expression of the AtTTM3

The AtGeneExpressvisualisation and development tools (Zimmermann *et al.*, 2004) were usedto obtain a comprehensive expressional profile of the AtTTM3 protein during the various developmental stages of the *A. thaliana* plant, by screening microarray data using the At2g11890 as a search query. The obtained results generated ten developmental stages during which AtTTM3 protein is expressed and indicated that the protein is highly expressedduring the three main stages of seed germination, rosette and senescence. The results also indicated that the protein is expressed at low levels during the seedling, young flower and maturesiliquesdevelopmental stages (Figure 3.6 below).

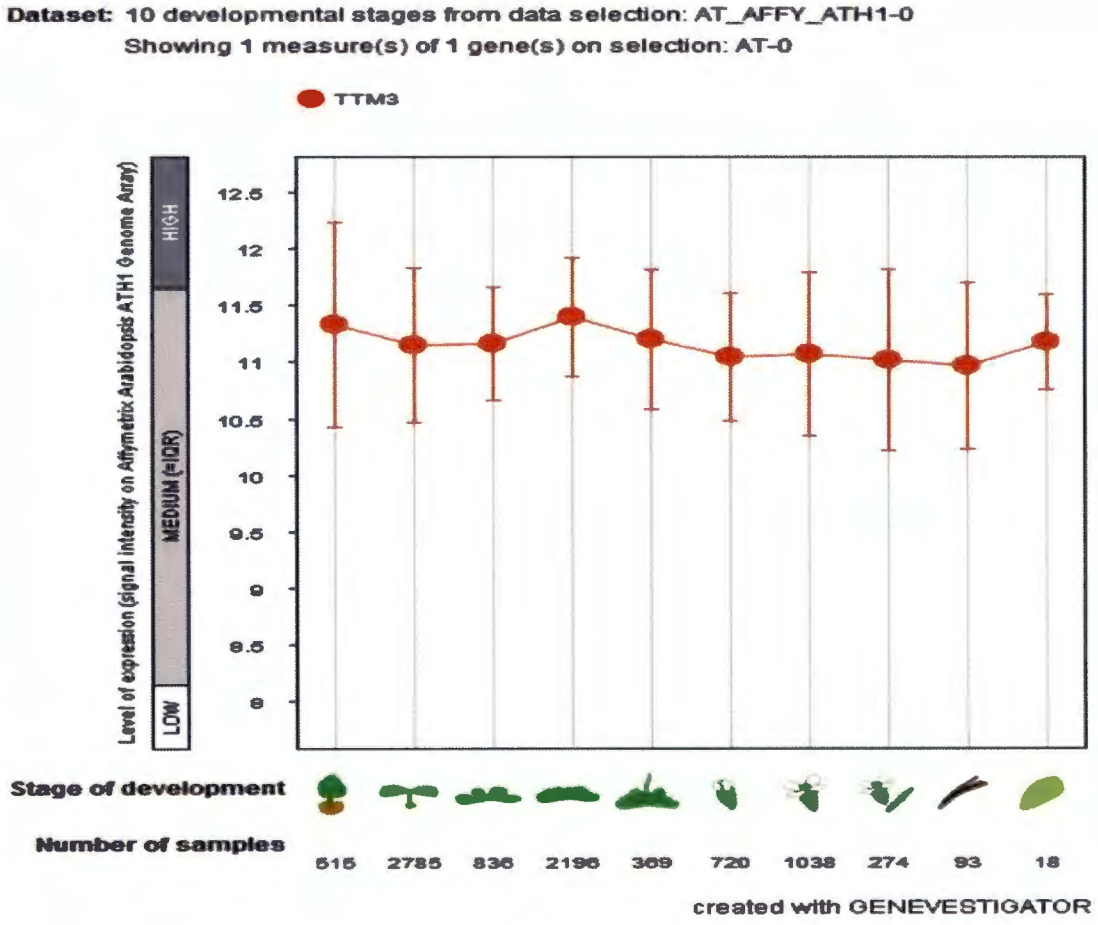


Figure 3.6:Expression profile of the AtTTM3 protein during the various developmental stages of the *A. thaliana* plant. Measurements of the expression levels of the AtTTM3 protein are shown above with the various stages of development, from the germinating seed up to senescence. The profile indicated that the AtTTM3 protein is highly expressed during the seed germination, the rosette developmentand senescence stages. It is also indicating that the protein is lowly expressed during the seedling, young flower and matured silique developments.

