



Molecular Characterization of the C-Terminus Kinase Containing Domain of a Triphosphate Tunnel Metallo-enzyme Family Protein (AtTTM2) from *Arabidopsis thaliana*

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DECLARATION

I, Keabetswe Lerato Patricia Molefe declare that this thesis titled **Molecular Characterization of a C-Terminus Kinase Containing Domain of a Triphosphate Tunnel Metallo-enzyme Family (AtTTM2) Protein from *Arabidopsis thaliana*** submitted to the Department of Biological Sciences for a Master of Science degree at the North West University, Mafikeng Campus, has never been submitted at this or any other institution. This is my own work, and all the sources used or quoted here have been properly indicated and sincerely acknowledged.

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DEDICATION

I would like to dedicate this research project to the following people who have been supportive and caring. My dearest mother, thank you very much for your daily encouragements and love that has seen me through in this project. To my brothers, Thabang and Kaizer, I appreciate your strong support system. My supervisor Dr Dave Kawadza, I sincerely thank you for your guidance, mentorship, time and for sharing knowledge of plant Biotechnology with me. Prof O. Ruzvidzo, thank you also for your guidance and being there throughout this project. A big appreciation to the Biological Science Department, North West University, Mafikeng Campus, for the opportunity they granted me to study for a Masters.

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DEFINITIONS OF TERMS

Abiotic stress: Is the negative impact of non-living factors on living organisms in a specific environment.

Agarose gel electrophoresis: Is a method used to separate a mixture of macromolecules such as nucleic acids or proteins in a matrix of agarose, exposed to a high level of electric field. one of the two main components of agar.

Biotic stress: Is the damaging impact of living organisms, such as bacteria, fungi, viruses, animals and weeds on other living organisms.

Cloning: Is a process of producing similar populations of genetically identical individuals that occurs in nature when organisms such as, bacteria, insects and plants reproduce asexually.

Crystallography: Is the experimental science of determining the arrangement of atoms in crystalline solids.

Cyclic 5',3'-adenosine monophosphate (cAMP): Is a second messenger in many biological processes used for most intracellular signal transductions systems in many different organisms.

Kinase: Is an enzyme that catalyses the transfer of phosphate groups from high-energy phosphate donating molecules to specific substrates.

Phosphorylation: Is the addition of a phosphate group to a protein or other organic molecule. Phosphorylation turns many protein enzymes 'on' and 'off', thereby altering their function and activity.

Polymerase chain reaction: Is a technology in molecular biology used to amplify a single copy or a few copies of a piece DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence.

Pyrophosphatase activity: Is the removal of a phosphate from a protein.

Pseudokinases: Are catalytically-deficient (usually inactive) variants of protein kinases that are broadly defined at the bioinformatics level, and represented in all kinomes that have been compiled across the kingdoms of life.

Primers: Are 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: Is 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): Is a molecular method used to convert a short RNA segment into DNA product termed copy DNA (cDNA) using reverse transcriptase.

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

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis: Is a common method for separating proteins by electrophoresis in a discontinuous polyacrylamide gel as a support medium and sodium dodecyl sulfate (SDS) to denature the proteins under a strong local electric field.

LIST OF ABBREVIATION

ABA	:	Abscisic acid
ACs	:	Adenosine cyclases
ATP	:	Adenosine 3',5'-triphosphate
ATPase	:	Adenosine triphosphatase
cAMP	:	Cyclic 3',5'-adenosine monophosphate
cDNA	:	Copy DNA
cGMP	:	Cyclic guanosine monophosphate
CK1	:	Casein kinase1
cNTs	:	Cyclic nucleotides
DAG	:	Diacylglycerol
ET	:	Ethylene
ETI	:	Effector-triggered immunity
GPCRs	:	G-protein-coupled receptors
HR	:	Hypersensitive response
IP3	:	Inositol 1,4,5-triphosphate
IPTG	:	Isopropyl- β -D-thiogalactopyranoside
JA	:	Jasmonic acid
MAPKs	:	Mitogen-activated protein kinases
MBS	:	Menadione sodium bisulphite
mRNA	:	Messenger ribonucleic acid
Ni-NTA	:	Nickel-nitrilotriacetic acid
OGAs	:	Oligouroides

PCR	:	Polymerase chain reaction
PIP3	:	Phosphophatidylinositol
PKA	:	Protein kinase A
PKC	:	Protein kinase C
PKG	:	Protein kinase G
PKI	:	Protein kinase inhibitors
PPA	:	Phosphatidic acid
PPPase	:	Phosphoprotein phosphatase
PR	:	Phathogen related
ROS	:	Reactive oxygen species
SA	:	Salicylic acid
Ser	:	Serine
T-DNA	:	Transfer-deoxyribonuceic acid
Thr	:	Threonine
ThTpase	:	Thiamine triphosphatase
TTM	:	Triphosphate tunnel metallo-enzyme
Tyr	:	Tyrosine

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ABSTRACT

Three loci, At1g73980, At1g26190 and At2g11890, are annotated as the CYTH domains in the *Arabidopsis* genome and coding for the AtTTM1, AtTMM2 and AtTTM3 proteins respectively. These proteins belong to the triphosphate tunnel metalloenzymes (TTM) family and members of this extended family act on triphosphate substrates and require a divalent cationic cofactor, usually Mg^{2+} and/or Mn^{2+} . They are therefore referred to as TTMs. TTMs are known as a group of enzymes that can hydrolyse a range of tripolyphosphates. Promoter swap analysis has shown that the AtTTM1-2 can functionally complement each other.

Moreover, all the TTMs display the same biochemical properties but distinct biological functions governed by their transcriptional regulation. Based on the fact that AtTTM2 has recently been annotated as a possible kinase, this work then primarily focused on the functional characterisation of its possible kinase activity in the *Arabidopsis thaliana* plant and possibly other related higher plants. The work involved germinating some *A. thaliana* seeds, followed by extraction of total RNA from the generated plants. Amplification of the targeted and desired AtTTM2 gene fragment was performed using a RT-PCR system. The amplified AtTTM2 coding region was ligated into a TrHis2-TOPO expression vector in preparation for recombinant protein expression. The ligated AtTTM2 coding region was then used to transform competent *E. coli* BL21 (DE3) pLysS cells followed by partial expression of the recombinant AtTTM2 protein. Using a crude extract of the recombinant protein, the predicted kinase activity of the AtTTM2 protein confirmed using the OmniaTM Recombinant system. After this, the protein was further assessed and analysed bioinformatically to determine its possible physiological and functional roles in *Arabidopsis* and other related higher plants. The generated information showed that the AtTTM2 protein is indeed a *bona fide* plant kinase that is highly expressed in various plant tissues during the different stages of plant growth and development.

CHAPTER ONE

Introduction and Literature Review

1.1 Introduction

In nature, plants are challenged by stresses. For plants to survive, they need to react to modest or severe, transient or permanent, single or co-occurring stresses (Osakabe *et al.*, 2013). These stress factors have been categorised into two main groups, namely biotic and abiotic. According to (Vranova *et al.*, 2002), biotic stress refers to the damaging impact of living organisms, such as bacteria, fungi, viruses, animals and weeds on other living organisms while abiotic stress includes diverse environmental conditions such as high temperatures, drought, salinity and UV light (Kitsios & Doonan, 2011). Biotechnology, the use of living systems and organism to develop or make products (Cieřla *et al.*, 2011), may be a possible route to developing plants with greater stress tolerance. For thousands of years, humankind has used biotechnology in agriculture, food production and medicine (Mueller *et al.*, 2015). Climate change is bringing about many changes in the weather and agricultural production. In this regard, how then can biotechnology assist plants in withstanding stress? Therefore, this gap thus needs to be filled in order to address this problem. Hence the development of new varieties, which could withstand the diverse weather effects, by improving features such as tolerance and resistance, has attracted the attention of many researchers (Edgerton, 2009). Among the many techniques related to biotechnology, recombinant DNA technology allows the manipulation of genes from various sources and insertion of such genes into other organisms to confer the characteristic of interest (Khan *et al.*, 2016). For example, drought and soil salinity resistance, herbicide toleration, disease and pest resistance, enhanced qualities, increased rate of photosynthesis and production of sugar, starch, and the production of medicines and vaccines in crop plants (Sharma *et al.*, 2002).

The possible role of cyclic adenosine monophosphate (cAMP) in plants has been an area of controversy for nearly 20 years (Bolwell, 1995). By the mid-1970s, cAMP has been firmly established as an essential signalling molecule and second messenger in both animals and lower eukaryotes (Gehring, 2010). This second messenger cAMP, together with kinases, play very critical and essential roles in several biochemical processes, including the regulation of glycogen, sugar and lipid metabolism (Ali *et al.*, 2016). The diversity and role of kinases in signalling and cell transduction system make them an interesting group of plant proteins to study. This diversity of processes mediated by kinases is shown by the viable conversation of some 50 distinct kinase

families, between yeast, invertebrate and mammalian kinomes (Manning *et al.*, 2002). Essentially, a kinase is an enzyme that mediates the transfer of a phosphate moiety from a high energy molecule such as ATP to a substrate molecule (Reiterer *et al.*, 2014).

Protein kinases are regulators of cell function that make up one of the largest functionally diverse gene families (Manning *et al.*, 2002). Kinases are prominent in signal transduction and also the coordination of complex processes such as the cell cycle (Wang *et al.*, 2016). Many different studies in yeast and other organisms have led to a model, where kinases act as major engines to promote progression throughout the different phases of the cell division cycle. These phases include two major periods of activity in which the genome is first duplicated (DNA synthesis or S phase) and the two newly replicated genomes are then distributed between the daughter cells (mitosis) (Hartwell & Weinert, 1989). The first protein to be recognised as catalysing phosphorylation of another proteins using ATP was observed in 1954 by Gene Kennedy (Krebs, 1983). He described a liver enzyme that catalysed the phosphorylation of casein (Krebs, 1983).

1.2 Relationship between kinases and cAMP in carbohydrate metabolism

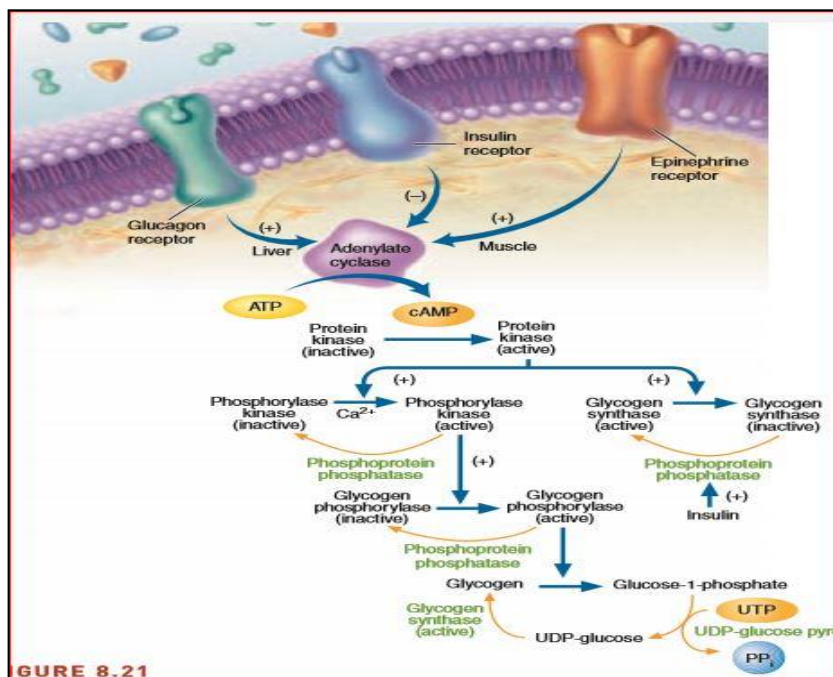


Figure 1.2: Kinases and cAMP relationship in carbohydrate metabolism (McKee & McKee, 2003).

