Characterization of the biological roles and biochemical properties of triphosphate tunnel metalloenzyme superfamily members in plants

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Cell and Systems Biology
University of Toronto

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ABSTRACT

The triphosphate tunnel metalloenzyme (TTM) superfamily comprises a group of enzymes that hydrolyze organophosphate substrates in a metal-dependent manner. Members of this family have diverse biological functions in different organisms. Arabidopsis encodes three TTM genes, called AtTTM1, AtTTM2, and AtTTM3. Previous work showed that AtTTM3 is a tripolyphosphatase requiring Mg$^{2+}$ where its crystal structure reveals the signature TTM catalytic tunnel. In this thesis, I present my findings on the biological and biochemical characterization of AtTTM1 and AtTTM2. A reverse genetics approach using T-DNA insertion knockout lines revealed that AtTTM2 is a negative regulator of the SA amplification loop in defense responses. ttm2 plants exhibit enhanced defense responses to virulent and avirulent pathogens, enhanced hypersensitive response (HR), elevated SA, and enhanced SAR. ttm2-mediated enhanced resistance requires the key defense regulators, PAD4, ICS1, and NPR1. Furthermore, AtTTM2 is transcriptionally downregulated in response to pathogen infection and flg22, SA, and BTH treatment. While ttm2 exhibits enhanced resistance, it is not an autoimmune mutant, suggesting that it is not a conventional negative regulator of defense responses. Phenotypic characterization of ttm1 did not reveal any alterations in defense responses. Instead, ttm1 exhibited delayed senescence by showing
enhanced chlorophyll retention after dark-induced senescence, which is concomitant with the delayed expression of senescence markers. Furthermore, \textit{AtTTM1} is transcriptionally upregulated in response to dark-induced senescence, suggesting that it is a positive regulator of senescence. Interestingly, functional complementation of \textit{ttm1} and \textit{ttm2} phenotypes in promoter swap analyses revealed that the distinct biological roles of \textit{AtTTM1} and \textit{AtTTM2} are governed by their expression patterns. These results indicate that their biochemical functions are likely highly similar or identical, which was supported by the observation that both AtTTM1 and AtTTM2 possess pyrophosphatase activity in the presence of Mg\textsuperscript{2+}. The findings presented in this thesis not only depict the first characterization of TTM superfamily members in plants, but they also represent one of the first accounts of the importance of TTM proteins in a biological context.
ACKNOWLEDGEMENTS

I would like to express my deepest appreciation for my supervisor, Dr. Keiko Yoshioka, whose philosophy regarding the practice of science reflects a true scholar with the utmost respect for the scientific method. Her relentless pursuit of good science combined with her enthusiastic spirit for research has taught me invaluable lessons that I will take with me in all my future endeavours. The successes that I have achieved since embarking on this journey are a product of her mentorship and constant support.

I would also like to thank the lab’s senior research associate, Dr. Wolfgang Moeder, for his continuous encouragement and guidance throughout my thesis project. He possesses an unbridled passion for solving the unsolved that has been instrumental for the successes achieved by the lab. The countless cases of troubleshooting we have worked through and fruitful discussions have helped shape my thesis to its finality.

I must also thank a past lab member and my dear friend, Dr. Kimberley Chin, who made daily life in the lab enjoyable and supported me throughout most of my graduate career. ‘Alley 3’ will always be memorable for unwritten reasons. I must further thank other past and current graduate members, Dr. William Urquhart, Christine Cao, Thomas DeFalco, and Alex Fortuna for the fun memories and constant support. I must also acknowledge the undergraduate students for their integral role in the maintenance of the lab with special thanks to Palak Patel for his assistance with the characterization of $ttm2$. To the best graduate administrator, Ian Buglass, I must thank you for your candor and advice in trying times. You must know that students are indebted to you.

I extend my deepest gratitude to the members of my supervisory committee, Dr. Daphne Goring and Dr. Nicholas Provart, for their invaluable input and guidance. Our annual meetings have always provided meaningful discussions that were helpful in developing my thesis project.

Lastly, I must thank my partner, my family, and my friends for their unwavering support throughout this challenging time. While many of you could not understand what has kept me so busy for the last several years, you continued to warm me with words of encouragement and provided me with the strength to persevere onwards. You cannot know how much I appreciate the small sacrifices that were made to accommodate my lifestyle. To show my gratitude, I dedicate this work to all of you.
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<table>
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<th>Abbreviation</th>
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<tr>
<td>°C</td>
<td>Degrees Celsius</td>
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<td>5'</td>
<td>5 prime</td>
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<td>α</td>
<td>Alpha</td>
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<td>A</td>
<td>Absorbance wavelength</td>
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<td>ABA</td>
<td>Abscisic acid</td>
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<td>AC</td>
<td>Adenylate cyclase</td>
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<td>Adenosine diphosphate</td>
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<td>Asp</td>
<td>Aspartic acid</td>
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<tr>
<td>ATG</td>
<td>Adenine-thymine-guanine</td>
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<td>Azelaic acid</td>
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<td>Base pair</td>
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<td>Brassinosteroid insensitive1</td>
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<td>BTH</td>
<td>Benzothiadiazole</td>
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<td>CAB</td>
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<td>CaMV 35S</td>
<td>Cauliflower mosaic virus 35S promoter</td>
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<td>CAPS</td>
<td>Cleaved amplified polymorphic sequences</td>
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<td>CC</td>
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<td>Term</td>
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<tr>
<td>cDNA</td>
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<td>CFU</td>
<td>Colony forming units</td>
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<td>CHAD</td>
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<td>CIAP</td>
<td>Calf intestine alkaline phosphatase</td>
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<td>g</td>
<td>Gram</td>
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<td>G3P</td>
<td>Glycerol-3-phosphate</td>
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<td>GC-MS</td>
<td>Gas chromatography-mass spectrometry</td>
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<td>Gm</td>
<td><em>Glycine max</em></td>
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mg  Milligram
Mg$^{2+}$  Magnesium ion
MgCl$_2$  Magnesium chloride
MgSO$_4$  Magnesium sulfate
ml  Millilitre
mM  Millimolar
Mn$^{2+}$  Manganese ion
MOPS  3-(N-morpholino)propanesulfonic acid
mRNA  Messenger ribonucleic acid
MS  Murashige-Skoog
n  Sample size
N$_2$  Nitrogen gas
NaCl  Sodium chloride
NADH  Reduced nicotinamide adenine dinucleotide
NaSA  Sodium salicylate
NBS-LRR  Nucleotide binding site-leucine-rich repeat
NDR1  Nonrace-specific disease resistance
Ni$^{2+}$  Nickel ion
NIM1  Noninducible immunity
nm  Nanometers
NPR1  Nonexpresser of pathogenesis-related
NPS  Sodium phosphate sulfate buffer
N-terminus/terminal  Amino terminus/terminal
NTPase  Nucleoside triphosphatase
NuK  Nucleotide kinase
OD  Optical density
OD595  Optical density at 595 nanometers
OD600  Optical density at 600 nanometers
OD630  Optical density at 630 nanometers
PAD4  Phytoalexin-deficient
PAMP  Pathogen-associated molecular pattern
PBS  Phosphate-buffered saline
PCD  Programmed cell death

xx
PCR  Polymerase chain reaction
PDB  Protein database bank
pH  Potential of hydrogen
PMSF  Phenylmethanesulfonyl fluoride (Serine protease inhibitor)
$P_i$  Free phosphate
PIP  Pipecolic acid
$PP_i$  Pyrophosphate
$PPP_i$  Tripolyphosphate
PR  Pathogenesis-related
PRR  Pattern recognition receptor
$Psg$  Pseudomonas syringae pathovar glycinea
psi  Pounds per square inch
psi-BLAST  Position-specific iterative basic local alignment search tool
$Psm$  Pseudomonas syringae pathovar maculicola
$Psph$  Pseudomonas syringae pathovar phaseolicola
$Pst$  Pseudomonas syringae pathovar tomato
PTI  Pathogen-associated molecular pattern-triggered immunity
pv  Pto
qRT-PCR  Quantitative real time-polymerase chain reaction
R  Resistance
rcf  Relative centrifugal force
RCS  RIN4 cleavage site
R gene  Resistance gene
RH  Relative humidity
RIN4  Resistance to Pseudomonas syringae pathovar maculicola1-interacting protein4
RNA  Ribonucleic acid
RNase A  Ribonuclease A
ROS  Reactive oxygen species
RP  Right border primer
rpm  Rotations per minute
RPM1  Resistance to Pseudomonas syringae pathovar maculicola1
RPP  Resistance to *Peronospora parasitica*
RPS2  Resistance to *Pseudomonas syringae*2
RPS4  Resistance to *Pseudomonas syringae*4
RT-PCR  Reverse transcriptase-polymerase chain reaction
SA  Salicylic acid
SAG  Salicylic acid glucoside
SAG12  Senescence-associated gene12
SAG13  Senescence-associated gene13
SAG101  Senescence-associated gene101
SAI1  Salicylic acid insensitive1
SAR  Systemic acquired resistance
SAV  Senescence-associated vacuole
SD  Standard deviation
SDG  Senescence-downregulated gene
SDS  Sodium dodecyl sulfate
SE  Standard error
Ser  Serine
ssi4  Suppressor of salicylic acid insensitive4
T-DNA  Transfer-deoxyribonucleic acid
TIR  Toll/Interleukin-like receptor
TM  Transmembrane
TTM  Triphosphate tunnel metalloenzyme
TE RNase  Tris/EDTA buffer with ribonuclease A
ThPP  Thiamine pyrophosphate
ThTPase  Thiamine triphosphatase
UBC21  Ubiquitin conjugating enzyme21
µg  Microgram
µl  Microlitre
UK  Uridine kinase
µm  Micron
µM  Micromolar
UTR  Untranslated region
Ws  Wassilewskija wild type
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<td>5-bromo-4-chloro-1H-indol-3-yl ( \beta )-D-glucopyranosiduronic acid</td>
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STATEMENT OF PUBLICATIONS

Ung H, Moeder W, and Yoshioka K. (2014) Involvement of Arabidopsis triphosphate tunnel metalloenzyme1 (AtTTM1) in leaf senescence indicates biological diversification of the TTM family in plants. Submitted to Plant Cell.

H. Ung performed most of the experiments and W. Moeder provided data for Table 4-1 and Fig. 5-2.


H. Ung contributed to experimental design and intellectual input.


H. Ung performed all experiments.


H. Ung provided data for Fig. 5 and created site-directed mutants for data presented in Table S1.


H. Ung provided data for Fig. 2.

_H. Ung contributed to Fig. 2._


_H. Ung contributed to Fig. 2._
CHAPTER 1

INTRODUCTION
1.1 Triphosphate tunnel metalloenzymes (TTMs)

The triphosphate tunnel metalloenzyme (TTM) superfamily was created to encompass a group of metal-dependent phosphohydrolases that act on substrates with triphosphate moieties (Gong et al., 2006). Despite a wide substrate range, these enzymes share a unique active site that is situated within a catalytic tunnel comprising antiparallel β sheets (Lima et al., 1999; Gallagher et al., 2006; Song et al., 2008). Within this tunnel are two glutamate-containing motifs that comprise the metal-binding site required for catalysis. Several members have been biochemically characterized; for example, RNA triphosphatase activity of Cet1 from *Saccharomyces cerevisiae*, adenylate cyclase activity of CyaB from *Aeromonas hydrophila*, which produces 3',5'-cyclic-adenosine monophosphate (cAMP) from adenosine triphosphate (ATP), triplyphosphatase activity from the TTMs of *Clostridium thermocellum* (CthTTM) and *Nitrosomonas europaea* (NeuTTM), and thiamine triphosphatase (ThTPase) activity from mammalian TTMs (Ho et al., 1998; Sismeiro et al., 1998; Keppetipola et al., 2007; Song et al., 2008; Delvaux et al., 2011; Delvaux et al., 2013; Bettendorff and Wins, 2013). Furthermore, the structure of a number of TTM proteins has been solved either by crystallography or by NMR spectroscopy, revealing the characteristic tunnel-shaped active site.