3.7Determination of the co-expression profile of the AtTTM3 protein

Measurements of the expression profile values for the At2g11890 gene using the Arabidopsis co-expression tool (ACT) (http://genevestigator.com) on available microarray data with about 359 diversetranscriptome experiments were taken to obtain protein lists that are co-expressed with AtTTM3. The various chip experiments indicated that the AtTTM3 protein is co-expressed with many other proteins and the50 most co-expressed proteins,having Pearson correlation values of between0.80and0.89 are listed in Table 3.1.

Table 3.1: A list of thetopmost50 proteinsco-expressed withthe *A. thaliana*AtTTM3protein.

Rank	r-value	Locus and GO	Description or Annotation
1	0.89	AT1G47970	Nucleolin
2	0.88	AT3G24490	Alcohol dehydrogenase transcription factor Myb/SANT
3	0.87	AT2G17350	Beta-mannosyltransferase-like protein
4	0.87	AT4G21140	Copper ion-binding protein
5	0.86	AT1G15200	Protein-protein interaction regulator family protein
6	0.85	AT3G24080	KRR1 family protein
7	0.85	AT3G19650	Cyclin-like protein
8	0.85	AT3G18790	Pre-mRNA-splicing factor ISY1-like protein
9	0.85	AT5G61310	Cytochrome c oxidase subunit Vc family protein
10	0.85	AT5G41770	Crooked neck protein, putative/ cell cycle protein
11	0.84	AT3G02760	Class II aaRS and biotin synthetases superfamily protein
12	0.84	AT1G54060 SoRP, MCoR, MoR, CPRMo, EsD, EaECD, MG, GG,FGG	Trihelix DNA binding protein family
13	0.84	AT2G40650	PRP38 family protein
14	0.84	AT4G03180	rRNA-processing protein
15	0.84	AT3G58660	Ribosomal protein L1/ L10e family
16	0.83	AT1G54440	Polynucleotidyltransferase, ribonuclease
17	0.83	AT5G55670	RNA –binding (RRM/RBD/RNP motifs) family protein
18	0.83	AT1G72440	NOC1/ Mak21 homology
19	0.83	AT2G27470	Nuclear factor Y, subunit B11
20	0.83	AT5G16950	Krueppel-like factor
21	0.83	AT3G50690	Leucine-rich repeat (LRR) family protein

22	0.82	AT1G12830[SoRP, MCoR, MoR, CPRMo, EsD, EsECD, MG, GG,FGG]	Nucleolin
23	0.82	AT3G20740	Polycomb group proteins
24	0.82	AT3G48120	Serine/ arginine-rich splicing factor
25	0.82	AT5G65720	Encodes a cysteine desulfurase
26	0.82	AT1G07660	Histone superfamily protein
27	0.82	AT4G12240	Zinc finger (C2H2 type) family protein
28	0.82	AT3G26640	LIGHT-REGULATED WD1 (LWD1)
29	0.82	AT3G62470 AT5G14820	Pentatricopeptide repeat (PPR) superfamily protein
30	0.82	AT5G44450	Alpha amino-terminal protein methyltransferase
31	0.82	AT5G25060	RNA recognition motif (RRM)-containing protein
32	0.82	AT1G11240[SoRP, MCoR, MoR, CPRMo, EsECD, MG, GG,FGG]	Ribosomal RNA-processing protein
33	0.82	AT2G25720	Hypothetical protein
34	0.82	AT5G09830	BolA-like family protein
35	0.81	AT5G58920	Homeoboxprospero protein
36	0.81	AT3G26420	Glycine-rich splicing factor
37	0.81	AT4G18593	Dual specificity phosphatase-like protein
38	0.81	AT1G68790	Little nuclei3
39	0.81	AT3G62450	DNA mismatch repair protein
40	0.81	AT3G50670	Encodes LACHESIS (LIS)
41	0.81	AT1G74250	DNAJ heat shock N-terminal domain-containing protein
42	0.81	AT5G61030	Glycine-rich binding protein
43	0.81	AT5G60030	Hypothetical protein
44	0.81	AT5G47090	Coiled-coil protein
45	0.80	AT5G51410	LUC7 N terminus domain-containing protein
46	0.80	AT3G49890[SoRP, MCoR, MoR, CPRMo, EsD, EsECD, MG, GG,FGG]	Hypothetical protein
47	0.80	AT5G23890	GPI-anchored adhesin-like protein
48	0.80	AT3G09980	Family of unknown function(DUF662)
49	0.80	AT5G36210	Alpha/ beta- hydrolases superfamily protein
50	0.80	AT3G49240[SoRP, MCoR, MoR, CPRMo, EsD, EsECD, MG, GG,FGG]	Pentatricopeptide repeat-containing protein