Kinases are classified into a broader group and by the substrate they act on (Reiterer *et al.*, 2014). Moreover, kinases act on small molecules such as lipids, carbohydrates, amino acids, and nucleotides (by transferring a phosphate group) for signalling or metabolic pathways (Reiterer *et al.*, 2014). Recent literature reports that kinases and pseudokinases are important signalling modulators in human cells - thus making kinases very important as drug targets (Byrne *et al.*, 2017).

1.2 Literature Review

1.2.1 The chemical nature of the extracellular signal

Biotic and abiotic stresses challenge plants. To cope with these environmental stresses, plants use phytohormones such as jasmonic acid (JA), salicylic acid (SA), ethylene (ET) and abscisic acid (ABA) to regulate their responses to both abiotic and biotic stresses, with signalling cross-talking (Fujita *et al.*, 2006). Aside from plant-pathogen interactions that are less defined, phytohormones pathways, knowledge of stress perception, and initial signalling events, have been well studied. It is now known that the application of purified oligouroids (OGAs) derived from the plant cell wall are capable of inducing plant defence responses (Chinnusamy *et al.*, 2006). This led to the hypothesis that the mechanical disruption of the cell wall may result in stress signalling (Schmelz *et al.*, 2006). The perception of cold stress has also been hypothesised to be mediated through the detection of changes in membrane fluidity and protein conformation (Xiong *et al.*, 2002). Lastly, second messengers such as cyclic nucleotides (cNTs), calcium ion (Ca^{2+}), reactive oxygen species (ROS), and phosphatidic acid (PPA) have been implicated in initial signalling cascades in response to both abiotic and biotic stresses (Apel & Hirt, 2004).

Menadione sodium bisulphite (MSB) is a water-soluble additive of the vitamin K3 (Borges *et al.*, 2003). Given its hydrophobic nature, MBS can easily cross biological membranes, allowing it to enter organelles and catalyse superoxide, hydrogen peroxide, and hydroxyl radical production (Lehmann *et al.*, 2012). A recent study in *Arabidopsis* roots using MBS as an oxidant shows that ROS are produced by the electron transport chain via mitochondria and plastids (Lehmann *et al.*, 2012). MBS has been demonstrated to function as plant defence elicitor against several pathogens in some plant species (Borges *et al.*, 2003).

1.2.2 Signal transduction

The ability of a cell to change behaviour in response to a ligand-receptor interaction is known as signal transduction. A ligand is the primary messenger. When these ligand binds to a receptor, other molecules or second messengers are produced within the target cell. Second messengers relay the signal from one location to another (such as from plasma membrane to nucleus). Often a cascade of changes occurs within the cell which results in a change in the cell's function or identity. Signal transduction pathways can act to amplify the cellular response to an external signal (Hardin *et al.*, 2012). Messenger molecules may be amino acids, peptides, proteins, fatty acids, lipids, nucleosides or nucleotides.

Signal transduction is the process by which a chemical or physical signal is transmitted through a cell followed by a series of molecular events, mostly protein phosphorylation catalysed by protein kinases, which ultimately results in a cellular response. Proteins that detect stimuli are generally termed receptors (Bradshaw & Dennis, 2009). The changes elicited by ligand binding in a receptor give rise to a signalling cascade, which is a chain of biochemical events along a signalling pathway. When signalling pathways interact with one another, they form networks, which allow cellular responses to be coordinated, often by combinatorial signalling events (Papin *et al.*, 2005).

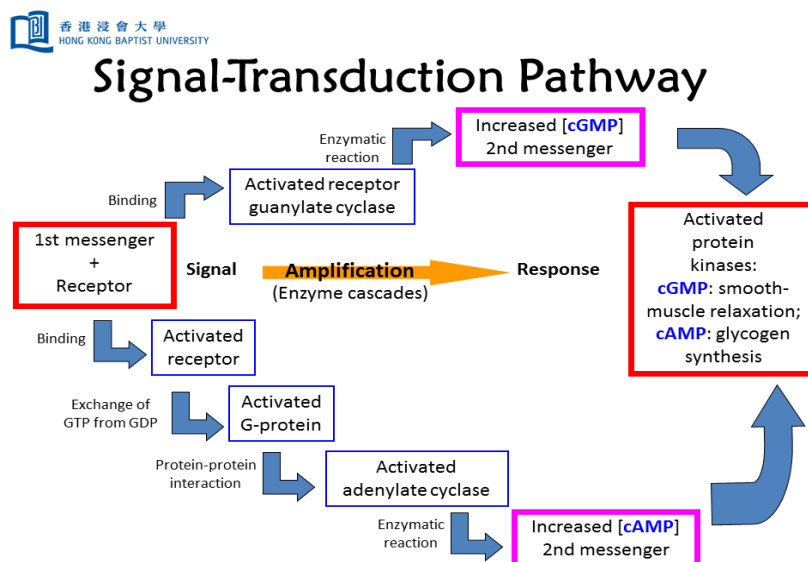


Figure 1.3: Signal transduction pathway indicating the roles of adenylate cyclase and protein kinases (Pray, 2008).

At the molecular level, such responses include changes in the transcription or translation of genes, and post-translational and conformational changes in proteins, as well as changes in their location.

These molecular events are the primary mechanisms controlling cell growth, proliferation, metabolism and many other processes (Zheng *et al.*, 2016). Ligands are termed first messengers, while receptors are the signal transducers, which then activate primary effectors. Such effectors are often linked to second messengers, which can activate secondary effectors, and so on. Depending on the efficiency of the nodes, a signal can be amplified (a concept is known as signal gain) so that one signalling molecule can generate a response involving hundreds to millions of molecules (Zaminpira & Niknamian)

1.2.3 Second messengers

Signals received by receptors at the cell surface or, in some cases, within the cell are often relayed throughout the cell via generation of small, rapidly diffusing molecules referred to as second messengers. These second messengers broadcast the initial signal that occurs when a ligand binds to a specific cellular receptor (Newton *et al.*, 2016). Ligand binding alters the protein conformation of the receptor such that it stimulates nearby effector proteins that catalyse the production or, in the case of ions, release or influx of the second messenger. The second messenger then diffuses rapidly to protein targets elsewhere within the cell, altering the activities as a response to the new information received by the receptor (Newton *et al.*, 2016).

Examples of second messengers include cyclic AMP (cAMP), cyclic GMP (cGMP), inositol 1, 4,5-triphosphate (IP3), diacylglycerol (DAG), and calcium. Three classic second messenger pathways are, the activation of adenylyl cyclases (ACs) by G-protein-coupled receptors (GPCRs) to generate the cyclic nucleotide second messenger cAMP, secondly the stimulation of phosphoinositide 3-kinase (PI3K) by growth factor receptors to generate the lipid second messenger phosphatidylinositol 3,4,5 triphosphate (PIP3), and lastly, the activation of phospholipase C by GPCRs to generate the two second messengers; membrane-bound messenger DAG and soluble messenger IP3, which binds to receptors on subcellular organelles to release calcium into the cytosol (Newton *et al.*, 2016).

In a cAMP-dependent pathway, the activated G subunit binds to and activates the enzyme ACs, which in turn, catalyses the conversion of ATP into cAMP (Hanoune & Defer, 2001). An increase in the concentration of the second messenger cAMP lead to the activation of an enzyme called protein kinase A (PKA), which activates many different molecules through phosphorylation (Meinkoth *et al.*, 1993). The protein kinase A then phosphorylates other proteins and influences the actions of such phosphorylated proteins.

1.2.4 Roles of kinases in controlling protein function

Protein kinases and phosphatases are enzymes catalysing the transfer of phosphate between their substrates. A protein kinase catalyses the transfer of γ -phosphate from ATP (or GTP) to its protein substrate while a protein phosphatase catalyses the transfer of the phosphate from a phosphor protein to a water molecule. Both groups of enzymes are phosphotransferases; they catalyse opposing reactions to modulate the structures and functions of many cellular proteins in prokaryotic and eukaryotic cells (Ubersax & Ferrell, 2007). Among the various types of posttranslational modifications, protein phosphorylation and dephosphorylation are the most prevalent modifications regulating the structures and functions of cellular proteins in a wide spectrum of cellular processes, ranging from cell fate control to regulation of metabolism. For example, even though protein kinase genes constitute only 2% of the genomes in most eukaryotes, protein kinases phosphorylate more than 30% of the cellular proteins (Ubersax & Ferrell, 2007). Phosphorylation regulates protein functions by inducing conformational changes or by disruption and creation of protein-protein interaction surfaces (Holt *et al.*, 2009). Conformational changes induced by phosphorylation are highly dependent on the structural context of the phosphorylated protein. Upon phosphorylation, the phosphate group regulates the activity of the protein by creating a network of hydrogen bonds among specific amino acid residues nearby. This network of hydrogen bonds is governed by the three-dimensional structure of the phosphorylated protein and therefore, is unique to each protein (Holt *et al.*, 2009).

The most notable example of the regulation of protein function by phosphorylation-induced conformational changes is glycogen phosphorylase (Johnson, 2009). Glycogen phosphorylase, having two identical subunits, is activated upon phosphorylation of its Ser-14 of each subunit by phosphorylase kinase (Johnson & Barford, 1994). Phosphorylation of the Ser-14 in one monomer creates a network of hydrogen bonds between the phosphate group and the side chains of Arg-43 of the same monomer as well as Arg-69 of the other monomeric subunit (Johnson & Barford, 1994). These networks induce significant intra- and inter-subunit configurational changes, allowing access of the substrates to the active sites and appropriately aligning the catalytically critical residues in the active sites for catalysis of the phosphorolysis reaction.

1.2.5 Kinases found in plants

1. Protein kinases

Protein kinases are found in bacteria and plants and include the pseudokinase subfamily, which exhibits unusual features (Reiterer *et al.*, 2014), including atypical nucleotide binding and weak or no catalytic activity (Murphy *et al.*, 2014). They are part of a larger pseudoenzyme group of degraded enzyme relatives that are found throughout life, where they take an active participation in mechanistic cellular signalling (Eyers & Murphy, 2016). When the first plant genome of *A. thaliana* was sequenced, more than 1000 protein kinases were identified (Hanks *et al.*, 1988).

2. Casein kinase 1

Casein kinase 1 (CK1) is an evolutionary conserved across eukaryotes and regulates a wide variety of cellular processes (Cheong & Virshup, 2011). Many plant lineages have more than twice the number of CK1 paralogues compared to humans. Most plant CK1s have not been functionally characterised; however, there is evidence that *A. thaliana* CK1 proteins are involved in microtubule organisation (Ben-Nissan *et al.*, 2008).