Two branches of enzymes comprise the TTM superfamily: 1) the metal-dependent RNA triphosphatases that are structurally similar to Cet1 and 2) proteins belonging to the CYTH superfamily, whose founding members are CyaB and human ThTPase (Gong et al., 2006). While the primary structure conservation is low between these enzymes, Shuman and colleagues recognized a striking similarity between the active site folds of Cet1 and several prokaryotic CYTH proteins. Thus, this superfamily was created to encompass proteins exhibiting these structural similarities and requiring a metal cofactor for activity, which include the Cet1-like RNA triphosphatases from fungi, protozoa, and several DNA viruses as well as members of the CYTH superfamily.

1.1.1 The CyaB-thiamine triphosphatase (CYTH) superfamily

The CYTH superfamily was named after its founding members, adenylate cyclase (AC) from *Aeromonas hydrophila* (CyaB) and thiamine triphosphatase from...
Homo sapiens (hThTPase) (Iyer and Aravind, 2002). Thiamine triphosphatases catalyze the hydrolysis of thiamine triphosphate to thiamine diphosphate, releasing inorganic phosphate (P_i) in the process. Thiamine triphosphate has been implicated as a second messenger in cell stress responses where ThTPase regulates these levels (Lakaye et al., 2004). Biochemical studies show that this enzyme functions at an alkaline pH optimum with a strict dependency on a divalent metal cofactor, such as Mg^{2+} (Song et al., 2008). ThTPase was first identified in rat brain, but has since been found in mammals, sea anemone (Nematostella vectensis), and zebrafish (Danio rerio) (Song et al., 2008; Delvaux et al., 2013; Bettendorff and Wins, 2013).

The other founding member of the CYTH superfamily, CyaB, was one of two isolated cya (adenylate cyclase) genes from an A. hydrophila genomic library that could functionally complement a mutant cya strain of Escherichia coli (Sismeiro et al., 1998). These gene products were named AC1 and AC2 (corresponding to CyaA and CyaB) for their ability to convert ATP into cAMP and pyrophosphate (PP_i). Based on structural similarity, it was determined that CyaA belongs to the enterobacterial class of AC enzymes (class I). This class includes ACs from E. coli and other gram-negative bacteria where these enzymes are tightly regulated by intracellular glucose levels (Danchin, 1993; Sismeiro et al., 1998). CyaB did not belong to previously existing classes because of its unusual biochemical properties. Thus, a distinct class was created.

As a result, ACs can be classified into four additional classes (Danchin, 1993). Class II ACs are extracellular toxins from certain pathogens, such as Bacillus anthracis and Bordetella pertussis, that generate supraphysiological levels of cAMP after activation by host-derived calmodulin. Class III is the largest class, comprising the mammalian ACs as well as others found in protozoa and some prokaryotes. While ACs from this class share several motifs for nucleotide binding, they are structurally diverse and can range in molecular weight. As a result, several types of ACs belong to this universal class and can be activated by different signals, such as calmodulin or the α-subunit of heterotrimeric G protein. Class IV was created specifically for CyaB (Sismeiro et al., 1998). This AC was reported to be significantly homologous to the gene products of three hyperthermophilic archaeabacteria as well as exhibiting activity at an unusual optimal high temperature (65°C) and alkaline pH (9.5). Similar to the case of CyaB, the
remaining two classes were created for ACs with distinct biochemical properties or divergent sequences from existing ACs (Cotta et al., 1998; Téllez-Sosa et al., 2002).

Interestingly, while ThTPase and AC are functionally unrelated, it was determined that they share a unique catalytic fold containing several conserved residues. Using CyaB as a query sequence for a position-specific iterative BLAST (psi-BLAST) analysis, Iyer and Aravind (2002) identified orthologs in various organisms across many phyla, such as bacteria, archaea, plants, and animals. The alignment of these sequences led to the identification of a conserved catalytic motif, ExExK (where x is any residue), along with six acidic residues for the coordination of two divalent cation cofactors and five basic residues for the binding of the β and γ phosphate moieties of a triphosphate substrate. In addition, the tertiary structures of several orthologs consistently revealed an active site comprising eight antiparallel β strands, forming a catalytic tunnel (Gallagher et al., 2006; Song et al., 2008). Collectively, these features defined the CYTH domain.

1.1.2 Additional domains in CYTH proteins

While the CYTH domain can be present as the sole domain of a protein, it can also be found fused to an additional domain. The most prevalent of these additional domains is the conserved histidine α-helical domain (CHAD) (Iyer and Aravind, 2002). Though no clear function has been elucidated for the CHAD, a set of conserved charged residues forming a polar surface is thought to participate in metal or phosphate binding. A hydrolase domain (HD) can also be found fused to the CYTH domain and typically confers phosphoesterase activity.

Among these additional domains is one comprising that of a phosphate-binding loop (P-loop) kinase domain, also referred to as a nucleotide kinase (NuK) domain. The P-loop kinase domain can be found N-terminal to the CYTH domain where this rare fusion is, so far, only found in plants and slime molds, such as Dictyostelium discoideum (Iyer and Aravind, 2002). The P-loop kinase domain is found in a class of proteins called the P-loop NTPases (Leipe et al., 2003). These enzymes are characterized by the presence of a Walker A motif, GxxxxGK(S/T) (where x is any residue), Walker B motif, hhhhhD (where h is any hydrophobic residue), and the lid module motif, Rx2-3R (where x is any residue). The Walker A motif binds the β and γ phosphate moieties of the substrate,
the Walker B motif coordinates metal cofactor binding, and the lid module helps to stabilize substrate binding (Leipe et al., 2003). The P-loop NTPases can be divided into 41 distinct families of kinases, each with consistent and distinct variations of the abovementioned motifs. For instance, the plant-derived CYTH proteins possess P-loop kinase domains belonging to the UCPP family of kinases, which includes the uridine-cytidine kinases, phosphoribulokinases, fructokinases, pantothenate kinases, and 2-phosphoglycerate kinases.

1.1.3 RNA triphosphatases

RNA triphosphatases are involved in eukaryotic mRNA capping, which is important for pre-mRNA processing and transport, recruitment of mature mRNA to the ribosome, and protection of mRNA from untimely degradation (Shuman and Schwer, 1995). The 5'-7-methylguanosine cap of eukaryotic mRNA is produced in three chemical reactions: 1) RNA triphosphatase removes the γ phosphate of the 5' triphosphate end of the nascent pre-mRNA; 2) guanylyltransferase transfers guanosine monophosphate (GMP) from guanosine triphosphate (GTP) to the exposed 5' diphosphate; and 3) RNA (guanine-N7) methyltransferase transfers a methyl group to the guanosine. Depending on the organism, this capping apparatus can be found as a single or multi-component system. For instance, *S. cerevisiae* possesses three individual proteins, Cet1, Ceg1, and Adp1, for each respective step (Lima et al., 1999). In contrast, the capping machinery of vaccinia virus comprises a single polypeptide, D1, capable of all three functions. Baculovirus, metazoans, and plants encode a two-component system with a bifunctional triphosphatase/guanylyltransferase enzyme and a separate methyltransferase protein (Ho et al., 1998). Thus, RNA triphosphatases can exist as a monofunctional or bifunctional protein, making them the most structurally and mechanistically divergent out of the three capping components.

RNA triphosphatases fall into two groups: 1) cysteine phosphatase enzyme superfamily and 2) metal-dependent phosphohydrolases (Gong et al., 2006). The first group of enzymes possess a conserved phosphate-binding loop containing the motif, H CxxxxxR(S/T) (where x is any residue), where the cysteine is critical for the triphosphatase reaction. The RNA triphosphatases of metazoans and plants belong to this group, and have no requirement for a metal cofactor (Changela et al., 2001). The
second group of enzymes is characterized by the presence of a catalytic ExExK (where x is any residue) motif, a strict dependency on a metal cofactor (Mg\(^{2+}\)) as well as the ability to hydrolyze NTPs into NDPs in the presence of Mn\(^{2+}\) or Co\(^{2+}\) (Ho et al., 1998). These RNA triphosphatases are found in fungi, protozoa, and several DNA and RNA viruses (Changela et al., 2001). For instance, Cet1 from *S. cerevisiae* belongs to this latter group, requiring Mg\(^{2+}\) for its RNA triphosphatase activity and Mn\(^{2+}\) for its ATPase activity (Ho et al., 1998).

### 1.1.4 The creation of the TTM superfamily

The tertiary structures of the CYTH proteins, CyaB from *Yersinia pestis* and ThTPase from *Mus musculus* were crystallized or solved by NMR spectroscopy and revealed a barrel shape composed of antiparallel β sheets (Fig. 1-1; Gallagher et al., 2006; Song et al., 2008). While the overall barrel shape was not unique, its composition of only eight or nine β sheets with inwardly facing hydrophilic residues was novel. Interestingly, no other known protein structure at the time resembled these characteristics except for Cet1 (Lima et al., 1999). As mentioned above, Cet1 is an ortholog of CyaB where its crystal structure revealed a catalytic fold resembling a tunnel comprising eight antiparallel β strands (Lima et al., 1999). This catalytic fold was found among other members of the CYTH superfamily, but also in another RNA triphosphatase from *Plasmodium falciparum*, called Prt1 (Gallagher et al., 2006; Gong et al., 2006). Prt1 also exhibits RNA triphosphatase activity in the presence of Mg\(^{2+}\) and ATPase activity in the presence of Mn\(^{2+}\) (Ho and Shuman, 2001). Because of the structural and biochemical similarities between the metal-dependent RNA triphosphatases and the CYTH proteins, a new superfamily was created to encompass these properties and aptly named the triphosphate tunnel metalloenzymes (TTMs; Gong et al., 2006).
Figure 1-1. Three-dimensional structures of representative proteins of the TTM superfamily.

Shown are TTM proteins from Homo sapiens (3TVL), Mus musculus (2TMV), Saccharomyces cerevisiae (1D8H), Mimivirus (3BGY), Yersinia pestis (3N10), Nitrosomonas europaea (3TYP), Arabidopsis thaliana (3V85), Pyrococcus furiosus (1YEM), and Vibrio parahaemolyticus (2ACA). Adapted from “Thiamine triphosphatase and the CYTH superfamily of proteins” by Bettendorff and Wins, 2013, FEBS Journal, 280, p. 6447. Copyright 2013 by John Wiley and Sons. Reprinted and adapted with permission.
To date, several TTMs have been identified and characterized, all possessing the unique catalytic tunnel and metal dependency. For example, a hypothetical protein from *Pyrococcus furiosus*, a putative adenylate cyclase from *Vibrio parahaemolyticus*, mimivirus capping enzyme (mimiCE), CthTTM from *Clostridium thermocellum*, and NeuTTM from *Nitrosomonas europaea* (Gong et al., 2006; Keppetipola et al., 2007; Benarroch et al., 2008; Delvaux et al., 2011). MimiCE was shown to be a trifunctional capping enzyme similar to that of vaccinia virus and poxvirus with additional Co\(^{2+}\)-dependent ATPase activity (Benarroch et al., 2008). CthTTM possesses tripolyphosphatase activity in the presence of Mn\(^{2+}\) or Mg\(^{2+}\), but strictly Mn\(^{2+}\)-dependent ATPase activity (Keppetipola et al., 2007). Similarly, NeuTTM also hydrolyzes tripolyphosphate in the presence of Mg\(^{2+}\), but was also shown to share CYTH-related properties, such as heat stability and an alkaline pH optimum (Delvaux et al., 2011). Thus, the ability of these proteins to act on different substrates suggests some level of versatility to the TTM catalytic tunnel in accommodating different phosphate species.