Abbreviations for the indicated GOs: SoRP=Single organism reproductive process; MCoR=Multicellular organism Reproduction;MoRP=Multicellular organism Reproduction Process; CPRMo =Cellular Process involved in Reproduction in Multicellular organisms; EsD= Embryo sac Development; EsECD= Embryo sac Egg Cell Development; MG= Megagametogenesis; GG= Gemete generation; FGG= Female Gamete Generation.

3.8Determination of the stimulus-specific expression of the AtTTM3 protein

After the co-expressional profile and the probable functional roles of the AtTTM3 was established there was anindication of its most top 50 co-expressed genes (ECGG-50) andthe whole set (AtTTM3:ECGG-50) was then exposed to an *in-silico*global expression analysis, where precise experimental conditions able to induce differential expression of these genes were identified in the set. In accordance with the co-expression and GO analysis, heat maps which were created from the microarray expression analysis showed that the transcriptional processes of the AtTTM3 protein and its related ECGG-50 proteins (the AtTTM3:ECGG-50 pool) are generally and collectively induced by a variety of biotic and abiotic factors (Figure 3.7).

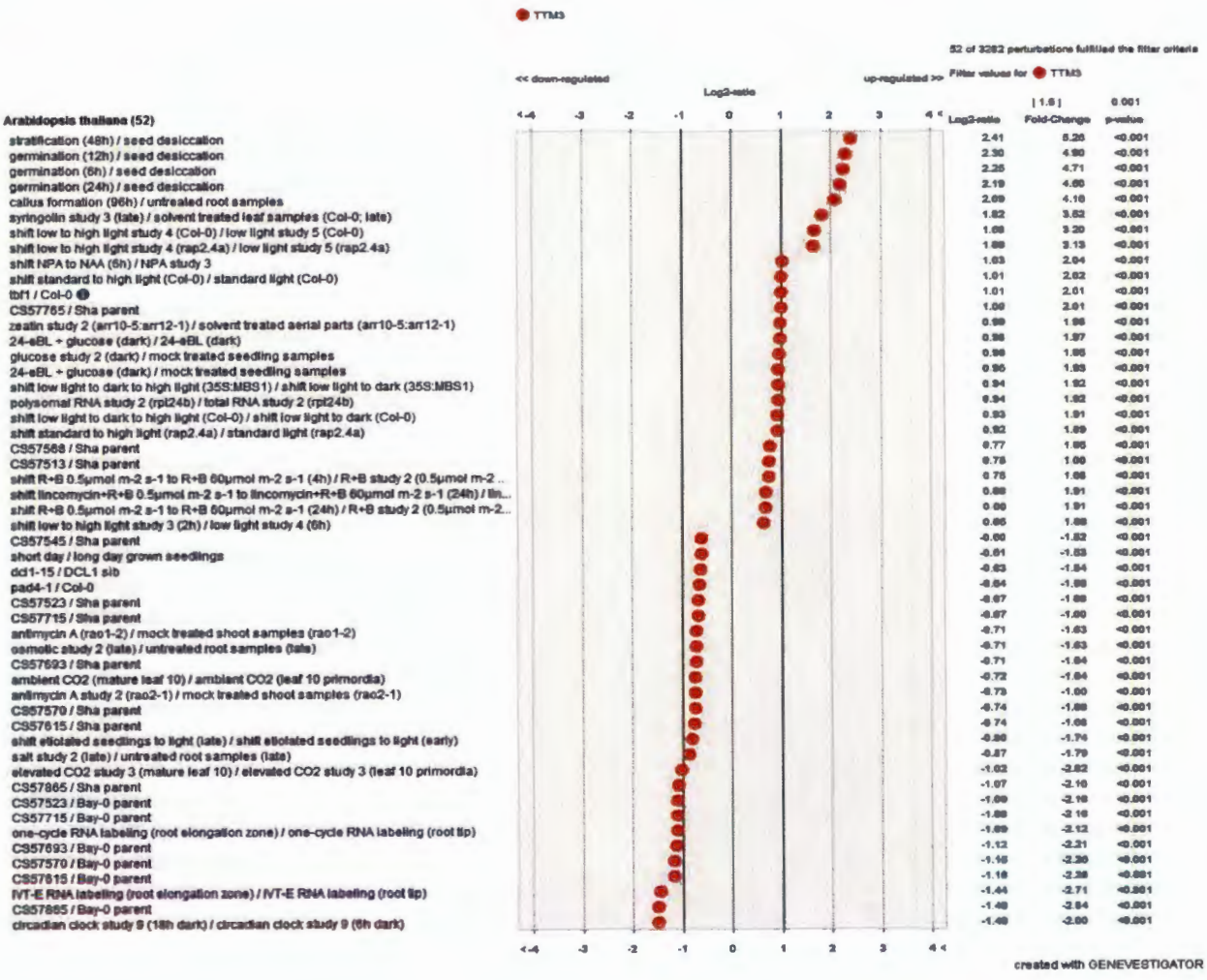


Figure 3.7:The AtTTM3 protein expression profiles in the *Arabidopsis thaliana*in response to various stimulus-specific conditions. A differential induced expression of the protein was observed in response to a wide array of biotic (*Pseudomonas bacteria*) and abiotic (desiccation and light) factors.

CHAPTER 4

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

4.1 Discussion

The At2g11890 gene, coding for the AtTTM3 protein, is annotated gene as an AC/kinase that is found in the *A. thaliana*. This gene is confirmed to be located in the roots of the plant, where it plays a significant role in root development (TAIR - The Arabidopsis Information Resource) (http://www.arabidopsis.org). Naturally, plants