3. Mitogen-activated protein kinases

Mitogen-activated protein kinases (MAPKs) in plants play diverse roles in development and response to biotic and abiotic stresses (Sinha *et al.*, 2011). Several signalling modules involving MAPs in plants have been defined. Though there is a high degree of conservation of structure of these modules, MAPK signalling pathways in plants are different from those in other eukaryotes (Sinha *et al.*, 2011). Furthermore, the MAPK family has expanded more in land plants (7-31 members) relative to yeast (6) and animals (6-14), suggesting more opportunities for diversification of functions in the plant lineage (Sinha *et al.*, 2011).

1.2.6 Triphosphate tunnel metallo enzyme (TTM)

The gene of interest for this study, triphosphate tunnel metallo enzyme 2 (TTM2), is found at the locus At1g26190 of the *A. thaliana* plant. There is a growing interest in understanding the role of TTM proteins in plants from the evolutionary and biological activity (Moeder *et al.*, 2013). *A. thaliana* encodes three TTM genes namely, AtTTM1 (At1g73980), AtTTM2 (At1g26190) and AtTTM3 (At2G11890) (Ung *et al.*, 2017). TTMs act on nucleotides and organic substrates

although their biological functions are unclear. It is reported that the activation of these TTM genes relies solely on covalent cationic cofactors such as Mg^{2+} , Mn^{2+} , Co^{2+} (Bettendorff & Wins, 2013). Recent research reveals that TTMs play important roles in cAMP signalling and mRNA capping (Ung *et al.*, 2017), whereby the RNA triphosphatase removes the γ -phosphate from the 5'-triphosphate, generating a diphosphate 5'-end and inorganic phosphate.

1.2.7 Structure of TTM proteins

The structure of TTM proteins was solved by X-ray crystallography or N-methylpyrrole (NMP) spectroscopy. It revealed a characteristic tunnel-shaped active site (Ung *et al.*, 2014). The TTM tunnel structure has eight antiparallel β -strand and seven alpha helices (Moeder *et al.*, 2013).

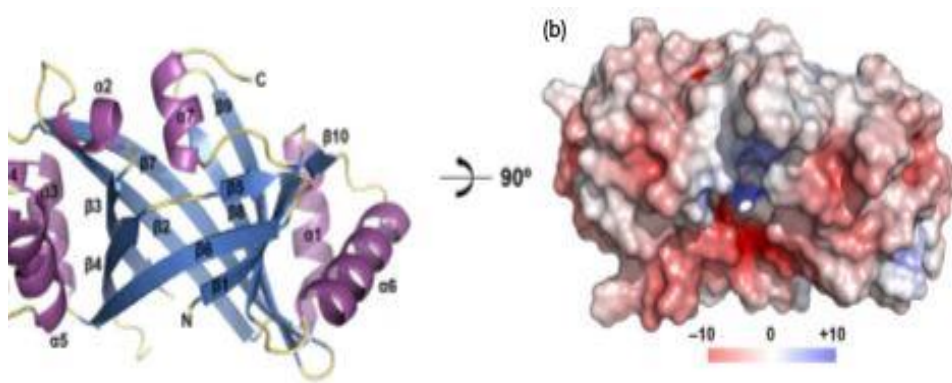


Figure 1.4: Structure of TTM (Moeder *et al.*, 2013).

1.2.8 Biochemically characterised members of TTM

Several members of TTMs have been characterized, for example, the RNA triphosphatase activity of *Saccharomyces cerevisiae* Cet1 (Gong *et al.*, 2006), the AC activity of *cyaB* from *Aeromonas hydrophila*, which is responsible for the production of cAMP (Gallagher *et al.*, 2006), the tripolyphosphatase activity of *Clostridium thermocellum* TTM (Moeder *et al.*, 2013), the *Nitrosomona seuropaea* NeuTTM (Delvaux *et al.*, 2011) and the thiamine triphosphatase (ThTpase) activity TTM2 from mammals. AtTTM2 is a negative regulator of the SA amplification loop in defence responses (Ung *et al.*, 2014).

In plants, two levels of resistance responses have been reported. There is the first line of defence, which is the basal immunity triggered by molecules that are conserved among many pathogens - the common name used being the pathogen-associated molecular patterns (PAMP)-triggered

immunity. The second line of defence is a stronger response to pathogen infection, which is mediated by resistance (R) genes that can recognise their similar effectors from the pathogens directly or indirectly and this is commonly known as the effector-triggered immunity (ETI) (Bent & Mackey, 2007). The hypersensitive response (HR) is characterised by apoptosis-like cell death around the site of pathogen entry, is a defence mechanism activated by R gene-mediated pathogen recognition (Hammond-Kosack & Jones, 1996). An increase in SA and the accumulation of pathogenesis-related (PR) proteins are observed during HR development (Vlot *et al.*, 2008).

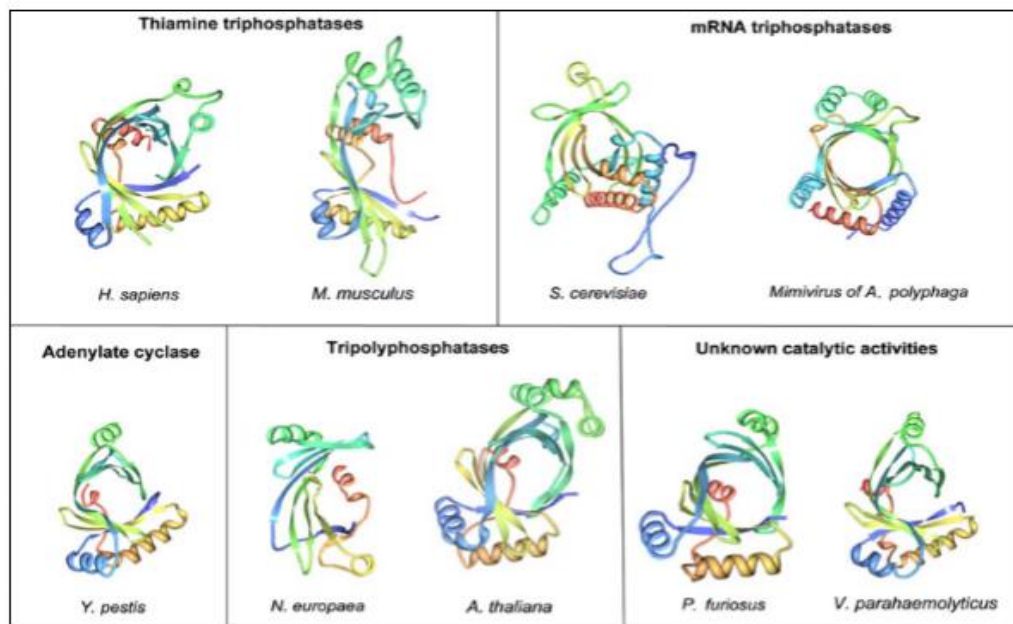


Figure 1.5: Three-dimensional structures of representative proteins of the TTM superfamily (Bettendorff & Wins, 2013).

1.2.9 Biological and biochemical characterisation of AtTTM2

By using transfer-deoxyribonucleic acid (T-DNA) insertion knockout lines, reverse genetics shows that AtTTM2 is a negative regulator of the SA amplification loop in defence responses (Moeder *et al.*, 2013). The study also revealed that TTM2 plants do exhibit an enhanced HR. Upon infection, AtTTM2 plants had highly elevated SA levels. In addition, the biological and chemical functions (expression patterns) of AtTTM1-AtTTM2 are the same (both possess pyrophosphatase activity in the presence of Mg^{2+} (Ung *et al.*, 2014).

Recent microarray data from the different expression patterns of both AtTTM1 and AtTTM2 suggest that both genes (AtTTM1&2) possess the same domain arrangement, meaning that they

have conserved parts of a given protein sequence and tertiary structure that can evolve, function, and exist independently of the rest of the protein chain (Ung *et al.*, 2017). TTM genes (AtTTM1&2) are said to both have a *CyaB*, the CYTH domain fused to a *p*-loop kinase domain. Proteins containing this domain act on triphosphorylated substrates and require at least one divalent metal cation for catalysis. Another striking similarity is that both of these genes (AtTTM1 and AtTTM2) do display pyrophosphatase activity (Ung *et al.*, 2017).

The AtTTM2 gene is located in the chloroplast, cytosol and is an integral component of membranes. Biological processes such as biosynthesis, defence response and phosphorylation are catalysed by this protein (Schneijderberg, 2012).

1.3 Problem statement

A number of protein molecules, including TTMs, have been bioinformatically identified, possibly as having synchronised kinase and AC activities in *A. thaliana*. Often both molecules (kinases and ACs) play essential roles in cell signalling and transduction systems. In turn, they are activated by the second messenger cAMP and thereafter, cAMP activates PKA, which then activates enzymes that are important in the growth and development of plants. Surprisingly, most of these annotated protein molecules have not yet been functionally characterised, particularly their kinase activity. This study, therefore, was designed to determine if the AtTTM2 has any functional kinase activity and possibly, its biological role in plants.

1.4 Aim of the Study

The study aimed to ascertain if the AtTTM2 has any functional kinase activity and possibly, to further determine its probable biological roles in *A. thaliana* and other closely related plants.

1.5 Objectives

1. To amplify and ligate the AtTTM2 coding region into an expression vector
2. To optimise strategies for expression of the recombinant AtTTM2 protein
3. To determine whether the recombinant AtTTM2 protein has kinase activity
4. To characterise the enzymatic activities of this protein kinase *in silico*.

1.6 Significance of the research study

Findings of this study would add to the existing body of knowledge on plant kinases in *A. thaliana* and other related higher plants. This study focused more on the function and physiological roles of this particular group of proteins in plants particularly the role of the predicted kinase may play in essential aspects of plant growth and development. The study would also identify yet another additional kinase to those currently known in *A. thaliana* and higher plants.

CHAPTER TWO

Research Methodologies

2.1 Germination of *Arabidopsis thaliana* plants

2.1.1 Seeds Sterilization and stratification

Arabidopsis seeds (~100) were transferred to a 1.5 mL Eppendorf tube and washed with 500 μ L ethanol through vortexing three times. The seeds were then vortexed in 500 μ L sterilisation buffer (0.1% SDS and 5% chlorine bleach) three times and then washed with 1 mL sterile water five times. The seeds were then suspended in 200 μ L water and stratified at 4°C for three days.

2.1.2 Plant re-generation and growth conditions

After the stratification, the seeds were then plated on 0.43% Murashige and Skoog (MS) medium composed of 3% sucrose, and 0.8% agar (pH 5.7) supplemented with 1 mL/L of Gamborgs vitamins. The plated seeds were then germinated and grown in the growth chamber for two weeks at a temperature of 23°C and light lux of 10000 (16 hrs light and 8 hrs darkness). The seedlings were then transferred into sterile potting soil composed of 50% vermiculite and 50% Canadian peat-based humus. The set conditions of the growth chamber were the same at 23°C and 16 hrs day/8 hrs night. The plants were then kept in the growth chamber for a further four weeks.