### 1.1.5 TTMs in plants

Thus far, only one plant TTM, *AtTTM3*, has been characterized (Moeder et al., 2013). Therefore, it is of great interest to examine the biological role of other plant TTMs. There are three TTMs in *Arabidopsis thaliana*, called *AtTTM1* (At1g73980), *AtTTM2* (At1g26190), and *AtTTM3* (At2g11890). *AtTTM3* is comprised solely of a CYTH domain, possessing the conserved ExExK motif as well as all other canonical acidic and basic residues (Moeder et al., 2013). It exhibits strong tripolyphosphatase activity in the presence of Mg\(^{2+}\) as well as some CYTH-related properties, such as an alkaline pH optimum between 9 and 10 and a higher optimal temperature of 45°C. Furthermore, crystal structure of *AtTTM3* also reveals the characteristic TTM tunnel (Fig. 1-1). Interestingly, *AtTTM3* is expressed in the root meristem and root primordia and null mutants of *AtTTM3* exhibit shorter roots and fewer lateral roots than wild type, suggesting a role for *AtTTM3* in root development.

While a CYTH domain is also present in *AtTTM1* and *AtTTM2*, these proteins also possess an N-terminal P-loop kinase domain. This rare fusion of domains appears to only be present in CYTH proteins that are missing canonical residues for substrate and metal binding (Iyer and Aravind, 2002). *AtTTM1* and *AtTTM2* orthologs can be found...
in almost all plant species. They display a high degree of amino acid sequence similarity (ranging between 52 – 94% identity) and also fall into a TTM1 clade and a TTM2 clade, suggesting functional conservation across species (Fig. 5-2; Table 5-2; Phytozome, Goodstein et al., 2012). Until recently, nothing was known about plant TTMs regarding their function or biological roles (Moeder et al., 2013). However, public microarray data from the Bio-Analytic Resource (BAR, Winter et al., 2007) reveals significant expression changes for \textit{AtTTM1} and \textit{AtTTM2} during abiotic and biotic stress, implicating a role for these genes in senescence and pathogen defense specifically.

\subsection{1.2 The plant immune system}

Pathogens have evolved diverse life strategies over their plant hosts. For example, bacteria can enter natural openings (stomata or hydathodes) and wounds to proliferate in the apoplastic space between plant cells, nematodes and insects use a stylet that directly pierces the cell, and fungi and oomycetes can parasitize the plant by forming an appressorium, which penetrates the cell wall by turgor pressure (Dou and Zhou, 2012). Since plants are sessile and lack mobile immune cells, the need for an adaptive and sensitive immune system is crucial for plant survival.

The plant immune system has been extensively studied over the last several decades. In the early 20\textsuperscript{th} century, field studies with flax (\textit{Linum usitatissimum}) and the flax rust fungus, \textit{Melampsora lini}, provided the early framework for our understanding of plant immunity today. This work was pioneered by Harold Henry Flor, who proposed that every gene that conditions a resistance response in plant hosts has a corresponding gene in the pathogen that conditions pathogenicity. This theory was based on the observation that varieties of flax displayed varying degrees of resistance or susceptibility to different races of flax rust fungus, and that these traits could be governed by Mendelian genetics, as shown through breeding studies. Flor’s work was first published in 1942 as the gene-for-gene hypothesis, which was later reviewed in 1971 (Flor, 1971). Today, the gene-for-gene hypothesis provides the basis for our current view on pathogen perception by plants and how these interactions can dynamically change over time.
In the lab, controlled studies of plant-pathogen interactions have been extensively conducted. This has led to the development of common pathosystems wherein experimental conditions and outcomes can be optimized and predicted, allowing for observations to be more easily interpreted. *Arabidopsis thaliana*, a dicot species of the Brassicaceae family, is a popular choice as a model plant for its short generation time (approximately one to two months), the availability of a sequenced genome, high seed production, and its ability to be easily transformed with *Agrobacterium tumefaciens*. Many ecotypes of *Arabidopsis thaliana* also exist owing to the wide range of terrains it can be found growing in; these include the frequently used Columbia (Col) and Wassilewskija (Ws) ecotypes. Together with the bacterial pathogen, *Pseudomonas syringae*, this pathosystem constitutes one of the most popular choices to probe the molecular events that occur upon infection. *P. syringae* requires a living plant host to survive, but infection eventually leads to host cell death. Thus, it is considered a hemibiotrophic pathogen (Xin and He, 2013). Biotrophic pathogens, such as the oomycete, *Hyaloperonospora arabidopsidis* (*Hpa*), require living tissue to survive and will attempt to maintain the uptake of nutrients without causing host cell death (Coates and Beynon, 2010). On the other hand, necrotrophic pathogens, such as fungi, thrive on dying or dead tissue. The successful colonization of virulent pathogens is called a compatible interaction, or disease susceptibility, leading to symptoms of leaf yellowing and brown-centered necrotic areas (Holub et al., 1993). In contrast, an incompatible interaction with an avirulent pathogen on the plant host can lead to disease resistance, sometimes resulting in a form of programmed cell death called the hypersensitive response (HR) (Jones and Dangl, 2006). This is an active and tightly regulated response that is mounted in order to wall off the pathogen at the site of entry to prevent further infection of neighbouring cells and manifests in the form of grey/silver patches on the leaf surface (Coll et al., 2011).

### 1.2.1 PAMP-triggered immunity (PTI)

Several works have indicated the existence of two layers of immunity in plants (Jones and Dangl, 2006). The first layer involves the recognition of conserved patterns on the surface of pathogens, called pathogen-associated molecular patterns (PAMPs), by plant pattern recognition receptors (PRRs). These PAMPs are highly conserved; for
instance, the flagellin protein of bacterial flagella or elongation factor-Tu (EF-Tu) of bacterial translation machinery can be recognized by different plant PRRs (Nicaise et al., 2009). Other examples include chitin from the cell walls of fungi and β-heptaglucan from the cell walls of oomycetes (Zipfel, 2014). By far, the most studied PAMP is flagellin, which is the protein subunit of the bacterial flagellum. It was determined that a 22-amino acid region of this protein, termed flg22, is the elicitor-active epitope sufficient to elicit defense responses in Arabidopsis (Felix et al., 1999; Zipfel et al., 2004). The plant PRR that determines the specificity of flg22 perception is FLAGELLIN SENSITIVE2 (FLS2), which is a leucine-rich repeat receptor kinase (LRR-RK) (Chinchilla et al., 2006). Activation of FLS2 by ligand binding also causes recruitment of and interaction with another LRR-RK called BRI1-ASSOCIATED KINASE1 (BAK1), which is also capable of binding flg22 at its C terminus (Chinchilla et al., 2007; Sun et al., 2013). The interaction of FLS2 and BAK1 triggers the transphosphorylation of each receptor kinase, activating a signaling pathway that includes a mitogen-activated protein kinase (MAPK) cascade, defense gene expression, reactive oxygen species (ROS) production, ion fluxes, hormone action, callose deposition, and stomatal closure (Fig. 1-2; Nicaise et al., 2009; Schulze et al., 2010).

PTI is fast acting and in most cases, sufficient to prevent further pathogen ingress (Nicaise et al., 2009). The MAPK cascade relays defense signals by protein phosphorylation in a successive manner. For instance, an unknown MAP kinase kinase kinase (MAPKKK) phosphorylates MAP kinase kinase 4 and 5 (MKK4, MKK5) in response to FLS2 activation, which in turn, phosphorylate MAP kinase 3 and 6 (MPK3, MPK6) to promote defense gene expression (Asai et al., 2002; Nicaise et al., 2009). An oxidative burst also occurs during PTI, resulting in the production of highly toxic ROS intermediates, such as superoxide anion and hydrogen peroxide, which can lead to strengthening of cell walls through cross-linking of glycoproteins, membrane damage through lipid peroxidation, and the transduction of signals that mediate defense activation (Lamb and Dixon, 1997; Torres et al., 2006). Further structural changes can also occur through the reinforcement of cell walls by callose deposition (Nicaise et al., 2009). Additionally, the closure of stomata through ion fluxes and hormone action prevents further pathogen entry.
Figure 1-2. Activation of PTI responses by flagellin perception.
Flagellin recognition by the FLS2 receptor induces the rapid phosphorylation of downstream targets leading to the activation of the MAPK cascade, ion fluxes, ROS production, and defense gene expression. Adapted from “Flagellin perception: a paradigm for innate immunity” by Gómez-Gómez and Boller, 2002, Trends in Plant Science, 7, p. 254. Copyright 2002 by Elsevier. Reprinted and adapted with permission.
PTI responses are effective against most invading pathogens, but not all. It has been known for some time that pathogens can produce a suite of effector proteins, which act to block PTI thereby subverting basal plant immunity. For example, the bacterial effectors from \textit{P. syringae} called AvrB and AvrRpt2 can evade defense responses by upregulating the expression of genes normally controlled by the phytohormones, jasmonic acid (JA) and auxin, which can inhibit defense responses to biotrophic pathogens (Dou and Zhou, 2012). Additionally, AvrB can induce the phosphorylation of RPM1-interacting protein4 (RIN4), a host membrane protein that can positively regulate the opening of stomata (Liu et al. 2009; Chung et al., 2011). Thus, it has been speculated that AvrB could enhance virulence by promoting stomatal opening to allow further pathogen entry. AvrRpt2 is a cysteine protease that can cause the cleavage of RIN4 at two sites called RIN4 cleavage site1 and 2 (RCS1 and RCS2) (Axtell and Staskawicz, 2003; Mackey et al., 2003; Kim et al., 2005). Interestingly, AvrRpt2-mediated cleavage of RIN4 at RCS2 results in two fragments – one that is degraded by the proteasome and another, which can prevent RPM1 activation by AvrB (Kim et al., 2005). Thus, secreted effectors can promote pathogen fitness by perturbing host defense responses in different manners.

1.2.2 Effector-triggered immunity (ETI)

The second layer of immunity involves the recognition of the actions of these effectors by the host, termed effector-triggered immunity (ETI). This is achieved by the presence of resistance proteins, products of \textit{R} genes, which monitor the effects of secreted pathogenic molecules within the host. The triggering of HR is often associated with the activation of ETI (Jones and Dangl, 2006). Interestingly, ETI also involves many of the same responses as PTI. For instance, ROS production occurs as a biphasic response to pathogen attack where an initial transient phase is activated by PTI in a low-amplitude manner (Lamb and Dixon, 1997; Torres et al., 2006). The second phase is sustained at a higher magnitude, occurring when ETI is activated. Another example is the role of hormones in both PTI and ETI, particularly the critical signaling hormone, salicylic acid (SA) (Gaffney et al., 1993; Delaney et al., 1994; Wildermuth et al., 2001). It was shown that the flg22-derived resistance in SA-deficient mutants was partially compromised, indicating a role for SA in PTI (Tsuda et al., 2009). In ETI, SA plays an
integral role in defense signaling, leading to the expression of \textit{pathogenesis-related} (\textit{PR})
genesis. There are 3 well-known \textit{PR} genes that are induced by SA signaling called \textit{PR1} (unknown function), \textit{PR2} (\textit{BGL2}; $\beta$-1,3-glucanase), and \textit{PR5} (thaumatin-like protein) (Uknes et al., 1992). Some of these \textit{PR} proteins (i.e. \textit{PR2}) can have a direct antimicrobial effect, breaking down the cell walls of pathogens. Another class of antimicrobials that is induced upon pathogen infection is the phytoalexins, such as camalexin (Glazebrook et al., 1996). These chemicals are actively synthesized at the site of pathogen entry to prevent further infection. Interestingly, the robustness of ETI has been demonstrated to be, in part, due to the prolonged effects of some of these responses, such as the prolonged duration of active MPK3 and 6 in the presence of an effector versus a PAMP (Tsuda and Katagiri, 2010).