are affected by various biotic and abiotic stress factors, affecting their yield and growth. Based on the fact that they are fixed in one position (sessile), they cannot move away for defence or protection when any dangers or environmental stress arises. Plants, therefore, need defence mechanisms to respond and/or overcome these situations.

For their defence or protection to take place where they are situation the defence mechanism called signal transduction mechanism is responsible. It is indicated in Figure 1.2 that the AC work hand in hand with the kinase where a transmembrane receptor activates AC which in turn activates PKA.

Other activated kinases are indicated within the signal transduction pathway which also plays a role in defense mechanism with the plant. It is indicated from other literature that kinase does play an essential role in plants defence or development. Also it is indicatated that AC plays an essential role in the activation of kinases so the adenylatecyclase system contains a group of proteins responsible for the generation of cyclic nucleotides. The complex is openly coupled to receptors which can inhibit or stimulate cyclase activity. The cyclase system's G proteins seem to impact the ligand affinity of receptors and might be openly involved in the process of receptor desensitisation (Gorelick, 1987).

It was indicated by Cohen, 2000 that the important role of protecting cells against cell damaging agents and infections is played by the protein kinase MAPK-activated protein kinase-2 (MAPKAP-K2). It is activated by stress-activated protein kinase-2 (SAPK2, also called p38) which is accompanied by the phosphorylation of three residues called T221, S272 and T334. The phosphorylation of only one residue is insufficient for activation whereby activation of the two from the three phosphorylated sites, are an achievement of a maximal activation (Cohen, 2000).