2.1.3 Extraction of total mRNA

Total RNA was extracted from an amount of 100 mg of plant leaf material harvested from the six-week-old *A. thaliana* ecotype Columbia (Col-0) plants. The RNA was extracted according to the manufacturer's protocol of the Gene Jet Plant RNA Purification Mini Kit (Thermo Scientific, Massachusetts, USA). Briefly, sterile and chilled pestle and mortar were used to grind the plant material under liquid nitrogen. The fine powder from the grinding was then immediately transferred to a 1.5 mL Eppendorf tube containing 500 μ L Plant Lysis Solution. The 1.5 mL tube was then vortexed for about 20 seconds and mixed thoroughly. The tube was centrifuged for 5 minutes at ~16300g (~14000 rpm), and the supernatant (~500 μ L) was transferred to a clean 1.5 mL microcentrifuge tube. Subsequently, a volume of 250 μ L 96% ethanol was added and mixed gently by pipetting. This mixture was then transferred to a purification column inserted within a

collection tube and centrifuged for a minute at 12000g (~11000 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 Wash Buffer 1 and centrifugation for a minute at 12000xg (~11000 rpm). The purification column was placed in a clean collection tube and washed again with 500 µL Wash Buffer 2 at 12000g (~11000 rpm) for a minute. Finally, the purification column was placed in a clean collection tube and 50µL of nuclease-free water was added onto the centre of the purification column membrane and centrifuged for a minute at 12000g (~11000 rpm) for elution of the RNA. The purification column was then discarded while the collection tube with its eluted RNA was stored at –80°C.

2.2 Amplification of the AtTTM2 coding region

2.2.1 Design of sequence-specific primers

The forward and reverse sequence-specific primers were manually designed based on the coding region of the AtTTM2-kinase gene sequence and sent for chemical synthesis and supply from the Inqaba Biotechnologies (Pretoria, South Africa). The primers were designed to flank the coding region of interest at about more than 100 bp away from the annotated kinase domain. The sequences of the annotated AtTTM2 gene and its manually designed specific primers are shown in Figure 2.1 below.

```

01 AATGTTACCT CCTCGTGGGT CTGAGATCTT TTTCCCCAGA TTCTCTACAA
51 ATCGCTCTCC CCGATAAAGA AGAAGCTCTC ACAAATTC TCTTTCTCTC
101 TCTCTCTCTG ATTCCCCATT ATTAGTTTCT GTGTTAAAT TGAATTGCGA
151 CATAACTCTG CCAAAGTGAT AAGCCCCGAT TCACACTAAT TCCGAGAGAT
201 TTTTCTGTAT TATTGTTTCAT AATGGGTCAA GACAGCAATG GAATTGAGTT
251 TCATCAGAAG AGACATGGTC TCTTGAAGGA TCAAGTCCAA TTGGTTAAGA
301 GAAGAGACTC TATTGGGTAT GAAATTGTTT CTATTCAAGA TCGGTTGTCA
351 TTTGAGAAGG GCTTCTTTGC GGTATCCGT GCTTGCCAAT TGCTTTCTCA
401 GAAGAATGAT GGGATCATAT TGGTTGGTGT TGCTGGACCT TCTGGTGCTG
451 GAAAGACTGT ATTCACTGAG AAGATACTCA ATTTTCTGCC AAGTGTGCT

```

Forward primer: 5'-GGTTGT ATC GTC TCT CTT AAA GCGAAG CCA-3'

Reverse primer: 5'GGGAAG TTT TCC TGACCGGAA AAC AGC AAA-3'

Figure 2.1: The complete nucleotide sequence of the At1g26190 gene from *A. thaliana*. The gene codes for the putative AtTTM2 protein that consists of a total of 2435 amino acids. The highlighted area (green) is the annotated kinase catalytic domain while the arrows are marking both the forward and reverse priming sites. The manually designed forward and reverse primers are presented below the sequences.

2.2.2 Reverse transcriptase polymerase chain reactions (RT-PCR)

From the extracted RNA, copy DNA (cDNA) was synthesised using reverse transcription as instructed by the manufacturer of Verso™ 1-step RT-PCR Reddymix™ kit (Thermo Fischer Scientific, Waltham, Massachusetts, USA). The cDNA was generated with a simultaneous combination of the total RNA and the specifically designed sequence-specific primers. The targeted AtTTM2 gene fragment was ultimately amplified using the reaction components listed in Table 2.1 below:

Table 2.1: Components of the PCR reaction mixture to amplify the AtTTM2 gene fragment.

COMPONENT	VOLUME	FINAL CONCENTRATION
Verso Enzyme Mix	1 µL	
1-Step Ready Mix (2X)	25 µL	1X
Forward Primer(10 µM)	2 µL	200 nM
Reverse Primer(10 µM)	2 µL	200 nM
RT Enhancer	2.5 µL	
Nuclease-Free H₂O	13.5 µL	
Total RNA	3 µL	1 ng
Total Volume	50 µL	

The RT-PCR procedure was carried out on a C1000 thermo-cycling system (Bio-Rad Laboratories Incorporated, California, USA), according to the Thermo Scientific Verso™ 1-Step RT-PCR ReddyMix™ kit. The used thermal cycling conditions are shown in Figure 2.2 below.

Table 2.2: The RT-PCR thermal cycling program used for amplification of the AtTTM2 gene fragment.

STEP	TEMPERATURE	TIME	CYCLES
cDNA Synthesis	50°C	15 min	1
Thermo-start Activation	95°C	15 min	1
Denaturing	95°C	20 sec	45 ↑ ↓
Annealing	60°C	30 sec	
Extension	72°C	1 min	
Final Extension	72°C	30 min	1

2.2.3 Agarose gel electrophoresis

Following RT-PCR, 5 µL of the PCR product was supplemented with 2 µL of the 6X DNA loading dye. The product was then resolved on a 1% agarose gel using a 1X TBE (62.5% Tris-HCl, 31.8% boric acid and 5.7% EDTA) buffer, which was also supplemented with 1% ethidium bromide. The product was resolved alongside on agarose gel along with a 0.5 µg/µL of Gene Ruler™ (100 bp) DNA ladder, and the samples were run at a voltage of 80 volts and a current of 250 mA for 45 minutes. The visualisation was done under a 2000 UV light using the UV trans-illuminator system (Bio-Rad Laboratories Incorporated., California, USA). The resultant images were finally captured with a gel documentation system, (Bio-Rad Laboratories Inc., California, USA).

2.3 Preparation of the pTrcHis2-TOPO: TTM2 construct

The amplified DNA gene fragment from section 2.2.3 was ligated into a commercially acquired pTrcHis2-TOPO expression vector (Figure 2.2) (Thermo-Fischer, Waltham, Massachusetts, USA). This vector allows for fast and efficient cloning of PCR products into a prokaryotic expression system. The vector also has an Amp^R gene, which is essential for the selection of transformed cells.

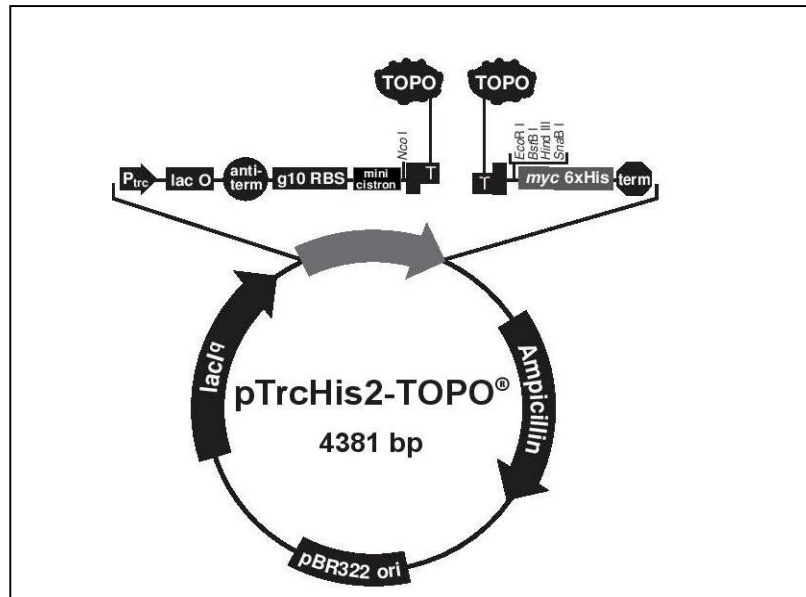


Figure 2.2: The commercially acquired pTrcHis-TOPO vector for cloning of the AtTTM2 PCR product: The illustration shows the expression and purification features of the plasmid such as the *trc* promoter for high-level expression. There is also a point of origin to facilitate replication of the plasmid in bacterial cells such as *E. coli*. In addition, there is an ampicillin resistant gene that allows for the screening of positive recombinants. For purification purposes, the vector expresses a 6-Histidine tag. (Adapted from www.lifetechnologies.com).

2.3.1 Addition of the 3'-adenine overhangs

In order to bring about the 3'-adenine overhangs into the amplified PCR product, (1) μL of *Taq* polymerase was added to 40 μL of the PCR product (the amplified AtTTM2 fragment) and the mixture incubated at 72°C for 10 minutes on a C1000 Thermal cycling system (Bio-Rad Laboratories Incorporated, California, USA). The resultant mixture was then kept on ice. This 3'-adenine tailing is crucial for efficient T-A cloning into the pTrcHis2-TOPO vector.

2.3.2 Ligation of the modified TTM2 gene fragment into the pTrcHis2-TOPO plasmid expression vector

A volume of 4 μL of the PCR product was pipetted into a new PCR tube, and 1 μL the pTrcHis2-TOPO expression vector (Figure 2.1) was added and mixed with a tip. The mixture was then left to stand at room temperature for 5 minutes. The ligation was verified by resolving contents on a 1% agarose gel.

2.3.3 Transformation of *E. coli* One Shot TOPO 10 competent cells with the pTrcHis2-TOPO: TTM2 construct

Immediately after the ligation process described above, an aliquot of 2 μL of the ligation mixture was pipetted out into a cold new tube, and 100 μL of *E. coli* One Shot TOPO 10 competent cells were added into the tube. The tube was then left to stand on ice 4°C at for 30 minutes. Thereafter, the mixture was supplemented with a volume of 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 placed on a shaker at 37°C for 30 minutes at 200 rpm. This step was undertaken to allow the cells to express the beta-lactamase enzyme, which would then detoxify ampicillin in transformants at a later stage. The mixture was subsequently 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 $\mu\text{g}/\text{mL}$ ampicillin and 0.5% glucose, pH 7.0. The plates were then incubated at 37°C until colonies were observed. The transformed colonies were then individually picked and inoculated into 5 mL 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 $\mu\text{g}/\text{ml}$ ampicillin and 0.5% of glucose. The prepared tubes were then incubated at 37 with shaking at 200 rpm overnight.