Activation of ETI depends on the recognition of an avirulence (Avr) gene product (effector protein) by its cognate resistance protein. This has been shown through the characterization of several \textit{R-Avr} gene pairs, validating Flor's theory of gene-for-gene resistance. For example, AvrRpt2 from \textit{P. syringae} is recognized by its cognate \textit{R} protein, \textit{RESISTANCE TO P. SYRINGAE2} (RPS2) (Kunkel et al., 1993). It was found that Arabidopsis mutants of \textit{RPS2} lost resistance to \textit{P. syringae} strains specifically carrying \textit{AvrRpt2}, but not to strains carrying other avirulence genes, and that this phenotype was due to a single mutation in the gene encoding RPS2 (Kunkel et al., 1993). Furthermore, it was observed that RPS2 can physically interact with RIN4, likely to monitor the elimination of RIN4 and subsequently activate disease resistance (Axtell and Staskawicz, 2003; Mackey et al., 2003). Thus, rather than directly interacting with AvrRpt2, RPS2 recognizes the proteolytic activity of AvrRpt2 on RIN4 in order to initiate defense signaling. In the case of AvrB, it was determined that its cognate \textit{R} protein is \textit{RESISTANCE TO P. SYRINGAE PV. MACULICOLA1} (RPM1) (Grant et al., 1995). AvrB phosphorylates RIN4 to subvert plant defense responses; however, the phosphorylation of RIN4 activates RPM1 to induce resistance (Mackey et al., 2003; Chung et al., 2011). Another example of an \textit{R-Avr} gene pair is \textit{AvrRps4} from \textit{P. syringae pv. pisi} and \textit{resistance to P. syringae4} (\textit{RPS4}) (Hinsch and Staskawicz, 1996; Gassmann et al., 1999). A cosmid DNA library from \textit{P. syringae pv. pisi} was expressed in virulent \textit{P. syringae pv. tomato (Pst)} DC3000 and disease resistance was observed in Arabidopsis by a particular cosmid expressing \textit{AvrRps4}. It was then determined that a single locus, \textit{RPS4}, was responsible for conferring resistance to \textit{Pst} carrying \textit{AvrRps4} (Hinsch and
Thus, host fitness can be regained when it can recognize the actions of such effectors.

### 1.2.3 Defense signaling

Microarray analyses have shed light on the transcriptional changes that occur during PAMP perception and effector recognition by the host. For example, the effects of flagellin perception in Arabidopsis were studied by comparing the changes in gene expression between wild type and the flg22 receptor mutant, \textit{fis2}, when treated with flg22. Approximately 1,000 genes were upregulated and over 200 genes were downregulated 30 minutes after treatment in wild type plants (Zipfel et al., 2004). Interestingly, minor changes in only 6 genes were observed in \textit{fis2} plants, suggesting that the transcriptional changes were specifically triggered by flg22 perception. Among the genes that were significantly upregulated were \textit{FLS2}, \textit{MAP/ERK kinase1 (MEKK1)}, and \textit{M KK4}. While the immediate events downstream of PAMP recognition are not yet known, it is clear that signaling through phosphorylation events plays an important role as a means of controlling protein activity. For example, the MAPK cascade can play a positive role in PTI through the MAPKKK (unknown)-MKK4/5-MPK3/6 pathway (Asai et al., 2002; Nicaise et al., 2009). In contrast, the MAPK cascade mediated by MEKK1-MKK1/2-MPK4 seems to act as a negative regulator in PTI (Suarez-Rodriguez et al., 2007; Nicaise et al., 2009).

A similar study examined the transcriptional changes that occurred in Arabidopsis seedlings in response to flg22 30 minutes after treatment and observed the altered expression of over 250 genes (Navarro et al., 2004). Of those genes that were upregulated, most were found to encode signaling components, such as transcription factors, protein kinases and phosphatases, and proteins involved in protein turnover. Interestingly, approximately 70% of these induced genes were also upregulated even when pretreated with cycloheximide, a protein synthesis inhibitor, before flg22 treatment, suggesting that the majority of these genes are negatively regulated by rapidly turned-over repressor proteins (Navarro et al., 2004).

A significant overlap in the transcriptional changes that occurred from flg22 elicitation and from effector recognition was also observed. Using already existing datasets collected from Arabidopsis at 3 hours post inoculation (hpi) with \textit{Pst} expressing
AvrRpt2 or AvrB and normalized against expression data from virulent Pst infection, it was discovered that approximately 45% of the genes that were significantly altered after flg22 treatment were also induced by AvrRpt2 or AvrB recognition (Navarro et al., 2004). These results suggest that both effector recognition and PAMP perception may trigger expression of similar subsets of genes.

Signaling hormones, such as SA, also play critical roles in mediating defense responses (Gaffney et al., 1993; Delaney et al., 1994; Wildermuth et al., 2001). While there is great complexity in the crosstalk between different hormones, it is generally regarded that resistance to biotrophic or hemibiotrophic pathogens is mediated by the SA signaling pathway whereas resistance against necrotrophic pathogens is mediated by the JA/ethylene pathways (Tsuda and Katagiri, 2010). Over the last decade, core components of the ETI pathway involving SA were determined: enhanced disease susceptibility1 (EDS1), phytoalexin-deficient4 (PAD4), nonrace-specific disease resistance1 (NDR1), isochorismate synthase1 (ICS1), and nonexpresser of pathogenesis-related1 (NPR1).

Two branches of ETI are mediated by either EDS1/PAD4 or NDR1 and are generally determined by the class of R gene in action (Fig. 1-3; Aarts et al., 1998). Distinct classes of R genes exist on the basis of tertiary structure where the largest is the nucleotide binding site leucine-rich repeat (NBS-LRR) class. This class comprises two subgroups, each defined by the motif present at the N terminus; either a Toll/interleukin-1 receptor (TIR) motif or a coiled coil (CC) motif, both of which are important for protein-protein interactions. Interestingly, R genes (i.e. RPS2, RPM1) recognizing bacterial effectors from P. syringae belong to the CC-NBS-LRR subclass with the exception of RPS4, which belongs to the TIR-NBS-LRR subclass (McHale et al., 2006). In contrast, R genes (i.e. RPP1, RPP4, RPP10, RPP14, RPP31) recognizing oomycetal effectors from Hpa belong to the TIR-NBS-LRR subclass with the exception of RPP27, which belongs to the CC-NBS-LRR class (McDowell et al., 2005). The dominance of an R gene subgroup existing in one pathogen over another may reflect some level of selection as host-pathogen relationships became more specialized over time.
Figure 1-3. Signal transduction during ETI responses.
It was determined that signals transduced by the TIR class of NBS-LRR \( R \) genes are mediated by \textit{EDS1/PAD4} (Aarts et al., 1998). \textit{EDS1} was first isolated in a mutagenesis screen induced by ethyl methanesulfonate (EMS) on Arabidopsis Ws ecotype for enhanced susceptibility to the avirulent \textit{Hpa} isolate, Noco2 (Parker et al., 1996). \textit{eds1-1} plants were also more susceptible to several other \textit{Hpa} isolates recognized by the TIR-NBS-LRR class of \( R \) proteins, but interestingly, not to \textit{Pst} carrying \textit{AvrB}, which is recognized by the CC-NBS-LRR \( R \) gene, \textit{RPM1} (Parker et al., 1996). \textit{eds1-1} was determined to be caused by a point mutation in a gene encoding a putative lipase (Parker et al., 1996; Falk et al., 1999). While the function of \textit{EDS1} remains to be solved, it is clear that it responds to the TIR-NBS-LRR class of \( R \) proteins, functions upstream of SA-dependent \textit{PR1} transcript accumulation, and is not required for JA-mediated responses (Falk et al., 1999). For example, \textit{RPS4}, which recognizes bacterial AvrRps4, signals through \textit{EDS1}, revealing specificity for \textit{EDS1} beyond \textit{Hpa} recognition (Aarts et al., 1998).

\textit{EDS1} function in plant defense is partially dependent on the lipase-like protein, \textit{PAD4} (Feys et al., 2001). \textit{PAD4} was identified in a mutagenesis screen induced by EMS on Arabidopsis Col ecotype for enhanced susceptibility to \textit{P. syringae} pv. \textit{maculicola} (\textit{Psm}) ES4326 (Glazebrook et al., 1996). \textit{pad4-1 (eds9)} was determined to be a point mutation, exhibiting enhanced susceptibility to \textit{Hpa} in addition to \textit{Psm} and reduced camalexin, SA, and \textit{PR1} levels (Glazebrook et al., 1996). Interestingly, \textit{pad4-1} shows reduced camalexin levels in response to \textit{Psm} ES4326, but not by infection with the ascomycete, \textit{Cochliobolus carbonum}, suggesting that \textit{PAD4} plays a regulatory role rather than one in biosynthesis of phytoalexins (Glazebrook et al., 1996; Glazebrook et al., 1997). It is thought that \textit{EDS1} functions both independently of \textit{PAD4} to promote early defense responses and HR formation, and with \textit{PAD4} to promote SA accumulation for a prolonged resistance response (Feys et al., 2001).

\textit{EDS1} was shown to interact with \textit{PAD4}, but also with \textit{SENESCENCE-ASSOCIATED GENE101} (\textit{SAG101}) to promote defense responses (Feys et al., 2001; Feys et al., 2005). It was determined that the \textit{EDS1-SAG101} complex occurs in the nucleus, which is different from the \textit{EDS1-PAD4} complex that exists in the nucleus and cytoplasm (Feys et al., 2005). Furthermore, that the combined activities of \textit{PAD4} and \textit{SAG101} are required for TIR-NBS-LRR \( R \) gene-mediated resistance to avirulent pathogens. Combined, these data indicate that dynamic \textit{EDS1} interactions in different
compartments are important for defense signaling. Indeed, it was determined that a nuclear pool of EDS1 is essential for transcriptional reprogramming in response to infection by biotrophic and hemibiotrophic pathogens and that the equilibration of nuclear and cytoplasmic pools of EDS1 is required for complete resistance (García et al., 2010).

Where EDS1/PAD4 mediates signals specified by TIR-NBS-LRR R proteins, NDR1 mediates resistance in response to signals transduced by CC-NBS-LRR R proteins. NDR1 was first isolated in a mutagenesis screen induced by fast neutron deletion in Arabidopsis Col ecotype for enhanced susceptibility to Pst DC3000 carrying AvrB (Century et al., 1995). ndr1-1 is a deletion mutant that exhibits enhanced susceptibility to several avirulent Pst strains and Hpa isolates (Century et al., 1995). NDR1 is post-translationally modified with a C-terminal glycosylphosphatidyl-inositol (GPI) anchor, suggesting its localization to the outer surface of the plasma membrane (Coppinger et al., 2004). Interestingly, it was shown that the N-terminal portion of NDR1 can interact with RIN4, and that this interaction is required for RPS2-mediated resistance against P. syringae expressing AvrRpt2 (Day et al., 2006). Furthermore, NDR1 possesses specific protein motifs that are found in mammalian integrins, which are involved in cell adhesion and signaling (Knepper et al., 2011). Thus, in addition to mediating R gene-specific signals, NDR1 is also thought to maintain the integrity of plasma membrane-cell wall adhesions.