Including the Bcl-2 and related cytoplasmic proteins, such as BAD (a Bcl-2/Bcl-XL antagonist which cause cell death), are known as the key regulators of apoptosis, and much interest has been focussed upon their regulation in recent years. BAD is known as a proapoptotic protein binding to the anti-apoptotic proteins Bcl-2 and Bcl-XL, the interaction is thought to neutralize the anti-apoptotic effects of Bcl-2 and Bcl-XL which may represent one of the mechanisms which BAD stimulates apoptosis (Cohen, 2000). A protein kinase that plays a key role in insulin signal transduction is known as Glycogen synthase kinase 3 (GSK3). It is subdued by PKB in response to insulin which leads to activation and dephosphorylation of proteins (glycogen synthase and eukaryotic protein synthesis initiation factor 2B (eIF2B)) also to the stimulation of glycogen and protein synthesis (Cohen, 2000).

Also according to Kwezi, *et al.*, 2010 it was stated that Phytosulfokines (PSKs) are sulfatedpentapeptides stimulating plant growth and variation mediated by the PSK receptor (PSKR1) it is a leucine-rich repeat receptor-like kinase.The wall-associated kinase-like 10 (WAKL10), has also been lately reported to have GC activity *in vitro* as well as being transcriptionally up-regulated in response to biotic stress. Also it is indicated that mutant studies of AtPSKR1 is involved in regulating root elongation (Kwezi*et al.,* 2010). The WAK/WAKL genes are typically predicted to encode a class of receptor-like protein kinases that possess a transmembrane (TM) domain, a cytoplasmic serine/threonine kinase (STK) domain and an extracellular region that is tightly associated with the cell wall and contains several epidermal growth factor (EGF) repeats that may act as ligand binding domains (Meier *et al.*, 2010).

Another kinase indicated by Wang et al., 2014 states that BAK1 is identified to be co-receptors of several ligand-binding LRR-RLKs on the plasma membrane such as BRASSINOSTEROID INSENSITIVE1 (BRI1) perceiving brassinosteroid (BR) signals [3–5], FLAGELLIN SENSING2 (FLS2) and EF-TU RECEPTOR (EFR) both recognizing specific pathogen-associated molecular patterns (PAMPs) to trigger innate defense programs upon biotic stresses. The heteromerization of BAK1 with different ligand-binding LRR-RLKs and subsequent phosphorylation–dephosphorylation events within the complexes provide the molecule basis for BAK1 regulatingmultiplesignaling pathways that contribute to plant development, innate immunity and cell death control (Wang *et al.,* 2014).

From the results it was indicated that the AtTTM3 is an annotated gene for AC and kinase functions, and therefore it was selected to be experimentally tested, using various biotechnological techniques. The AtTTM3 sequence and encoded protein sequence was

retrieved from the TAIR and Expasy websites. Following this retrieval, some sequence-specific primers were then designed (Figure 2). As is shown in Figure 3.2A, the expected 423 bp long fragment was successfully obtained. After isolation, the obtained AtTTM3 gene fragment was then ligated into pTrcHis2-TOPO vector, where its successful cloning was then confirmed under standard PCR. Protein expression was induced by adding 1 mM IPTG to the transformed cells, followed by analysis by SDS-PAGE. As is shown in Figure 3.3, a recombinant protein product of approximately 15.51 kDa was obtained.

Compared to other known Arabidopsis kinases such as AtWAKL-10 (Meier *et al.*, 2010), AtPSKR-KD2 (Kwezi*et al.*, 2011) and AtPSK-R1 (Muleya*et al.*, 2014), whose activities were also similarly determined as that of AtTTM3, the activity of AtTTM3 was somewhat higher. The relative activity for AtWAKL-10 was found to be 5000 RFUs (Meier *et al.*, 2010), that of AtPSK-KD2 1400 RFUs (Kwezi*et al.*, 2011) while the one for AtPSK-R1 was 700 RFUs (Muleya*et al.*, 2014). The possible reason why AtTTM3 had a higher enzymatic activity than its other Arabidopsis counterparts could be because AtTTM3 was tested in the form of a crude extract (in its natural native form) while the rest of the other recombinants were tested after passing through the purification and refolding processes. Both the purification and refolding processes severely expose recombinant proteins to excessive salts and buffers that somewhat might affect their standard physiological properties and thus function (Bornhorst&Falke, 2000).