2.3.4 Extraction of the pTrcHis2-TOPO: AtTTM2 plasmid construct from the transformed *E. coli* One Shot TOPO 10 cells

The cells from step 2.3.3 above were harvested by centrifugation at a speed of 6 800*g* for 5 minutes, and the supernatant was discarded. The plasmid was extracted using the Gene-Jet plasmid miniprep kit (Thermo Fisher Scientific, Inc., California, USA). The pelleted cells were re-suspended until there were no more clumps by pipetting gently up and down 250 µL of Resuspension Solution (clear cap), which was supplemented with RNase. The resuspension was followed by addition of 250 µL Lysis Buffer (blue cap) to the tube and mixed completely by inverting the tube 4-6 times for 2 minutes or until the solution changed from opaque to clear, indicating complete lysis. Then after, 350 µL Neutralization Solution was added to the lysate and mixed thoroughly by inverting the tube. The solution turned yellow when the neutralisation was completed and a yellowish precipitate formed. Complete neutralisation was ensured by inverting the sample an additional 2-3 times. Centrifugation was performed at 11000*g* for 4 minutes. The supernatant of 900 µL was transferred into the provided Zymo spin column and centrifuged at 11,000*g* for 15 seconds. The flow-through was discarded, and the column placed back into the same collection tube. About 200 µL Wash Solution was added to the column and centrifuged at 11,000*g* for 30 seconds. The Zippy Wash Buffer (400 µL) (pink cap) was added to the column and centrifuged at 11,000*g* for 1 minute. The column was then transferred into a clean 1.5 mL microcentrifuge tube, and 30 µL of the Zippy Elution Buffer was added directly to the column matrix and left to stand at room temperature for 1 minute. Centrifugation was then performed for 30 seconds to elute the plasmid DNA. The DNA was then stored -80°C till needed.

2.3.5 Confirmation of successful cloning

In order to confirm positive clones, conventional PCR on a C1000 thermo-cycler system (Bio-Rad Laboratories Inc., California, USA) was carried out following the Standard MyTaq™ Mix protocol (Bioline, London, UK). Firstly, to confirm whether the TTM2 gene insert had been successfully ligated into the pTrcHis2-TOPO™ plasmid expression vector and secondly, whether such a ligation outcome was in the correct orientation. Successful ligation was established with the amplification of the insert using both specific primers (forward and reverse primers for the coding region) while 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 Table 2.3 and 2.4 below while the associated thermal cycling conditions for both processes are shown in Table 2.5 (below).

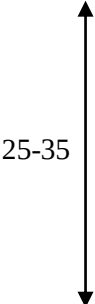
Table 2.3: PCR reaction mixture to confirm the successful ligation of the kinase insert.

COMPONENT	VOLUME
Plasmid Template	3 μ L
Primer Forward (insert)	2 μ L
Primer Reverse (insert)	2 μ L
MyTaq Red mix (2X)	25 μ L
SdH ₂ O	18 μ L

Table 2.4: A PCR reaction mixture to confirm the correct orientation of the kinase insert into the pTrcHis2-TOPO expression vector.

COMPONENT	VOLUME
Plasmid Template	1 μ L
Forward Insert Primer and Reverse Vector Primer (20 μ M each)	2 μ L
MyTaq Red Mix, (2X)	25 μ L
Water (sdH ₂ O)	22 μ L

Table 2.5: The reaction thermal cycling program for confirmation of the successful ligation and correct orientation of the AtTTM2 kinase insert 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	60°C	15 seconds	
Extension	72°C	10 seconds	

2.3.6 Agarose gel electrophoresis of the correctly cloned AtTTM2 gene fragment

The successfully ligated PCR products were resolved on a 1% agarose gel supplemented with 10 μ L ethidium bromide for every 100 mL agarose gel and run at 80-volts for 30 minutes. The visualisation was done under UV light using the UV-trans-illuminator 2000. The images were then captured using the Gel Documentation system (Biorad, California USA).

2.4 Transformation of *E. coli* BL21 pLysS cells

A 10 μ L aliquot of ligation mix of the correctly cloned construct (AtTTM2 gene) was transferred into a cold, sterile 1.5 mL microcentrifuge tube with 100 μ L of the *E.coli* BL21 pLysS cells. The cells were mixed gently and carefully kept on ice for 20 minutes. The tube was transferred to a 42°C heating block for 90 seconds. Subsequently, the contents were supplemented with 500 μ L SOC media and incubated for 90 minutes at 37°C. Aliquots of 50, 100 and 200 μ L were then plated on LB agar plates containing 100 μ g/mL ampicillin. The plates were incubated at 37°C overnight.

2.5 Protein expression

Individual cell colonies harbouring the pTrcHis2-TOPO: AtTTM2 expression construct were inoculated into 10 mL 2YT medium supplemented with 20% glucose, 34 μ g/mL chloramphenicol

and 100 µg/mL ampicillin in a 15 mL falcon tube and incubated overnight at 37°C while shaking at 200rpm. The following day, a volume of 400 µL of the overnight transformation culture was inoculated into 20 mL fresh 2YT medium containing 34 µg/mL chloramphenicol, 100 µg/mL ampicillin and 20% glucose. The culture was then incubated at 37°C, shaking 200 rpm and until an OD₆₀₀ of 0.5 was reached (measured by the Helios spectrophotometer (Merck, Gauteng, RSA)). After that, the culture was split into two 15 mL falcon tubes, each containing about 7 µL. One culture was then induced to express the intended AtTTM2 recombinant by adding 1 Mm of isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma-Aldrich Corp, Missouri, USA) while the other culture was left un-induced and to serve as a control. The two cultures were shaken in an incubator (200 rpm) at 37°C for 3 hours. After shaking, the cultures were centrifuged at 8000g for 5 minutes to pellet out the cells. The pelleted cells were stored at -20°C while part of them (500 µL) analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.5.1 Determination of the kinase activity of the recombinant AtTTM2 protein

The pelleted cells were sonicated to generate crude protein extracts, and the generated crude protein extracts were then used to assess the probable kinase activity of the recombinant AtTTM2 protein. This assessment was carried out *in vitro*, whereby the TTM2's ability to direct the phosphorylation of a particular substrate peptide as described by the OmniaTM 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, 1 mM ATP and, 0.2 mM DTT, and 25 µM of the Ser/Thr substrate 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 the 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.

2.5.2 Statistical analysis of the *in vitro* kinase activity assays

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

2.6 Bioinformatic expression analysis of the AtTTM2 gene in *Arabidopsis thaliana*

The gene of interest, At1g26190 or AtTTM2, was further analysed by bioinformatics. This approach provided us with a further and clearer understanding of the AtTTM2 protein and thereby also further augmenting the biochemical work that had already been undertaken on the protein.

2.6.1 Anatomical expression analysis of the AtTTM2 gene

The Microarrays database and Expression data analysis tool, GENEVESTIGATOR Version V3 (www.genevestigator.com/gv/) (Zimmermann *et al.*, 2004; Grennan, 2006) was used to obtain expression levels of the AtTTM2 gene in various tissues of the *A. thaliana*. The tool uses the Affymetrix Arabidopsis genome array platform of the 260011_At probe to provide information about the genomic transcriptome information of a specific selected gene. In this regard, the At1g26190 gene was used as the query term before the arbitrary values of its expression intensities.

2.6.2 Developmental expression analysis of the AtTTM2 gene

The AtGeneExpress visualization and developmental tool (www.arabidopsis.org) was used to determine the expressional levels of the AtTTM2 gene in the Arabidopsis plant at the different stages of its development, and in this instance, the At1g26190 gene was queried against 10 different developmental stages of the plant (Zimmermann *et al.*, 2004; Grennan, 2006). Such developmental stages were set from the radicle emerging from the seed coat up to the release of mature seeds from the pod.

2.6.3 Co-expressional Analysis of the AtTTM2 gene

The Arabidopsis co-expression tool (ACT) (<http://www.genevestigator.com>) was used to determine the co-expressional profile of the AtTTM2 gene with the other related genes in the Arabidopsis. The At1g26190 (AtTTM2) was used as the search gene, to carry out analysis across all microarray experiments, and obtained a full correlation list and leaving the gene list limit blank. Pearson correlation coefficients (r-values), which indicate the linear associations of various expressions between a reference gene (At1g26190, AtTTM2) and all other Arabidopsis genes represented on the selection chip were obtained through a tool that makes use of signal intensities from microarray experiments. Both the positive and negative correlations (ranges from -1 to +1) were calculated by this tool, which is measures F statistical significance, expressed as a probability (P) and expectation (E) values.

2.6.4 Stimuli specific analysis of the AtTTM2 gene

The expression profiles of the AtTTM2 and its topmost 50 co-expressed genes (ECGG-50) (the AtTTM2:ECGG-50) were screened by using a perturbation tool, over the ATH1:22K array Affymetrix public microarray data in the GENEVESTIGATOR Version V3 (<http://www.genevestigator.com>). The microarray data were then downloaded and analysed by GEO (NCBI) (www.ncbi.nlm.gov/geo), the TAIR GeneExpress ([www.ebi.ac.uk/microarrays-as\(ac\)](http://www.ebi.ac.uk/microarrays-as(ac))) and the NASC Arrays (www.affymetrix.arabidopsis.info/narrays/experimentbrowse.pl) for experiments that induced differential expression of the co-expressed genes. The fold change ($\log_2$) value was measured for each experiment that induced expression, and consequently, providing expression values through the Multiple Array Viewers program of the Multiple Viewer (MeV) software package (version 4.2.01) (The Institute for Genomic Research (TIGR)).

CHAPTER THREE

Results

3.1 Generation of the *Arabidopsis* plants

Arabidopsis seeds were germinated on MS media in Petri dishes. The generated seedlings were then allowed to grow for two weeks in a growth chamber. This was done under greenhouse conditions which were adjusted to a temperature of 23°C and a day/night setting of 16/8 hours light/darkness at 10000 light lux.



Figure 3.1: Germinated *Arabidopsis* seedlings on MS media under growth chamber conditions.

3.2 Growth and maintenance of the *Arabidopsis* plants

The germinated seedlings were transplanted into sterile potting soil and then allowed to grow for a further 2-4 weeks under growth chamber conditions. These 2-6 week old plants were then harvested to provide material for isolation of total RNA which was necessary for the subsequent experimental procedures.



Figure 3.2: Some six week old *Arabidopsis thaliana* plants from which the total RNA for isolation of the targeted AtTTM2 gene fragment was extracted.

3.3 Isolation of the AtTTM2 gene fragment

The isolated total RNA was used to amplify the kinase coding region of the AtTTM2 gene. Using the generated cDNA together with the manually designed sequence-specific primers, the targeted AtTTM2 gene fragment was amplified using a specific 1-Step RT-PCR system.

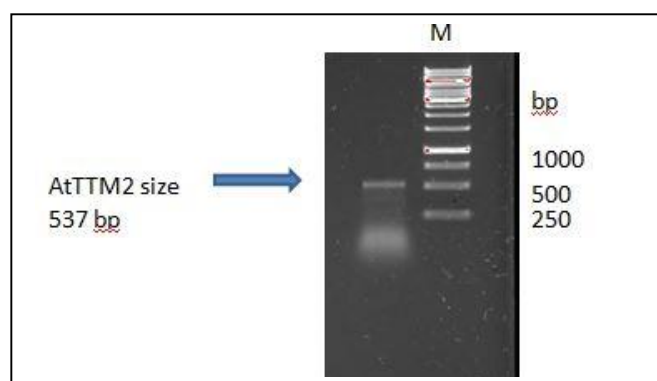


Figure 3.3: A 1% agarose gel resolution of the 537 base pair AtTTM2 gene fragment amplified from total RNA extracted from the *Arabidopsis* plants via a 1-Step RT-PCR, where the first well is representing the amplified AtTTM2 gene fragment and the second well a low molecular weight marker (M). The arrow is pointing at the amplified AtTTM2 gene fragment.