While specificity exists for the initiation of ETI, signal transduction eventually converges onto the biosynthesis of SA. One of the most important breakthroughs to occur in the last two decades was the discovery of one of the biosynthetic genes for SA, ICS1 (Wildermuth et al., 2001). There are two proposed pathways for the biosynthesis of SA in plants. One pathway involves the enzyme phenylalanine ammonia lyase (PAL), which converts phenylalanine into the immediate SA precursor, benzoic acid. The other pathway involves ICS1, which encodes isochorismate synthase, converting chorismate into the immediate SA precursor, isochorismate. SA accumulates in both local tissue and systemic tissue after pathogen infection, and has shown to be required for defense responses (Gaffney et al., 1993; Vernooij et al., 1994; Wildermuth et al., 2001). A mutant of ICS1, SA induction-deficient2 (sid2), was identified in a mutagenesis screen induced by EMS in Arabidopsis Col ecotype for altered levels of total SA after infection with avirulent Pst carrying AvrRpm1 (Nawrath and Métraux, 1999). Another mutant of ICS1 was later identified as eds16 from a separate screen (Dewdney et al., 2000). sid2-1 is
caused by a point mutation, exhibiting enhanced susceptibility to virulent \textit{Hpa} and \textit{Pst} and significantly reduced levels of the pathogen marker, \textit{PR1}, but not \textit{PR2} or \textit{PR5}, suggesting that SA plays a critical role in signaling defense responses (Nawrath and Métraux, 1999). The interesting observation that only \textit{PR1} levels were altered, but not \textit{PR2} and \textit{PR5} suggest that there must be an independent pathway of induction for \textit{PR2} and \textit{PR5} from \textit{ICS1}. This also indicates that \textit{ICS1}-derived SA production leading to \textit{PR1} accumulation plays a major role in limiting pathogen growth. Indeed, it was shown that SA produced by the ICS pathway is critical for induction of \textit{PR1} and systemic defense responses whereas SA produced by the PAL pathway seems to be important for the induction of HR against pathogen invasion (Mauch-Mani and Slusarenko, 1996; Huang et al., 2010).

The involvement of \textit{PR} genes in pathogen defense has long been known where they accumulate in local tissue after pathogen infection to act as antimicrobials (Uknes et al., 1992; Cao et al., 1994; Durrant and Dong, 2004). While the function of \textit{PR1} is currently unknown, many studies still employ the use of \textit{PR1} as a reliable marker for defense activation. In the 1990s, three independent research groups discovered the gene responsible for regulating the expression of \textit{PR1}, called \textit{NPR1}, \textit{noninducible immunity1 (NIM1)}, or \textit{SA-insensitive1 (SAI1)}, though \textit{NPR1} remains the common name (Cao et al., 1994; Delaney et al., 1995; Shah et al., 1997). \textit{NPR1} was isolated in a mutagenesis screen induced by EMS in transgenic Arabidopsis Col ecotype plants expressing $\beta$-glucuronidase (GUS) under the \textit{PR2} promoter for insensitivity to SA or 2,6-dichloroisonicotinic acid (INA) (Cao et al., 1994). Out of several lines, only one line showed a dramatic decrease in GUS activity and \textit{PR1}, \textit{PR2}, and \textit{PR5} expression. This line was determined to be caused by a point mutation in the \textit{NPR1} locus. \textit{npr1-1} plants also exhibited enhanced susceptibility to \textit{Psm ES4326} and displayed defective systemic defense responses. These data suggest that SA, INA, and the activation of systemic defense responses share a common pathway mediated by \textit{NPR1}. Interestingly, \textit{EDS1}, \textit{PAD4}, and \textit{NPR1} are all transcriptionally induced as rapidly as 30 minutes after treatment with flg22, suggesting that they also function during PTI responses (Zipfel et al., 2004).

Recently, a rather elegant study was conducted to explore the network properties of PTI and ETI through bacterial growth curve assays (Tsuda et al., 2009). Pretreatment of plants with flg22 followed by infection with virulent \textit{Pst} DC3000 revealed that
approximately 80% of the PTI signaling network is defined by delayed dehiscence 2 (DDE2), ethylene insensitive 2 (EIN2), ICS1, and PAD4. Where single mutants of the above genes exhibited varying, but largely significant levels of resistance due to flg22 pretreatment, the quadruple mutant, dde2/ein2/sid2/pad4, exhibited only 20% difference in bacterial growth between mock-treated and flg22-treated plants. Signaling allocation analysis was also conducted to determine the output of various networks by comparing the effects of all genes on growth curve analysis. Interestingly, it was discovered that part of the robustness of ETI is due to compensation of function between signaling sectors, particularly those controlled by PAD4 and ICS1 in the case of AvrRpt2. In other words, PAD4 can compensate for the loss of the SA signaling sector mediated by ICS1. On the other hand, PTI involves the synergistic actions of these signaling sectors. For example, there exists a positive feedback loop comprising PAD4 and ICS1 that maintains an SA signal during flg22-mediated PTI. While the network explored by this study is small, it highlights the ability of the plant to bolster immune responses by utilizing the same networks in different manners.

1.2.4 Systemic acquired resistance (SAR)

In addition to PTI and ETI responses, plants possess the ability to mount a systemic defense response, termed systemic acquired resistance (SAR). This level of defense is induced upon activation of ETI against an avirulent pathogen. As a result, long distance signaling of potentially several mobile signals occurs to protect systemic tissues against further pathogen infection. SAR is considered to be long lasting and effective against a broad spectrum of pathogens, including viruses, bacteria, fungi, and oomycetes (Durrant and Dong, 2004). Therefore, plants that have been infected with an avirulent pathogen will exhibit fewer disease symptoms upon subsequent infection with a virulent pathogen, and this effect may last up to the lifetime of the plant.

Like with ETI, SA has been shown to be necessary for the establishment of SAR. Early studies were conducted using tobacco (Nicotiana tabacum) expressing the salicylate hydroxylase (NahG) gene from Pseudomonas putida driven by a constitutive cauliflower mosaic virus 35S (CaMV 35S) promoter. Salicylate hydroxylase converts SA to catechol, rendering SA biologically inactive, and making it a useful tool for the study of the effects of SA. The leaves of tobacco plants exhibit lesions in response to infection
with tobacco mosaic virus (TMV) where the size of the lesions reflects the extent of viral spread. It was observed that challenge infection of previously infected tobacco plants resulted in smaller lesions than challenge infection of mock-treated plants (Gaffney et al., 1993). This SAR effect was abolished if the plants expressed *NahG*, indicating that SA plays a critical role in establishing SAR.

The importance of SA in the establishment of SAR combined with the detection of SA in both local and systemic tissue spurred the belief that it could be the mobile signal (Métraux et al., 1990; Vernooij et al., 1994). However, this was shown not to be the case in a classical grafting experiment involving transgenic tobacco expressing *NahG*. Reciprocal grafts of *NahG*-expressing rootstocks (lower half; primary infection) with wild type scions (upper half; challenge infection) or wild type rootstocks with *NahG*-expressing scions showed that SAR could only be established if scions were wild type regardless of the rootstock (Vernooij et al., 1994). These results suggested that SA is required for the establishment of SAR, but is not the mobile signal. Since then, several candidates for mobile signals have since been postulated, including DEFECTIVE IN INDUCED RESISTANCE1 (DIR1), methyl salicylate (MeSA), glycerol-3-phosphate (G3P), azelaic acid (AzA), pimelic acid (Pip), JA, and dehydroabietinal (DA) (Durrant and Dong, 2004; Dempsey and Klessig, 2012; Shah and Zeier, 2013). However, it appears that rather than relying on one mobile signal, SAR is established by the concerted perception and integration of many signals.

The ability of plants to resist further pathogen attack subsequent to initial infection prompted the research of chemical inducers of SAR other than SA. One of the most common inducers is the biological SA analog, benzothiadiazole (BTH). Using tobacco plants, BTH was found to act either at the site or downstream of SA in the defense pathway, since BTH application did not cause SA accumulation (Friedrich et al., 1996). Additionally, BTH could induce disease resistance and SAR as well as the accumulation of *PR1*. A similar study in Arabidopsis showed BTH to be a potent activator of SAR against turnip crinkle virus, *Pst*, and *Hpa* (Lawton et al., 1996). Furthermore, BTH could induce the expression of *PR1*, *PR2*, and *PR5* where *PR1* induction and resistance could be seen even in Arabidopsis plants expressing *NahG*, indicating that BTH does not require SA accumulation. However, BTH does require *NPR1* to activate SAR, since *PR1* induction and resistance could not be triggered by BTH application in *nim1* plants. While BTH can mimic many of the effects of SA related to SAR, it has been
shown that it cannot mimic other effects of SA outside of defense responses, such as water loss (Moeder et al., 2010). Still, BTH continues to be used as a common SA analog among others, such as INA and 5-chloro-SA, in the study of defense responses (Durrant and Dong, 2004).

1.3 Model pathosystems

The field of plant-pathogen interactions has dramatically expanded over the last two decades. We now have a better understanding of the molecular events that occur not only at the interface between host and pathogen, but also within the host and the signaling events that occur. Without model pathosystems, the advancement of this field would not be possible. In this thesis, the extensive use of *Arabidopsis thaliana* with the biotrophic oomycete, *Hyaloperonospora arabidopsis*, and the hemibiotrophic bacterium, *Pseudomonas syringae*, were used.

1.3.1 *Hyaloperonospora arabidopsis* as a model pathogen

*Hyaloperonospora arabidopsis* was formerly known as *Hyaloperonospora parasitica* (formerly *Peronospora parasitica*). This pathogen is the causal agent of downy mildew and belongs to the Oomycota, which belong to the kingdom Chromalveolata. Oomycetes are not true fungi – their vegetative bodies are diploid and their cell walls are primarily made of cellulose, not chitin. In addition, they are obligate biotrophs and as such, must be continually propagated on living tissue in cool and moist conditions (Coates and Beynon, 2010).

The life cycle of *Hpa* progresses through asexual and sexual states. When an asexual conidiospore lands on leaf tissue, it germinates and forms an appressorium, which rests above epidermal cells. A penetration hypha then emerges from the appressorium, penetrating the host cell wall and eventually forming a haustorium. The formation of this structure leaves the host plasma membrane intact and acts as the site of nutrient uptake by the pathogen. More hyphae and haustoria are subsequently generated across mesophyll cells until asexual reproductive structures called sporangiophores (conidiophores) are formed, which house the conidiospores (Coates and Beynon, 2010). Sexual reproduction occurs when hyphae intertwine to form
antheridia and oogonia, which eventually results in the formation of the oosphere and the oospores that lay inside it. Completion of this life cycle can take between 5 – 7 days depending on the host genotype and *Hpa* isolate (Fig. 1-4).
Figure 1-4. Life cycle of *Hyaloperonospora arabidopsidis* (*Hpa*).