4.2 Conclusion

Findings from this study clearly showed that the AtTTM3 protein from *A. thaliana*is a *bona fide*functional higher kinase with key and essential roles in plant growth and development. The AtTTM3, therefore, becomes the fourth ever kinase molecule to be identified in *A. thaliana.*

4.3 Recommendations

Based on findings generated from this work, the following recommendations are proposed for future studies:

1. A crude protein was used during the assays of this study,therefore, further and future assays should be carried out using a purified version of the AtTTM3 protein.

2. The kinetic assays (measuring the K_m and V_{max} values)and further characterisation of the AtTTM3 need to be undertaken so that a better picture of the enzymology and/or enzymatic properties of this novel plant protein are ascertained and established.

3. This undertaken work was carried out *in vitro*so, it could be better to further attempt it *in situ* or *inplanta* so that a clear picture of the functional roles of this AtTTM3 protein in Arabidopsis and other related higher plants is deduced and ascertained.

4.The possible interaction of this AtTTM3 and ACs (cAMP signalling) in plants must be explored further, particularly with regards to plant growth and developments.

REFERENCES

Agrawal GK, R.R., Iwahashi H.2002. Isolation of novel rice (*Oryza sativa* L.) multiple stress responsive MAP kinase gene, OsMSRMK, whose mRNA accumulates rapidly in response to environmental cues. *BiochemBiophys Res Commun***294:** 1009- 1016.

Alberts B, Lewis J, Morgan D, Raff, Roberts K, Walter P. 2014.*Molecular biology of the Cell.* 6thEdition, New York: Garland Science.

kjassociations of Gene Ontology terms with groups of genes. [Bioinformatics] (http://www.ncbi.nlm.nih.gov/pubmed/14990455) 20(4):578-80.

Biochemistry online: Signal transduction at cell membranes: Available at http://biochem-vivek.tripod.com/id57.html on 8 November 2018.

BornhorstJA ,Falke JJ. 2000. Purification of proteins using polyhistidine affinity tags. *Methods Enzymol***326:** 245- 254.

Chang C, Kwok SF, Bleecker AB, Meyerowitz EM. 1993. Arabidopsis ethylene-response gene ETR1: similarity of product to two-component regulators. *Science-York then Washington***262:** 539.

Dissmeyer N, Schnittger A. 2011. The age of protein kinases. In: DISSMEYER, N. & SCHNITTGER, A. (eds.) Plant kinases: Methods and protocols. *Molecular Biology* **779:** 7- 52.

Gallagher DT, Smith NN, Kim SK, Heroux A, Robinson H, Reddy PT. 2006. Structure of the class IV adenylyl cyclase reveals a novel fold. *Journal of Molecular Biology***362:** 114-122.

Gasteiger E, Gattiker A, Hoogland C, Ivanyi I, Appel RD, Bairoch A. 2003. ExPASy: the proteomics server for in-depth protein knowledge and analysis *Nucleic Acids Res.* **31:** 3784- 3788.

Gong C, Smith P, Shuman S. 2006. Structure-function analysis of Plasmodium RNA triphosphatase and description of a triphosphate tunnel metalloenzyme superfamily that includes Cet1-like RNA triphosphatases and CYTH proteins. *RNA***12:** 1468- 1474.

Grennan AK. 2006. Genevestigator, facilitating web-based gene-expression analysis. *Plant Physiology* **141:** 1164- 1166.

Hatfield JL, Prueger JH. 2015. Temperature extremes; effect on plant growth and development. *Weather and Climate Extreme***10:** 4- 10.