3.4: Confirmation of the successful ligation of the AtTTM2 gene fragment into the pTrcHis-TOPO expression vector

The amplified PCR product was then cloned into a pTrcHis2-TOPO vector and transformed into *E. coli* BL21 (DE3) pLysS cells for the subsequent intended recombinant protein expression. However, before expression was initiated, the recombinant plasmid vector (pTrcHis2-TOPO:AtTTM2 construct) was first checked if it was indeed carrying the desired AtTTM2 fragment by re-amplifying the PCR product with its own set of primers. As is shown in Figure 3.4, the AtTTM2 gene fragment could be re-amplified.

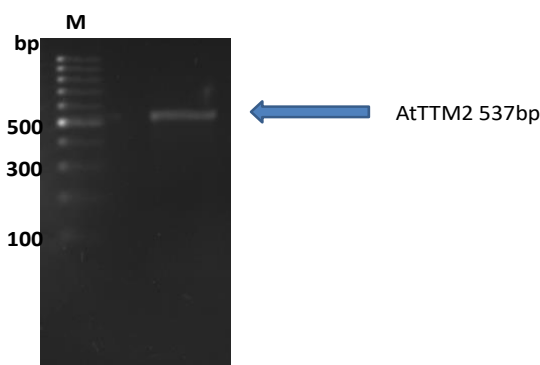


Figure 3.4: An agarose gel showing the PCR product yielded after a reamplification of the AtTTM3 gene fragment cloned in the pTrcHis-TOPO: AtTTM2 construct. Lane 1 is the 100 bp MW ladder (Catalog# SM1143-Fermentas International Inc., Burlington, Canada) while lane is the AtTTM3 gene fragment re-amplified with its own set of primers. The arrow marks the amplified gene fragments.

3.5: Confirmation of the correct orientation of the AtTTM2 gene fragment in the pTrcHis-TOPO expression vector

The correct orientation of the cloned AtTTM2 gene fragment in the generated pTrcHis-TOPO: AtTTM2 construct was ascertained by re-amplifying it using the reverse primer for the coding regions and the reverse vector primer. As is shown in Figure 3.5 below, the cloned AtTTM2 gene fragment could be successfully re-amplified using the selected set of primers.

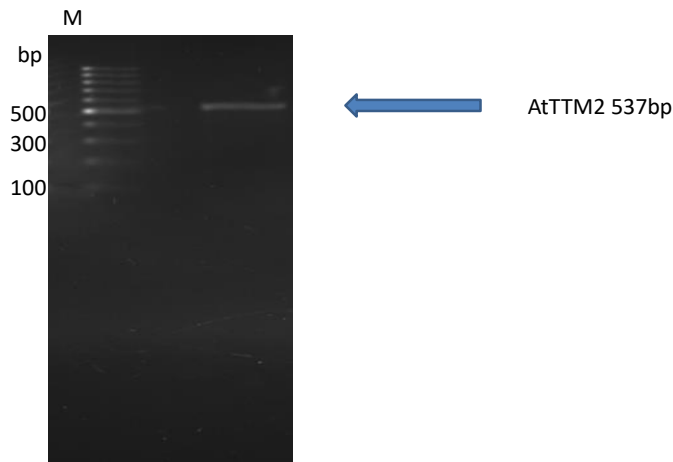


Figure 3.5: Agarose gel showing confirmation of the correct orientation of the AtTTM2 gene fragment in the generated pTrcHis2-TOPO:AtTTM2 construct. Lane 1 (M) is the 100 bp MW ladder (Catalog# SM1143-Fermentas International Inc., Burlington, Canada) while lane 2 represents the AtTTM2 gene fragment re-amplified with the specific reverse primer for the coding region and the vector forward primer. The arrow marks the re-amplified AtTTM2 gene fragment.

3.6 Protein expression

After the cloning of the AtTTM2 gene fragment into the pTrcHis2-TOPO vector was successfully confirmed, the resultant pTrcHis2-TOPO: AtTTM2 expression construct was then used to transform competent *E. coli* BL21 (DE3) pLySs DUOs cells. Protein expression was then induced with 1 mM IPTG in order to partially express the targeted and desired AtTTM2 recombinant protein (Figure 3.6).

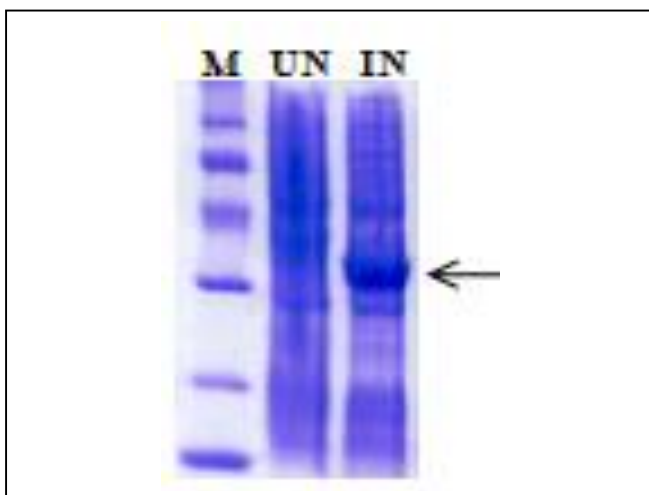


Figure 3.6: Expression of the recombinant AtTTM2 protein. An SDS-PAGE of protein fractions expressed in BL21 (DE3) plysS cells transformed with the pTrisHis2-TOPO:AtTTM2 fusion construct, where lane 1 (M) is the unstained low molecular weight marker (Catalog# SM0431Fermentas International Inc., Burlington, Canada), lane 2 (UN) is the un-induced cell culture (control) and lane 3 (IN) the cell cultures induced with 1 mM IPTG. The arrow marks the expressed recombinant AtTTM2 protein.

3.7 Determination of the kinase activity of the recombinant AtTTM2 protein

After recombinant protein expression, the generated crude protein extract was assessed for its probable kinase activity. The assessment was carried out *in vitro* measuring phosphorylation activity in the form of fluorescence signals at 485 nm emission (λ_{em} 485) using the OmniaTM Recombinant system.

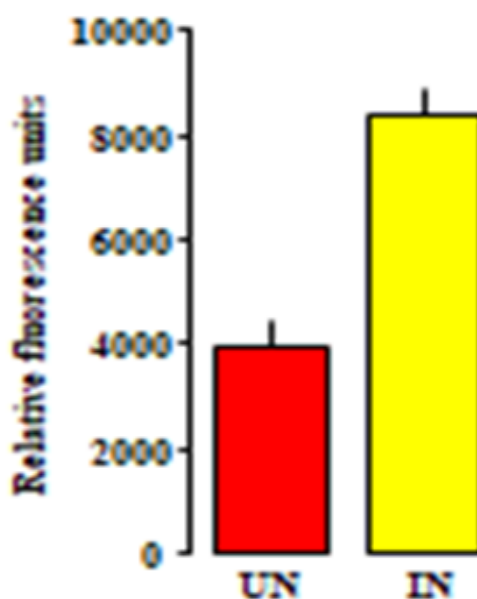


Figure 3.7: Determination of the kinase activity of the recombinant AtTTM2 protein. Phosphorylation activity levels of the un-induced (UN) and induced (IN) cell cultures of the recombinant AtTTM2 protein.

3.8 Determination of the anatomical expression profile of AtTTM2

Genevestigator was used to analyse the At1g26190 gene. The analysis revealed that the AtTTM2 protein is expressed in various tissues (105) of the *A. thaliana* plant. The AtTTM2 protein is expressed in the leaf, stamen, roots, stem, cell embryo, seedling, flower carpel, meristem and stem. Moreover, a closer analysis also showed that AtTTM2 is highly expressed in the stigma, pistil and callus but lowly expressed in the abscission zone, inflorescence, cell cultures and cotyledons (Figure 3.8).

Dataset: 105 anatomical parts from data selection: AT_AFFY_ATH1-0
Showing 1 measure(s) of 1 gene(s) on selection: AT-0

● AT1G26190

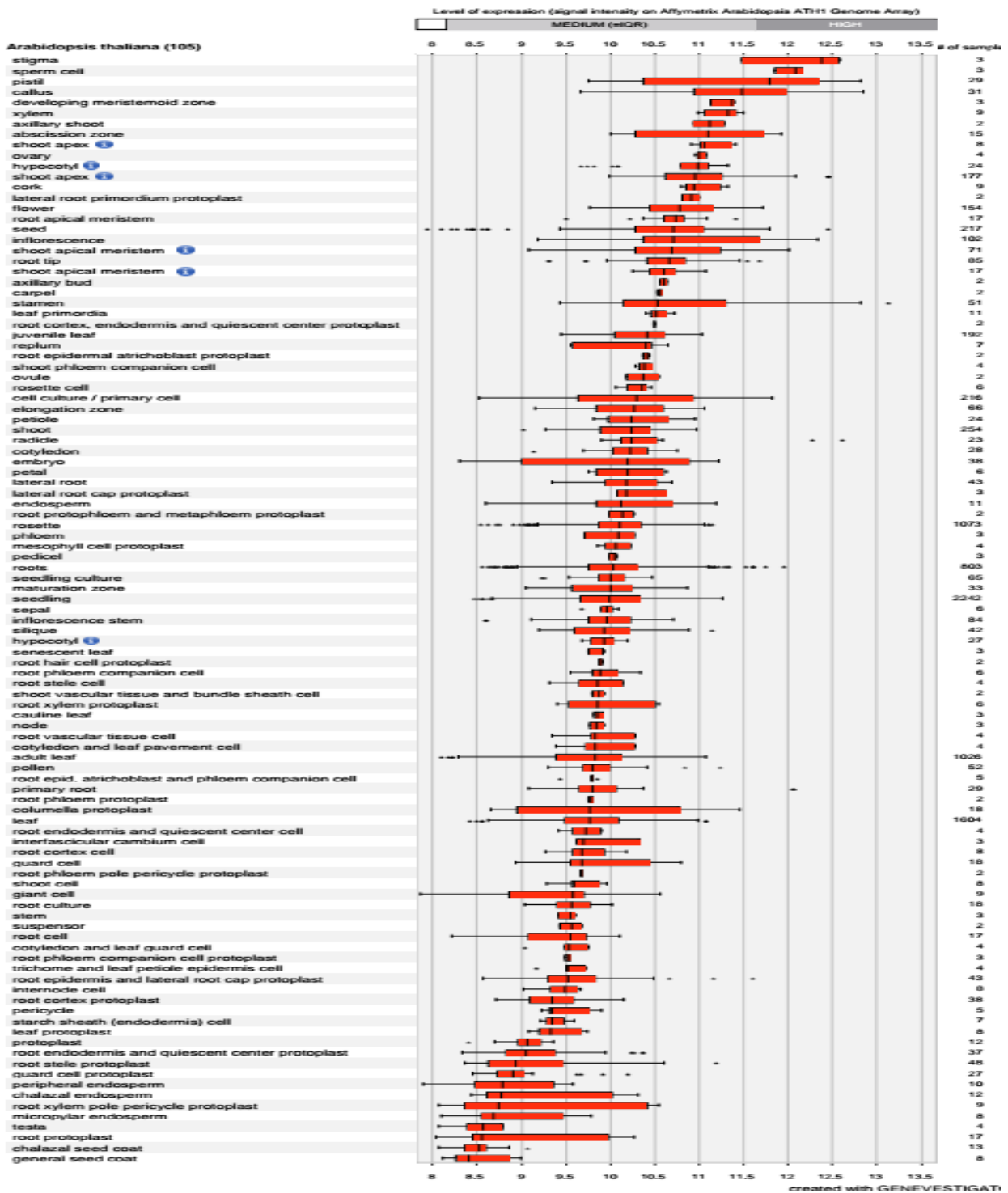


Figure 3.8: Intensity expression levels of the AtTTM2 protein in various tissues of the *A. thaliana*. The AtTTM2 protein is expressed in various parts of the plant but mostly in the stigma, pistil callus, cell culture and cotyledon, and lowly in the seed coat (Retrieved from the Genevestigator anatomy tool).