Shown is a cross-section of a leaf infected with *Hpa*. A compatible interaction is depicted on the left-hand side where *Hpa* progresses through its life cycle. An incompatible interaction is depicted on the right-hand side where *Hpa* ingress ceases at the first layer of mesophyll cells. a, asexual spore; b, short germ tube; c, appressorium; d, haustorium; e, sporangiophore. Adapted from “*Hyaloperonospora arabidopsidis* as a pathogen model” by Coates and Beynon, 2010, Annual Review of Phytopathology, 48, p. 332. Copyright 2010 by Coates and Beynon. Reprinted and adapted with permission.
There exist both compatible and incompatible isolates of *Hpa* where the outcome of infection depends largely on the host genotype (Holub et al., 1993). For example, Noco2 is a widely used isolate that is virulent on the Arabidopsis Col ecotype, but is avirulent on the Ws ecotype. The resistance exhibited by Ws was determined to be a result of the complex *R* gene locus *RPP1*, which comprises *RPP1*, *RPP10*, and *RPP14* (Reignault et al., 1996; Botella et al., 1998). *Hpa* isolate Emco5 is virulent on both Col and Ws ecotype, but Col exhibits developmentally regulated resistance at later stages. This resistance was determined to be a result of the *RPP31* locus (McDowell et al., 2005). In contrast, *Hpa* isolate Emwa1 is avirulent on Col ecotype, but is virulent on Ws ecotype. The resistance of Col was determined to be conferred by the *RPP4* locus (van der Biezen et al., 2002). The products of these *R* gene loci directly or indirectly recognize the products of *A. thaliana recognized (ATR)* genes (avirulence genes) produced by *Hpa* (Hein et al., 2009).

The outcome of such interactions manifests itself in different forms. In the case of a compatible interaction, susceptibility is manifested by the expansion of hyphae, followed by sporangiophore formation, oospore production, and restriction of plant growth (Holub et al., 1993). Incompatible interactions result in necrotic flecks (from regions of penetrated host cells), programmed cell death as a result of the activation of HR, and sometimes limited hyphae growth (remnants of unsuccessful colonization).

### 1.3.2 *Pseudomonas syringae pv. tomato* as a model pathogen

*Pseudomonas syringae* is a gram-negative bacterial pathogen that causes bacterial blight on different hosts. The tomato pathovar (*Pst*) is capable of infecting both tomato and Arabidopsis, and is one of several widely used pathovars (pv.) of *P. syringae*, such as pv. *phaseolicola* (*Psp*), pv. *maculicola* (*Psm*), or pv. *glycinea* (*Psg*) (Xin and He, 2013). *Pst* causes bacterial speck disease in tomato plants, manifesting itself as small necrotic flecks on the leaves and fruit of the plant. Arabidopsis leaves infected with *Pst* show disease symptoms of localized necrosis along with diffuse chlorosis. Strict biotrophic pathogens obtain nutrients from living cells without causing host cell death. However, *Pst* eventually causes extensive necrosis of host tissue at later stages of infection. Therefore, it is regarded as a hemibiotroph.
*Pst* multiplies in the apoplastic space where it can form a syringe-like supramolecular complex that is capable of piercing through the cell wall and plasma membrane of the host cell (Xin and He, 2013). This complex is called the Type III secretion system, which delivers a suite of effectors into the plant cell, comprising a crucial tool for virulence and survival of this pathogen. While several strains of *Pst* are available, the most widely used is *Pst* DC3000. This strain was originally isolated by Dr. Cuppels for its ability to easily accept foreign DNA as well as being pathogenic (Cuppels, 1986). *Pst* DC3000 has since been used as a surrogate strain for the study of the virulence function of several effectors by expressing individual effector genes from other pathovars, such as *AvrRpt2*, *AvrB*, and *AvrRps4* (Xin and He, 2013).

### 1.4 The impact of defense activation on development

The result of effective deployment of defense responses is resistance. However, this can be costly to the plant, resulting in significant growth inhibition (Heidel et al., 2004; Spoel et al., 2007). This highlights the energy balance that must be achieved by the plant when responding to stress stimuli as well as growth and developmental cues.

One class of mutants that exhibits such defects is called autoimmune mutants. They are mutants that display constitutive activation of defense responses, such as elevated SA accumulation and elevated *PR* gene expression. Frequently, these mutants also display spontaneous cell death formation without pathogen infection. These mutants belong to a subclass of autoimmune mutants, called lesion mimic mutants (Lorrain et al., 2003; Moeder and Yoshioka, 2008). Examples of these mutants are *constitutive expresser of PR genes*22 (*cpr22*), *defense no death*1 (*dnd1*), and *suppressor of SA-insensitive*4 (*ssi4*) (Yoshioka et al., 2001; Yu et al., 1998; Clough et al., 2000; Moeder and Yoshioka, 2008). While these autoimmune mutants show aberrant signaling at different points of the defense network, they all exhibit stunted growth, varying degrees of leaf curliness, and yellowing around leaf edges, similar to senescence-like programmed cell death (Yoshioka et al., 2001; Lorrain et al., 2003).

Indeed, one autoimmune mutant that exhibits early leaf senescence as well as constitutively active pathogen defense responses in Arabidopsis is called *hypersenescence*1 (*hys1*) or *constitutive expresser of PR genes*5 (*cpr5*) (Kirik et al., 2001; Yoshida et al., 2002). *hys1-1* shows rapid onset of senescence with leaves
yellowing earlier than wild type, which is correlated to the early appearance of senescence marker genes (Yoshida et al., 2002). While the function of HYS1 in regulating senescence and pathogen defense remains to be solved, it is believed that HYS1 might participate in the hexokinase-dependent sugar sensing pathway, since hys1-1 plants exhibit hypersensitivity to glucose. Interestingly, disruption of hexokinase1 (HXK1) can partially suppress this glucose hypersensitivity, but acts synergistically with the hys1 mutation towards elevated SA levels (Aki et al., 2007). As a result, it was proposed that HYS1 and HXK1 both act to repress SA accumulation while HYS1 also represses senescence.

1.5  Leaf senescence

Senescence is an active process whereby nutrients are mobilized from older parts of the plant to actively growing tissue. This process can also be induced by a variety of external factors, such as pathogen infection, darkness, drought, and exogenous hormone application (Lohman et al., 1994; Weaver et al., 1998). In the absence of external stress, senescence is activated in an age-dependent manner, termed natural senescence. Macroscopically, the typical symptom of a plant undergoing senescence is patterned leaf yellowing due to the loss of chlorophyll, since chloroplasts are the first organelle to be disassembled (Woolhouse, 1984; Lohman et al., 1994). On the cellular level, dismantling of organelles through the degradation of membranes, proteins, lipids, and nucleic acids also occurs to actively recycle these nutrients to other growing tissues (Lohman et al., 1994). The result of this is yellowing of the leaf starting from the tip and outer edges towards the vasculature and petiole. Thus, senescence is a highly regulated process that occurs not only within each cell, but also across an individual leaf due to the concerted responses of many cells (Fig. 1-5).
Figure 1-5. Numerical labeling of leaves within the Arabidopsis rosette.

To facilitate the study of leaf senescence, individual leaves are numbered according to age beginning with the first true leaves. Adapted from “Identification of a transcription factor specifically expressed at the onset of senescence” by Hinderhofer and Zentgraf, 2001, Planta, 213, p. 470. Copyright 2001 by Springer-Verlag. Reprinted and adapted with permission.
Leaf senescence refers to the responses that occur across an individual leaf rather than the entire plant. Since natural senescence is activated in an age-dependent manner, there are varying degrees of senescence occurring in all leaves after senescence is activated in the first leaf in a plant. Thus, it can be difficult to study the molecular aspects of senescence when such a high degree of variation exists across the entire plant. To overcome these difficulties, external stressors, such as darkness or exogenous hormone application, have been used to induce senescence, as they have been shown to induce a more rapid and uniform response (Quirino et al., 2000).

1.5.1 The identification of senescence markers

In the last two decades, several genes have been identified that are thought to play a role in senescence because of their transcriptional responses to various treatments that induce senescence (Lohman et al., 1994; Weaver et al., 1998; Quirino et al., 2000). A cDNA library screen comprising mRNA from senescing leaves of Arabidopsis was conducted and identified a group of genes, called senescence-associated genes (SAGs), which were upregulated compared to nonsenescent tissue (Lohman et al., 1994). The expression of these SAGs was then monitored over several leaves according to their stage, ranging from nonsenescent to varying levels of senescence. Most SAGs were shown to gradually increase with more senescent leaves with low levels of transcript detected in nonsenescent leaves as well. Among these were SAG13, 14, and 17. Interestingly, SAG12 also gradually increased with older leaves, but was not detected in nonsenescent leaves (Lohman et al., 1994). A similar study was conducted, but experimentally devised so that the same leaf from a plant was sampled over time rather than sampling multiple leaves at different stages of senescence in a single plant (Weaver et al., 1998). Effectively, this allowed researchers to monitor age-dependent senescence rather than stage-dependent senescence. For example, SAG13 expression increased in aging plants similarly to stage-dependent senescence, but its induction was more clearly defined to occur approximately 2 to 5 days prior to visible symptoms of senescence. Interestingly, SAG12 also behaved similarly in aging plants to stage-dependent senescence with transcripts only detectable when visible symptoms of senescence had occurred. Due to the reliability of the induction of these genes, SAG12 has become a notable senescence-specific marker and SAG13 is also widely used as
an early marker for senescence (Lohman et al., 1994; Weaver et al., 1998; Quirino et al., 2000).

While the function of most SAGs is currently unknown, it is believed that they play a wide variety of roles up to and during senescence based on their promoter sequences and the putative enzymes that they encode (Quirino et al., 2000). For example, through promoter deletion analysis, it was determined that the promoter of SAG12 contains a highly conserved region that is responsible for its specificity for senescence (Noh and Amasino, 1999). Furthermore, through gel shift assays, it was found that this senescence-specific promoter element is differentially bound by transcription factors from young leaves versus senescent leaves. This promoter element was not similar to any known consensus binding sequences of transcription factors, indicating that the senescence-specific regulation of SAG12 may involve a new class of factors. This indicates the possibility of a transcriptional repressor that is modified upon the induction of senescence or simply, that different complexes are formed in an age-dependent manner. Interestingly, SAG12 encodes a cysteine protease that was found to accumulate in senescence-associated vacuoles (SAVs), which only form during senescence in guard and mesophyll cells of Arabidopsis and soybean (Otegui et al., 2005). It is hypothesized that SAG12 may be involved in the hydrolysis of chloroplast-derived molecules since SAVs are only present in chloroplast-containing leaf cells.

In contrast, not much is known regarding SAG13. It encodes a putative tropinone reductase, which is an enzyme that is known to be involved in alkaloid biosynthesis. A study of the promoter of SAG13 from Arabidopsis showed that its promoter activity in tomato is similar to that in Arabidopsis (Swartzberg et al., 2006). In both plant species, the SAG13 promoter exhibited some level of senescence-independent expression and stronger senescence-dependent expression, which was found to be regulated by the phytohormone, cytokinin.

1.5.2 A comprehensive view into a complex process

cDNA library screens that aided in the search for SAGs and consequently, senescence-downregulated genes (SDGs), were able to provide reliable markers for senescence (Lohman et al., 1994). However, what remains unsolved is the global regulation of processes that contributes to the senescence program. To study this, a
transcriptome analysis was conducted from samples taken from the same leaf of aging plants (Breeze et al., 2011). By comparing the transcription of many genes over a time course of senescence development, many patterns can be observed (Breeze et al., 2011; Penfold and Buchanan-Wollaston, 2014). For example, in leaves harvested between 19 – 23 days after sowing (DAS), there is induction of genes involved in autophagic transport, JA and abscisic acid (ABA) signaling, and responses to ROS whereas a downregulation of genes involved in amino acid metabolism and chlorophyll biosynthesis occurs (Breeze et al., 2011). There is induction of genes involved in caspase and esterase activities as well as DNA and protein binding and a downregulation of genes involved in carbon utilization and photosynthesis in leaves harvested between 25 – 31 DAS. Lastly, leaves harvested between 33 – 39 DAS exhibited induction of genes involved in catalytic activity, transport, and lipid metabolism. Interestingly, photosynthesis-related genes are also maintained even in senescing leaves, presumably to keep up with the energy demands of an active recycling process.