Hegedus DD, Gerbrandt K, Coutu C. 2016. The eukaryotic protein kinase superfamily of the necrotrophic fungal plant pathogen, *Sclerotiniasclerotiorum*. *Molecular Plant Pathology***17:** 634- 647.

Ho HL. 2015. Functional roles of plant protein kinases in signal transduction pathways during abiotic and biotic stress. *Journal of Biodiversity, Bioprospecting and Development***2:** 1- 8.

Hruz T, Laule O, Szabo G, Wessendorp F, Bleuler S, Oertle L, Widmayer P, Gruissem W and P Zimmermann (2008) Genevestigator V3: a reference expression database for the meta-analysis of transcriptomes. *Advances in Bioinformatics* 420747.

Hughes DA. 1994. Signal-transduction-histidine kinases; Hog the limelight. *Nature***369:** 187-188.

Ishida T, Tabata R, Yamada M, Aida M, Mitsumasu K, Fujiwara M, Yamaguchi K, Shigenobu S, Higuchi M, Tsuji H. 2014. Heterotrimeric G proteins control stem cell proliferation through CLAVATA signalling in Arabidopsis.*EMBO Reports***15:** 1202-1209.

Iyer LM, Aravind L. 2002. The catalytic domains of thiamine triphosphatase and CyaB-like adenylyl cyclase define a novel superfamily of domains that bind organic phosphates. *BMC Genomics***3:** 33.

Johnson SA, Hunter T. 2005. Kinomics: methods for deciphering the kinome. *Nature Methods* **2:** 17- 25.

Klimecka M, Muszynska G. 2007. Structure and functions of plant calcium-dependent protein kinases. *ACTA BiochimicaPolonica***54:** 219.

Korneeva NL, Song A, Gram H, Edens MA, Rhoads RE. 2016. Inhibition of mitogen-activated protein kinase (MAPK)-interacting kinase (MNK) preferentially affects translation of mRNAs containing both a 5′-terminal *cap* and hairpin. *Journal of Biological Chemistry***291:** 3455- 3467.

Kwezi L, Ruzvidzo O, Wheeler JI, Gowender K, Iacuone S, Thompson PE, Gehring C, Irving HR. 2011. The phytosulfokine (PSK) receptor is capable of

guanylatecyclaseactivity and enabling cyclic GMP-dependent signalling in plants. *Journal of Biological Chemistry***286:** 2258- 2258.

Lemmon MA, Schlessinger J. 2010. Cell signalling by receptor tyrosine kinases. *Cell***141:** 1117- 1134.

Lima CD, Wang LK, Shuman S. 1999. Structure and mechanism of yeast RNA the mRNA capping apparatus. *Cell***99:** 533- 543.

Lucas KA. 2000. Guanylylcyclases and signalling by cyclic GMP. *Pharmacological Reviews***52:** 375- 414.

Martinez J, Truffault V, Hothorn M. 2015. Structural determinants for substrate binding and catalysis in triphosphate tunnel metalloenzymes. *Journal of Biological Chemistry***290:** 23348- 23360.

Medical dictionary accessed at (http://medical-dictionary.thefreedictionary.com/mitogen) on 7 November 2016.

Meier S, Ruzvidzo O, Morse M, Donaldson L, Kwezi L, Gehring C. 2010. The Arabidopsis Wall Associated Kinase-Like 10 gene Encodes a Functional GuanylylCyclase and is Co-expressed with Pathogen Defense-Related Genes. *PLoS ONE***5 (1):** 1- 17

Mutwil M, Obro J, Willats WGT. 2008. GeneCAT-novel webtools that combine BLAST and co-expression analyses. *Nucleic Acids Research***36:** W320- W326.

Pearce LR, Komander D, Alessi DR. 2010. The nuts and bolts of AGC protein kinases. Nature reviews. *Molecular Cell Biology***11:** 9.

Petrov V, Hille J, Mueller- Roeber B, Gechev TS. 2015.ROS-mediated abiotic stress-induced programmed cell death in plants. *Frontiers in Plant Science***6:** 69.