3.9 Determination of the developmental expression profile of AtTTM2

The AtGeneExpress visualisation and developmental tool were used to obtain expressional profiles of the AtTTM2 protein during the various stages of development in the Arabidopsis plant (Zimmermann *et al.*, 2004) in which the microarray data were screened using AtTTM2 as the search query. The results, yielded nine developmental stages during which the AtTTM2 protein is expressed and also highlighting that the protein is expressed mostly when the plant begins to produce pods (fruiting) and least expressed when the plant begins to flower (Fig3.5).

Dataset: 9 developmental stages from data selection: AT_AFFY_ATH1-0
Showing 1 measure(s) of 1 gene(s) on selection: AT-0

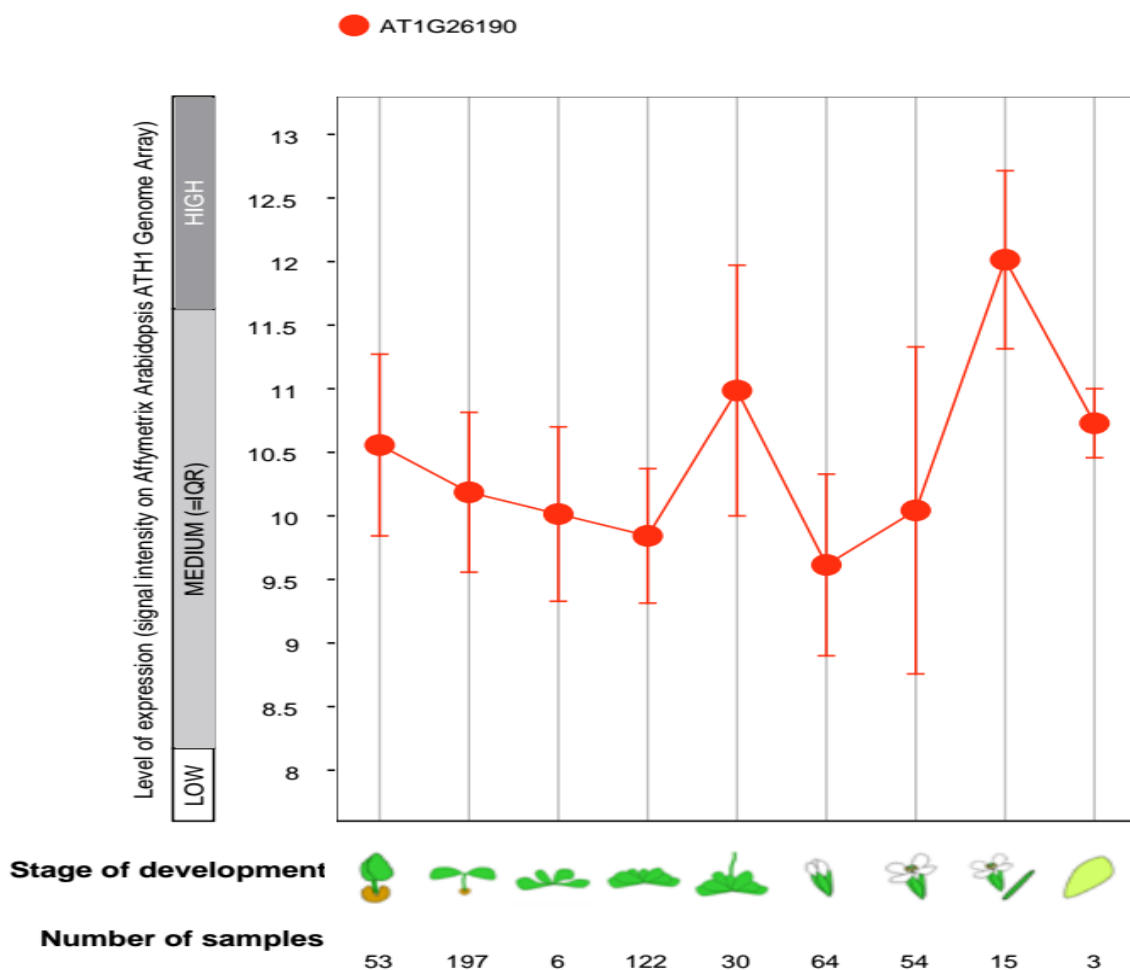


Figure 3.9: Expression profile of the AtTTM2 protein during the different developmental stages of *A. thaliana*. The above data shows measures of expression levels in relation to the various stages of development, beginning from the time when the radical emerges from the seed coat up to the time when mature seeds are released from the pods. The profile indicates that the AtTTM2 protein is highly expressed during the late flowering stages and least expressed during the early flowering stage.

3.10 Determination of the co-expression profile of AtTTM2

The expression profile of the At1g26190 gene were measured using the Arabidopsis co-expression tool (ACT) in order to obtain a list of other proteins that are co-expressed with AtTTM2 protein in the *A.thaliana* plant (<http://www.genevestigator.com>) on the available microarrays data with about 359 diverse transcriptome experiments. The AtTTM2 protein was found to be co-expressed with many other proteins and the 50 top most co-expressed proteins having correlation values of 0.65 to 0.78 (Table 3.1).

Table 3.1: List of the top 50 genes that are co-expressed with the AtTTM2 gene (At1g26190).

Gene	GO	Score	Description
AT3G48250		0.78	Encodes a pentatricopeptide repeat protein
AT1G27420		0.77	Galactose oxidase/Kelch repeat superfamily protein
AT5G64630	DMP,DR, RDD,HA	0.77	Chromatin Assembly factor-1 (CAF-1) p60 subunit
AT5G37020	RSSD	0.77	Auxin response Factor 8
AT5G37630		0.76	ARM repeat superfamily protein
AT3G18524	DMP,DR,RDD,FOM	0.76	Encodes a DNA mismatch repair homolog of human
AT1G64790		0.73	ILITYHIA
AT5G17410		0.73	GAMMA-tubulin complex component2
AT5G37530		0.72	NAD(P)-binding Rossmann-fold superfamily protein
AT4G38150		0.72	Pentatricopeptide repeat (PPR) superfamily protein
AT1G10490		0.72	GNAT acetyltransferase (DUF699)
AT3G13150		0.72	Tetratricopeptide repeat (TPR)-like superfamily protein
AT2G18850		0.71	SET domain-containing protein
AT3G20240		0.71	Mitochondria substrate carrier family protein
AT1G09800		0.71	Pseudouridine synthase
AT4G29910	DMP,DR	0.70	Origin recognition complex protein 5
AT1G55540		0.70	Nuclear pore complex protein
AT2G17270		0.69	Encodes a mitochondrial phosphate transporter

AT3G56900	FOM	0.69	Encodes ALADIN, a component of the nuclear pore
AT1G63160	DMP,DR	0.69	Replication factor C2
AT2G05210	DMP,DR	0.69	Encodes AtPOT1a
AT5G45300	RSSD	0.69	Beta-amylase 2
AT2G28330		0.68	Hypothetical protein)
AT3G18630	DMP,DR,RDD	0.68	Encodes a uracil-DNA glycosidase (UDG)
AT5G52860		0.68	ABC-2 type transporter family protein
AT5G53620		0.68	RNA polymerase II degradation factor
AT5G20040		0.68	Encodes tRNA isopentenyl transferase AtPT9
AT1G06590	RD	0.68	Anaphase-promoting complex subunit
AT4G30150		0.68	Urb2/Npa2 family protein
AT3G04860		0.67	Hypothetical protein (DUF868)
AT5G26680	DMP,DR,RDD	0.67	5-3 exonuclease family protein
AT5G44500		0.67	Small nuclear ribonucleoprotein family protein
AT1G64450	FOM	0.67	Glycine-rich protein family
AT3G04770		0.67	40s ribosomal protein SAB
AT1G53530		0.67	Mitochondrial ATP-independent protease
AT2G31320	DMP,DR,RDD,RDMP	0.67	Encodes a poly (ADP-ribose) polymerase
AT2G46470		0.66	Inner membrane protein OX1-like protein
AT4G38760		0.66	Nucleoporin (DUF3414)
AT5G06940		0.66	Leucine-rich repeat receptor-like protein kinase family protein
AT3G13222		0.66	Encodes a protein that binds to G-box
AT1G79490		0.66	Pentatricopeptide repeat (PPR) superfamily protein
AT3G27740		0.66	Encodes carbamoyl phosphate synthetase (CPS)
AT1G34010		0.65	Hypothetical protein
AT3G55160	AB	0.65	Thyroid adenoma-associated-like protein
AT1G33410		0.65	Suppressor of Auxin resistance1
AT5G26180		0.65	S-adenosyl-L-methionine-dependent methyltransferases

AT2G40720		0.65	Tetratricopeptide repeat (TPR)-like superfamily protein
AT5G10920		0.65	L-Aspartase-like family protein
AT3G20440		0.65	Encodes BE1 a putative glycoside hydrolase
AT4G33200		0.65	Myosin

Abbreviations for the indicated GOs: **DMP** = DNA metabolic process; **DR** = DNA Repair; **RDD** = Response to DNA Damage Stimulus; **HA** = Heterochromatic Assembly; **RSSD** = Regulation of shoot system development; **FOM** = Floral organ morphogenesis; **RD** = Regulation of DNA; **RDMP** = Regulation of DNA metabolic process; **AB** = Arginine biosynthesis.

3.11 Determination of the stimulus-specific expression profile of AtTTM2

The co-expressional profile of the AtTTM2 protein was established and the indicative functional roles together with its 50 top most co-expressed genes (ECGG-50), the whole set (AtTTM2:ECGG-50), was then subjected to an *in-silico* global expression analysis in which specific experimental conditions that could induce the differential expression of all genes in the set were identified. In accordance with the co-expression and GO analysis, the heat maps generated from the microarray expression analysis revealed that the transcriptional process of the AtTTM2 protein and its related ECGG-50 proteins (the AtTTM2: ECGG-50 pool) are generally and collectively induced in response to a wide range of biotic stress factors.