As mentioned above, many studies on leaf senescence are conducted using dark treatment or phytohormone application to induce rapid and consistent responses across the leaf. Interestingly, it appears that the molecular events that occur between natural senescence and dark-induced senescence in detached leaves are overlapping, but not identical (Buchanan-Wollaston et al., 2005; van der Graaff et al., 2006). Through transcriptome analysis, a consistent 3-fold upregulation of 827 genes was observed in senescent leaves compared to nonsenescent leaves. Of these genes, approximately 53% were also upregulated in dark-induced senescent leaves (Buchanan-Wollaston et al., 2005). Many of these genes either have putative or determined roles in macromolecule degradation, carbohydrate metabolism, membrane transport, secondary metabolism, and autophagy. Interestingly, 34% of the 827 genes were not upregulated at all, suggesting that there are molecular differences between natural and dark-induced senescence. In a similar study, 2,000 genes were observed to be upregulated during natural senescence whereas just over 1,000 genes were induced during dark-induced senescence (van der Graaff et al., 2006). Of these, approximately 800 genes are shared between both treatments, indicating that these genes may constitute a core senescence pathway of components required for the execution of senescence.
My doctoral thesis was carried out on the basis of two main objectives:

1) Identify and characterize TTM family members from *Arabidopsis thaliana*
2) Characterize the biochemical properties of these TTM family members

As mentioned above, there are three TTM members in *Arabidopsis thaliana*, which we named *AtTTM1*, *AtTTM2*, and *AtTTM3*. *AtTTM3* encodes a single CYTH domain and exhibits tripolyphosphatase activity in the presence of Mg$^{2+}$ (Moeder et al., 2013). Crystal structure analysis revealed a three-dimensional structure depicting a β barrel comprising eight antiparallel β strands. Furthermore, the tunnel-shaped active site contains inwardly facing acidic and basic residues shown to be essential for enzymatic activity. These characteristics are hallmarks of TTM superfamily members, particularly resembling those of CthTTM and NeuTTM because of their ability to act on tripolyphosphate (Keppetipola et al., 2007; Delvaux et al., 2011). Although *AtTTM3* was biochemically characterized, the biological role of *AtTTM3* remains to be specified. Tissue expression analysis revealed that *AtTTM3* is expressed in root meristems and lateral root primordia. Thus, T-DNA insertion knockout lines of *AtTTM3* were used in root growth assays, revealing fewer and shorter lateral roots in *ttm3* plants compared to wild type. While it appears that *AtTTM3* plays some role in root development, the mechanism by which this is achieved remains unclear. Thus, my thesis goals aimed to define the biochemical properties and the biological roles of the remaining TTM family members in *Arabidopsis* in order to understand their biological importance.

A reverse genetics approach using T-DNA insertion knockout lines was taken to study the biological roles of *AtTTM1* and *AtTTM2*. Analyses of public microarray data from the BAR led to the hypothesized involvement of *AtTTM1* in leaf senescence and *AtTTM2* in pathogen defense responses (Toufighi et al., 2005; Winter et al., 2007). Characterization of *AtTTM1* knockout mutants revealed that *ttm1* plants exhibit delayed senescence in the form of enhanced chlorophyll retention and delayed expression of senescence markers upon dark-induced senescence. On the other hand, *AtTTM2* knockout mutants displayed enhanced resistance that requires the key defense
components, \textit{PAD4}, \textit{ICS1}, and \textit{NPR1}. Furthermore, \textit{AtTTM2} is transcriptionally downregulated in response to pathogen infection and flg22, salicylic acid, or benzothiadiazole treatment, suggesting its involvement in an SA-mediated amplification loop for defense responses. Interestingly, \textit{ttm1} does not exhibit alterations in defense responses and \textit{ttm2} does not exhibit delayed leaf senescence while the loss of both \textit{AtTTM1} and \textit{AtTTM2} does not lead to an additive effect. This indicates that \textit{AtTTM1} and \textit{AtTTM2} are involved in distinct biological processes. Promoter swap analyses revealed that \textit{AtTTM1} and \textit{AtTTM2} can functionally complement each other, suggesting that the differences in their transcriptional regulation govern their distinct biological functions.

Enzymatic characterization revealed that both \textit{AtTTM1} and \textit{AtTTM2} possess \textit{in vitro} pyrophosphatase activity. This activity is dependent on a metal cofactor (Mg$^{2+}$) and is optimal at an alkaline pH range, which are common biochemical properties of TTM family proteins. Overall, this thesis provides the first detailed descriptions of both the biochemical and biological roles of TTM s in plants. These discoveries add to our current understanding of the TTM superfamily and also highlight the biological importance of TTM s.
CHAPTER 2

MATERIALS AND METHODS
2.1 Plant growth conditions

*Arabidopsis thaliana* (*Arabidopsis thaliana*), *Brassica napus* (*Brassica napus* var. Westar), *Glycine max* (*Glycine max* var. Harasoy) plants were grown on Sunshine Mix in a growth chamber at 22°C and 60% relative humidity (RH) with a 9hr-photoperiod.

2.2 *Hyaloperonospora arabidopsidis* propagation and infection

*Hyaloperonospora arabidopsidis* (*Hpa*; downy mildew) isolates Emwa1, Noco2, and Emco5 were propagated weekly onto Ws, Col, and Ws plants, respectively. Each propagation generation was incubated in a growth chamber at 16°C and 60% RH with a 16hr-photoperiod for a maximum of 2 weeks. Conidiospores for infection experiments were harvested between 5 – 7 days after propagation to concentrations of 2x10^5 cells ml^-1, 1x10^5 cells ml^-1, and 8x10^5 cells ml^-1 for Emwa1, Noco2, and Emco5 isolates, respectively. Infections were conducted on 10 – 12 day-old seedlings and analysis was carried out 7 – 14 days later either by quantitative real-time PCR (qPCR), visualization of sporangiophore density (and subsequent binning into numerical categories), or by Trypan blue staining to visualize pathogen structures or HR cell death.

2.3 *Pseudomonas syringae* infection

*Pseudomonas syringae* (*P. syringae*) pv. *tomato* DC3000 carrying *AvrRps4* was used to infect 4 – 5 week-old plants. An inoculum of 1x10^5 CFU ml^-1 in 10mM MgCl₂ was used to syringe-infiltrate the abaxial side of leaves. Leaves were collected from 3 – 4 plants for each genotype and 3 leaf cores were collected for each replicate (n = 3). Each sample was subjected to mechanical grinding in 500μl of 10mM MgCl₂ followed by the addition of another 500μl of 10mM MgCl₂. For plating, 100μl of serial dilutions of 1X and 10X were spread for day 0 and 1000X and 10,000X were spread for day 3. Plates were incubated at 28°C for 2 nights before counting.

2.4 Trypan blue staining

Leaf samples were syringe-infiltrated and then boiled in Trypan blue stain (1:1:1:0.93 ddH₂O : glycerol : lactic acid : phenol, 0.025% (w/v) Trypan blue dye) for 4mins. Samples were stained overnight at room temperature and destained overnight
the following day with chloral hydrate solution (250g/100ml). Tissue was then mounted on 80% glycerol for light microscopy.

2.5 GUS staining

Seedlings or leaf tissue was incubated at 37°C in GUS buffer (100mM sodium phosphate buffer pH 7.5, 10mM EDTA pH 7.5, 0.5mM potassium ferricyanide, 0.5mM potassium ferrocyanide, 2mM X-gluc) for 16 – 18hrs. Samples were then destained at room temperature overnight with GUS fixing buffer (1:3 glacial acetic acid : ethanol). Tissue was then mounted on 80% glycerol for light microscopy.

2.6 DNA extraction

DNA extraction was carried out by grinding leaf samples in extraction buffer (200mM Tris pH 8, 250mM NaCl, 250mM EDTA pH 8, and 0.5% SDS) followed by addition of 1:1 phenol : chloroform solution. Following centrifugation, the supernatant was collected and an equal volume of isopropanol was added. Following precipitation, the dried pellet was resuspended in TE RNase buffer (10mM Tris pH 8, 1mM EDTA pH 8, and 0.5µl/ml RNase A) and stored at −20°C.

2.7 RNA extraction and reverse transcriptase (RT)-PCR

Small-scale RNA extraction was carried out by grinding frozen leaf samples in a total of 1ml of TRlzol reagent each (Life Technologies). Samples were incubated at room temperature for 5min and 200µl of chloroform was added to each sample. Each sample was then vortexed and left at room temperature for 3min before centrifugation at 11,500rpm for 15min at 4°C. The aqueous portion of the supernatant was collected into a new tube and 500µl of isopropanol was added. Each sample was then inverted gently to mix and left at room temperature for 15min. Following incubation, each sample was centrifuged at 11,500rpm for 20min at 4°C and the supernatant was decanted. The remaining pellet was washed with 75% ethanol, vortexed to mix, and centrifuged at 9,000rpm for 15min at 4°C. The supernatant was decanted and the pellet was left to dry in the fumehood for 15min before resuspension in 20.5µl of diethyl pyrocarbonate (DEPC)-treated ddH₂O. RNA quality was checked by first incubating 2µl of RNA mixed with 18µl RNA loading buffer (1.9µl DEPC-treated ddH₂O, 3.5µl formaldehyde, 10µl
formamide, 1μl of 10X MOPS buffer, 0.1μl ethidium bromide, 1.5μl RNA loading dye) at 65°C for 10min. The sample was then subjected to gel electrophoresis using a 1.5% agarose formaldehyde gel (15g/L agarose, 30ml/L formaldehyde, 1X MOPS buffer) in 1X MOPS buffer (41.35g/L MOPS, 6.8g/L sodium acetate, 0.37g/L EDTA, pH 7). RNA samples were then subjected to DNasel treatment at 37°C for 30min by the addition of 2.5μl of 10X DNasel buffer (Invitrogen), 3μl of DNasel (Invitrogen), and 1μl of RNaseOUT (Invitrogen) to the remaining 18.5μl of RNA. Following incubation, 175μl of DEPC-treated ddH2O and 200μl of chloroform were added to each sample before centrifugation at 14,000rpm for 5min at 4°C. The aqueous portion of the supernatant was then collected into a new tube. RNA was precipitated overnight at -20°C by the addition of 500μl of 95% ethanol and 20μl of 3M sodium acetate to each sample. The next day, samples were centrifuged at 14,000rpm for 20min at 4°C followed by a 70% ethanol wash. The samples were centrifuged again at 14,000rpm for 20min at 4°C and the supernatant was decanted. The remaining pellet was left to dry for 15min in the fumehood before resuspension in 10μl of DEPC-treated ddH2O.

For cDNA synthesis, the total RNA amount for each sample was determined by spectrophotometric reading at A260. RT-PCR was then conducted in two steps using 2μg of total RNA. Firstly, 1μg of oligo dT primer was added to a total volume of 11μl containing 2μg of total RNA. Annealing of the primer was performed by incubation of the reaction tube at 65°C for 10min followed by 5min incubation on ice. Secondly, the following was added to the reaction tube for a total of 20μl: 4μl of 5X FirstStrand (FS) buffer (Invitrogen), 2μl of 0.1M dithiothreitol (DTT), 1μl of 10mM deoxyribonucleoside triphosphates (dNTPs), 1μl of RNaseOUT, and 1μl of SuperScript II Reverse Transcriptase (Invitrogen). The reaction was carried out at 42°C for 90min followed by another 15min at 65°C. Following RT-PCR, 20μl of DEPC-treated ddH2O was added and the cDNA was stored at -20°C until use.