Pitelka LF. 2017. Plant migration and climate change. *JSTOR***85:** 464- 473.

Poole R. 2007. The TAIR database. *Plant Bioinformatics: Methods and Protocols***1:** 179-212.

Popov KM, Kedishvili NY, Zhao Y, Gudi R, Harris RA. 1994. Molecular cloning of the p45 subunit of pyruvate dehydrogenase kinase. *Journal of Biological Chemistry***269:** 29720- 29724.

Rodríguez-Concepción M, Boronat A. 2015. Breaking new ground in the regulation of the early steps of plant isoprenoid biosynthesis. *Current Opinion in Plant Biology***25:**17- 22.

Song J, Bettendorff L, Tonelli M, Markley JL. 2008. Structural basis for the catalytic mechanism of mammalian 25-kDa thiamine triphosphatase. *Journal of Biological Chemistry* **283:** 10939- 10948.

Stone JM, Walker JC. 1995. Plant protein kinase families and signal transduction. *Plant Physiology***108:** 451- 457.

Suzuki M,Shibuya M, Shimada H, Motoyama N, Nakashima M, Takahashi S, Suto K, Yoshida I, Matsui S, TsujimotoN. 2016. Autophosphorylation of specific threonine and tyrosine residues in Arabidopsis CERK1 is essential forthe activation of chitin-induced immune signalling. *Plant and Cell Physiology***57:** 2312- 2322.

Tanenbaum ME, Stern-Ginossar N, Weissman JS, Vale RD. 2015. Regulation of mRNA.*Elife***4:** 07957.

Tejos R, Sauer M, Vanneste S, Palocious- Gomez M, Li H, Heilmann M, Van Wijk R, Vermeer JEM, Heilmann I, Munnik T. 2014. Bipolar plasma membrane distribution of phosphoinositides and their requirement for auxin-mediatedcell polarity and patterning in Arabidopsis. *ThePlant Cell* **26:** 2114- 2128.

The Arabidopsis Information Resource (TAIR) accessed at http://www.arabidopsis.org on 7 July 2016.

Ung H, Karia P, Ebine K, Moeder W, Ueda T, Yoshioka K. 2017. Triphosphate tunnel metalloenzyme function in senescence highlights a biological diversification of this protein superfamily. *Plant Physiology***00700:** 2017.

Ung H, Moeder W, Yoshioka K. 2014. Arabidopsis triphosphate tunnel metalloenzyme2 (AtTTM2) is a negative regulator of the salicylic acid-mediated feedback amplification loop for defense responses. *Plant Physiology***166:** 1009- 1021.

Walker JC. 1994. Structure and function of the receptor-like protein kinases of higher plants.*Plant Molecular Biology***26:** 1599- 1609.

Wheeler JI, Wong A, Marondedze C, Groen AJ, Kwezi L, Freihat L, Vyas J, Raji MA, Irving R, Gehring C. 2017. The brassinosteroid receptor BRI1 can generate cGMP-dependent downstream signalling. *The Plant Journal***19:** 590- 600.

Wong WS, Scott J D. 2004. AKAP signalling complexes: Focal points in space and time. *Cell Biology***5:**: 959- 970.

Yan F, Quan L, Wei Z, Cong W. 2015. Regulating phosphoenolpyruvate carboxylase activity by copper-induced expression method and exploring its role of carbon flux distribution in Synechocystis PCC 6803. *Journal of AppliedPhycology***27:** 179- 185.

Zhu X, Dahlmans V, Thali R, Preisinger C, Viollet B, Voncken JW,Neumann D. 2016. AMP-activated protein kinase up-regulates mitogen-activated protein (MAP) kinase-interacting serine/threonine kinase 1a-dependent phosphorylation of eukaryotic translation initiation factor 4E. *Journal of Biological Chemistry* **291:** 17020- 17027.

Zimmermann P, Hirsch- Hoffmann M, Hennig L, Gruissem W. 2004. GENEVESTIGATOR. Arabidopsis microarray database and analysis toolbox. *Plant Physiology***136:** 2621- 2632.