Dataset: 37 perturbations from data selection: AT_AFFY_AGRONOMICS-3
Showing 1 measure(s) of 1 gene(s) on selection: AT-4

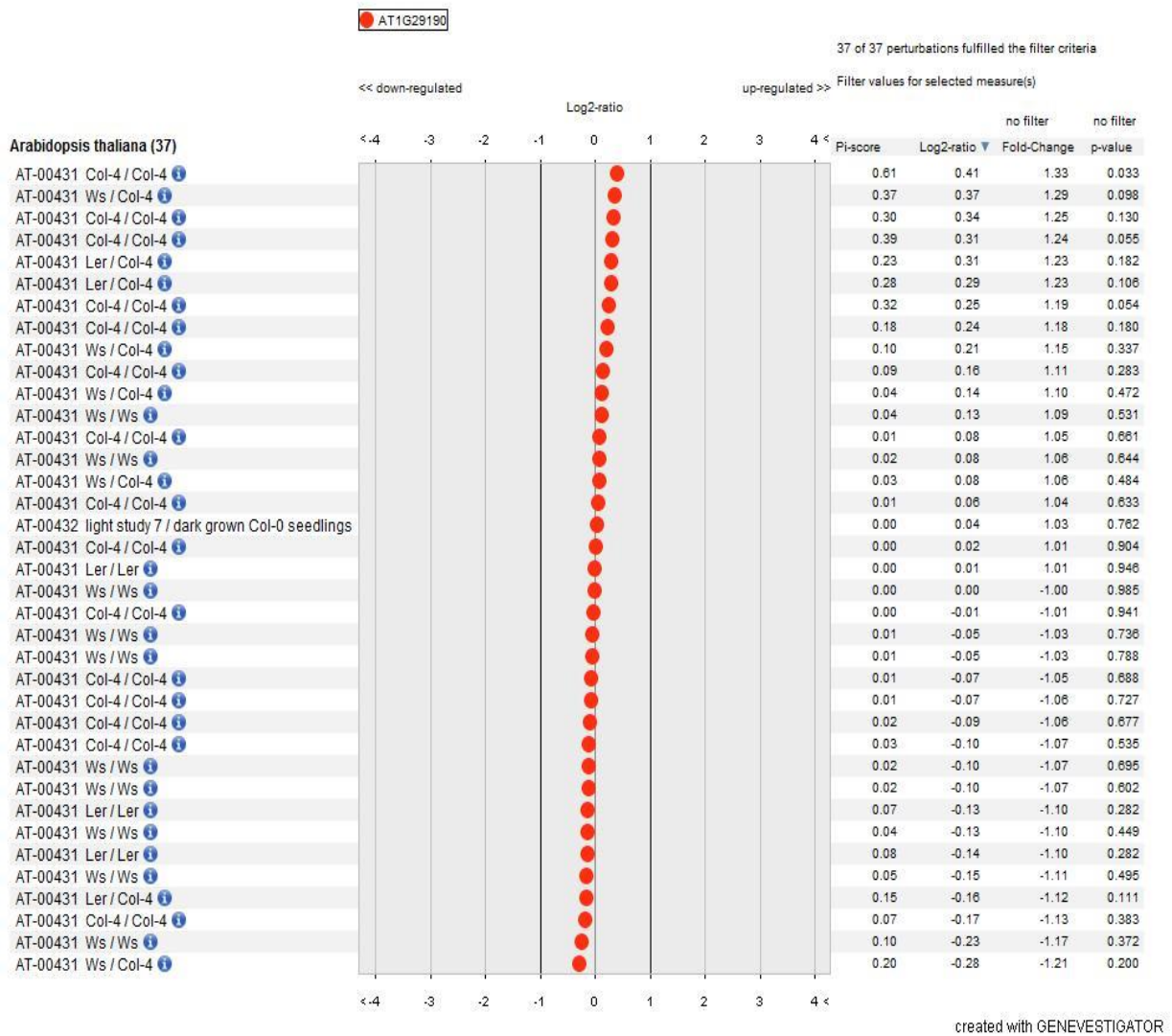


Figure 3.10: Expression profiles of the AtTTM2 protein in *A. thaliana* in response to the various stimulus-specific conditions. The profile shows a differential induced expression of the protein in response to a wide array of biotic factors.

CHAPTER FOUR

Discussion, conclusion and recommendations

4.1 Discussion

The AtTTM2 protein, encoded by the At2g26190 gene, is one of the putative candidate molecules that have been annotated to contain a kinase catalytic core motif, and as has already been mentioned in Chapter 1. Even though it has been annotated as a putative kinase, this protein has never been experimentally demonstrated to function as a kinase. Hence in this study, the AtTTM2 protein was targeted for functional characterisation. In order to achieve the set objectives for the study, some gene-specific primers were manually designed, with the help of the nucleotide sequences retrieved from the TAIR (The Arabidopsis Information Resource) website (Figure 2.1) to isolate the targeted gene segment. In order to isolate the targeted AtTTM2 gene fragment, *A. thaliana* ecotype Columbia plants were generated (Figure 3.1) and maintained under controlled greenhouse conditions in a growth chamber (Figure 3.2). From these generated plants, total RNA was isolated, cDNA synthesised, followed by amplification of the intended AtTTM2 gene fragment (Figure 3.3) via a specialised 1-Step RT-PCR system in the presence of both the cDNA and sequence-specific primers.

The amplified AtTTM2 gene fragment was then cloned into a pTrcHis2-TOPO[®] expression vector (Figure 2.2), which was commercially acquired to generate a pTrcHis-TOPO expression construct. Before this construct could be used for any further experimental assays, both the presence and correct orientation of the AtTTM2 gene fragment in the pTrcHis2-TOPO vector were checked and ascertained. The AtTTM2 gene fragment in the construct was re-amplified with its own sets of primer (Figure 3.4) while for correct orientation, the gene fragment was re-amplified with its own reverse primer and the vector forward primer (Figure 3.5). After confirming that the AtTTM2 was indeed properly cloned into the pTrcHis-TOPO vector, the resultant construct was then used to transform some competent *E. coli*[®] EXPRESS BL21 (DE3) pLysS DUOs cells for protein expression. Protein expression was then induced through the addition of 1 mM IPTG at an OD₆₀₀ of 0.6 resulting in expression of the expected C-terminus His-tagged fusion protein product of approximately 22 kDa (Figure 3.6).

After successful expression, the obtained protein product was then assessed for its ability to phosphorylate a substrate peptide in the Omnia[™] Recombinant system. As is seen in Figure 3.7, the recombinant AtTTM2 had ~9000 RFUs activity, which was about 2.5 times more than its

control activity (~4000R RFUs). Relating to other known kinases from *A. thaliana*, which among others, include AtWAKL-10 (Meier *et al.*, 2010), AtPSKR-KD2 (Kwezi *et al.*, 2011) and AtPSK-R1 (Muleya *et al.*, 2014), the activity of AtTTM2 was somewhat higher. The relative activity of AtWAKL-10 was ~5000 RFUs (Meier *et al.*, 2010), that of AtPSK-KD2 ~1400 RFUs (Kwezi *et al.*, 2011) while that of AtPSK-R1 was ~700 RFUs (Muleya *et al.*, 2014). The reason why AtTTM2 had a higher activity than its related counterparts could be attributed to the fact that AtTTM2 was tested in the form of its natural and native form (crude extract) while the rest of the other proteins were tested after passing through the purification and refolding processes. Both processes severely expose recombinant proteins to excessive salts and buffers that somehow affect the general physiological and physical properties of proteins (Bornhorst & Falke, 2000).

After establishing the functional kinase activity of the AtTTM2 *in vitro*, bioinformatics was used (*in silico*) to bring about a more explicit functional analysis of the AtTTM2 protein in the *A. thaliana* plant and possibly other related plants. These analyses were achieved by using computer-based programs and also a variety of web-based software (Zimmermann *et al.*, 2004). Firstly, using genevestigator analyses, it was revealed that anatomically, the AtTTM2 protein is highly expressed in tissues such as the stamen, cell culture and cotyledon (Figure 3.8). Expressional profiles of the AtTTM2 protein during its various stages of development were then obtained using the AtGeneExpress visualisation tool (Zimmermann *et al.*, 2004). The results demonstrated nine developmental stages during which this protein is transiently expressed with its expression being high during the late flowering stage but low during the early flowering phase (Figure 3.9).

Furthermore, a list of other proteins that are co-expressed with AtTTM2 was also obtained on the microarrays data (www.genevestigator.com). From this analysis, AtTTM2 was shown to be co-expressed with several other proteins of which the 50 top most of such proteins had correlation values between 0.78 and 0.65 (Table 3.1). Such proteins were found to be principally involved in essential cellular processes such as DNA metabolic processes, DNA repair response to biotic stimuli, and any process that would results in a change of state or activity of a cell (in terms of movement, secretion, enzyme production, gene expression, etc.) as a result of a stimulus indicating damage to its DNA from environmental insults or errors during metabolism (Table 3.1). Lastly, a global expression analysis of the AtTTM2 and its co-expressed list (ECGG-50) revealed that, and in accordance with the co-expressional and GO analysis tool, revealed that the transcriptional process of this AtTTM2 protein and its related ECGG-50 proteins are collectively induced in response to a variety of biotic stress factors (Figure 3.10).

Therefore, by summing up all the findings in this study, it is clear that the studied AtTTM2 protein is indeed a critical higher plant signalling molecule with a central role in responses to a variety of pathogenic fungal stress factors (Figure 3.10), and whose mechanism of action may be mediated through kinase activity. Thus the study has managed to experimentally confirm and establish AtTTM2 as a *bona fide* higher plant kinase.

Since the first publication concerning protein phosphorylation in plants, analogies with well-understood animal signal transduction systems have been used to guide plant signal transduction studies (Verhey, 1994). Such analogies are useful in suggesting outlines of potential signal transductions systems, informing researchers of further possibilities, and shaping the designs of experiments. Animal signal transduction systems have a broad diversity in the system components. There are literally hundreds of different protein kinases, at least 16 G α subunits, and more than a hundred G-protein-linked receptors. The most widespread protein kinases, PKA and PKC, comprise a total of at least 13 different subspecies. All this diversity is part of a system of interlocking signal transduction chains, which link phosphorylation-dephosphorylation-based control of metabolism and gene function with signals from outside the cell. The diversity presumably allows interactions including potentiation, cooperation, synergism, and antagonism between many different pathways.

4.2 Conclusion

Outcomes of this study have demonstrated both biochemically and bioinformatically that the AtTTM2 protein from *A. thaliana* is a *bona fide* functional plant kinase with critical roles in plant growth and development. The AtTTM2, therefore, becomes the fourth ever kinase molecule to be identified in *A. thaliana*.

4.3 Recommendations

1. This study used a crude protein, therefore, further and future work should be carried out using the pure version of AtTTM2.
2. The kinetic measures (K_m and V_{max} values) and further characterisation of the AtTTM2 need to be undertaken so that a better picture of its enzymology and/or enzymatic properties is ascertained.

3. The undertaken work was carried out *in vitro* so, it could be better to further attempt *in situ* and/or *in planta* experiments so that a clear picture of the functional roles of this AtTTM2 in Arabidopsis and other related higher plants is established.
4. The possible interaction of this protein and ACs in plants must be explored further, particularly with regards to plant growth and developments.

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