Gene expression was detected by PCR after 28 cycles followed by gel electrophoresis. All genes were normalized to β-tubulin (AtTub-F/R) for gene expression analyses carried out by PCR. All primers used are listed in Table A1.
2.8 Quantitative real-time PCR (qPCR)

qPCR was performed with a total reaction volume of 15µl containing 0.25µl of 10µM forward primer, 0.25µl of 10µM reverse primer, 7µl of diluted cDNA (94% ddH₂O, 6% cDNA), and 7.5µl of Power SYBR Green Master Mix (Life Technologies). Reactions were carried out in triplicate (three technical repeats) using the following thermal cycling protocol (50 cycles): 95°C/3min, 95°C/15sec (denature), 58°C/25sec (anneal), plate read, 72°C/20sec (extension), and a final plate read. Fold gene expression was calculated by the Livak method (relative expression). Gene expression was normalized to the expression of \textit{AtEF1a} (\textit{elongation factor1-alpha}) for Arabidopsis; \textit{GmEF1b} (\textit{elongation factor1-beta}) for \textit{Glycine max}; \textit{BnUBC21} (\textit{ubiquitin conjugating enzyme21}) for \textit{Brassica napus}; and \textit{AtEF1a} for \textit{Hpa ITS2}. All primers used are listed in Table A1.

Prior to experiment, all primer combinations were tested with cDNA in order to ensure successful amplification of a single product and to determine efficiency (E) values. This was carried out with a total reaction volume of 15µl containing 0.25µl of 10µM forward primer, 0.25µl of 10µM reverse primer, 7µl of diluted cDNA (serial dilutions of 10X increments in ddH₂O), and 7.5µl of Power SYBR Green Master Mix (Life Technologies). Reactions were carried out in triplicate (three technical repeats) using 5 serial dilutions of cDNA and the same thermal cycling protocol as above with the addition of a melting phase (65°C to 90°C, incrementing at 0.2°C with a plate read every 2sec) at the end to determine primer specificity and efficiency. Independent experiments were conducted to verify results.

2.9 Confirmation of T-DNA insertion knockout lines

T-DNA insertion lines were obtained from the SALK Institute Genomic Analysis Laboratory (SIGnAL, http://signal.salk.edu/cgi-bin/tdnaexpress) and the Max Planck Institute of Plant Breeding Research (https://www.gabi-kat.de) for \textit{AtTTM1} (\textit{ttm1-1}, SALK_079237; \textit{ttm1-2}, GABI_672E02) and \textit{AtTTM2} (\textit{ttm2-1}, SALK_145897; \textit{ttm2-2}, SALK_114669). Homozygous individuals were isolated by using gene-specific primers (\textit{ttm1-1}, 229RP/LP; \textit{ttm1-2}, 980-Seq-F/SK73980R1; \textit{ttm2-1}, 897RP/LP; \textit{ttm2-2}, 244RP/LP) to confirm the absence of the wild type gene and by using T-DNA specific primers (\textit{ttm1-1}, 229RP/LBb1-F; \textit{ttm1-2}, GABIKAT-TDNA-F/SK73980R1; \textit{ttm2-1}, 897RP/LBb1-F; \textit{ttm2-2}, 244RP/LBb1-F) to detect the presence of the T-DNA insertion.
Insertion positions were determined by sequencing where insertions in *ttm1-1* and *ttm1-2* were found to be located at 1067bp (end of exon 3) and 2693bp (middle of exon 9), respectively, and at 1204bp (end of exon 3) and 1819bp (beginning of intron 5) in *ttm2-1* and *ttm2-2*, respectively. The knockout status of each insertion line was confirmed by RT-PCR using cDNA generated from total RNA extracted from leaf tissue and the following primers: 980RT-F/732RT-R (*ttm1-1*, *ttm1-2*) and 190RT-F/244RT-R (*ttm2-1*, *ttm2-2*). All primers used are listed in Table A1.

### 2.10 Plasmid construction

Several vectors were used for conventional restriction enzyme-based cloning: plant complementation analysis (pORE-O1), promoter-GUS analysis (pORE-R2), promoter swapping analysis (pORE-R2), overexpression analysis (pBI121), and biochemical analysis (pGEX-6P-1; pET15b). For plant complementation, full length genomic *AtTTM1* was amplified from the promoter region (905bp upstream of the ATG start codon) to the 3’ untranslated region (UTR) region. XhoI and PstI sites were used to ligate the insert into the pORE-O1 vector. For promoter-GUS constructs, the promoter region and 5’ UTR were cloned from genomic DNA for *AtTTM1* (905bp upstream + 5’ UTR) and *AtTTM2* (1680bp upstream + 5’ UTR). XbaI and EcoRI sites were used to ligate this region into the pORE-R2 vector upstream of the *GUS* gene (*uidA*). For the promoter swap analysis, existing promoter GUS constructs were utilized to introduce full-length *AtTTM1* and *AtTTM2* cDNA downstream of the promoter regions using NotI and SpeI sites to replace the *GUS* (*uidA*) gene. The reverse primer was designed to include a C-terminal hemagglutinin (HA; YPYDVPDYA) tag. For the overexpression analysis, full-length *AtTTM1* and *AtTTM2* cDNA was cloned into the pBI121 vector using SmaI and SacI sites under control of the strong cauliflower mosaic virus 35S (CaMV 35S) promoter.

Plasmids for protein expression in *E. coli* and subsequent biochemical analyses were constructed by ligating truncated inserts (TTM1, Met1 to Ser621; TTM2, Met1 to Asp648) into the protein expression vector, pGEX-6P-1, using Sall and NotI sites. TTM1 mutagenesis constructs were created using a 3-step PCR technique: 1) a short PCR product is created with a restriction site appended to the 5’ end of the amplicon and a 3’ end sequence containing the mutation of choice using a template plasmid; 2) a second short PCR product is created with a 5’ end sequence containing the mutation of choice
and a restriction site appended to the 3' end of the amplicon using the same template plasmid; and 3) a final PCR product is created using the products from the first and second PCRs as a template where the final product has both 5' and 3' end restriction sites intended for ligation into the destination vector. By this technique, 3 final PCR products were generated, each containing one of the following mutations: K78A, T250E-I252E, or R299A. These inserts were used to replace selected regions within the existing truncated TTM1 (Δ622-643; transmembrane domain) construct in pGEX-6P-1 such that three protein expression plasmids were generated, each containing one of K78A, T250E-I252E, or R299A mutations. For protein expression and biochemical analyses of AtUKL1, NdeI and BamH I sites were used to amplify the coding region of AtUKL1 (At5g40870) and inserted into the pET15b vector. For plant expression studies, NotI and SpeI sites were used to amplify the coding regions of the K78A or R299A constructs and inserted into existing pORE-R2 plasmids such that each point mutated version of TTM1 (K78A or R299A) lies downstream of the TTM1 native promoter. All primers used are listed in Table A1.

2.11 Agrobacterium-mediated stable transformation

Stable transformation of Arabidopsis plants was carried out using the floral dip method. A single colony from a culture plate of Agrobacterium tumefaciens GV3101 carrying the plasmid of interest was used to start an overnight test tube culture at 28°C. This was subcultured into a larger volume (5ml into 250ml) the following morning for approximately 6 – 7hrs. Cells were pelleted and resuspended to a final inoculum (10mM MgCl₂, 50g/L sucrose, 50µl/L Silwet L-77) for floral dip at OD600 = 0.8. Each plant was clipped of all siliques, dipped for 3 minutes, and left overnight under Saran wrap before returning to the growth chamber for seed maturation.

2.12 SA, BTH, and flg22 treatments

Arabidopsis seedlings (7 – 10 day-old) and canola and soybean plants (6 week-old) were spray-treated with 100µM salicylic acid (SA) and 0.025% (v/v) Silwet L-77 or 200µM benzothiadiazole (BTH) and 0.025% (v/v) Silwet L-77. Leaf tissue was collected at 24hrs and 48hrs post treatment for SA- and BTH-treated plants, respectively, and stored at –80°C. flg22 treatment was carried out by syringe-infiltration of 4 week-old
plants with 5µM flg22. Leaf tissue was collected 4hrs post treatment and stored at –80°C. All mock treatments were carried out with ddH₂O and 0.025% (v/v) Silwet L-77.

2.13 Dark-induced senescence assay

A combination of non-senescent leaves 3, 4, 5, and 6 of 4 – 5 week-old plants were detached and floated on ddH₂O in petri dishes in the dark for the specified amount of time. Leaf samples were then weighed, frozen in liquid N₂, and crushed in 80% acetone (v/v), 25mM HEPES pH 7.5 buffer. Total chlorophyll content was quantified by measuring the absorbance of chlorophylls A and B using a spectrophotometer, as defined by the Porra method: total chlorophyll content = 17.76 (A₆₄₆) + 7.34 (A₆₆₃), followed by normalization to fresh weight (Porra et al., 1989).

2.14 Protein expression

Full length, truncated, and mutant versions of AtTTM1 and AtTTM2 were heterologously expressed using E. coli BL21 codon plus cells carrying genes of interest in the pGEX-6P-1 expression vector. A single colony was used to start an overnight culture at 37°C. This was used to subculture a larger volume (3ml into 300ml, 2 flasks) of LBP-5052 medium (LB with 1X 5052, 1X NPS, 1mM MgSO₄) for approximately 4 – 5hrs at 37°C until OD600 = 0.4. At this time, the temperature was gradually reduced to 18°C overnight. Cells were then pelleted and resuspended in extraction buffer (1X PBS pH 7.5, 2mM DTT, 5µM E-64, 1mM PMSF). Lysis of cell suspensions was carried out by French press twice at 1,000psi. Soluble and insoluble fractions were then separated by centrifugation at 14,000rpm (over 20,000rcf). Affinity chromatography was then carried out by first equilibrating the glutathione agarose resin with 1X PBS pH 7.5 and then passing the soluble fraction through. The resin was then washed with 50mM Tris pH 7.5. Protein was eluted by incubation of the resin with 10mM reduced glutathione pH 9.5 for 15 minutes on ice before elution. Protein was quantified by the Bradford reagent assay at OD595 and then stored at –80°C.

The Arabidopsis uridine kinase gene (AtUKL1, At5g40870) was expressed with a C-terminal 6xHis tag in the pET15b vector. Protein was heterologously expressed using E. coli BL21 codon plus cells. A single colony was used to start an overnight culture at 37°C. This was used to subculture a 1L culture of LBP-5052 medium (LB with 1X 5052,
1X NPS, 1mM MgSO$_4$) for approximately 4 – 5hrs at 37°C until OD$_{600} = 0.4$. At this time, the temperature was gradually reduced to 18°C overnight. Cells were then pelleted and resuspended in extraction buffer (100ml of 50mM Tris pH 7.5, 1mM DTT, 1mM PMSF, 10µg/ml DNaseI). Lysis of cell suspensions was carried out by French press twice at 1,000psi followed by the addition of 500mM NaCl, 5mM imidazole, and 5% glycerol. Soluble and insoluble fractions were then separated by centrifugation at 14,000rpm (over 20,000rcf). Affinity chromatography was then carried out by first equilibrating Ni-NTA agarose with 50mM Tris pH 7.5, 500mM NaCl, 5mM imidazole, and 5% glycerol and then passing the soluble fraction through. The resin was then washed with 50mM Tris pH 7.5, 500mM NaCl, 30mM imidazole, and 5% glycerol. Protein was eluted with 50mM Tris pH 7.5, 500mM NaCl, 300mM imidazole, and 5% glycerol. Protein was quantified by the Bradford reagent assay at OD$_{595}$ and then stored at –80°C.

### 2.15 Malachite green assay

Free phosphate (P$_i$) release was measured with the Malachite green assay (Bernal et al., 2005). The reaction was carried out in a volume of 500µl containing 25mM glycine buffer pH 9, 2.5mM MgCl$_2$, and 0.5mM PP$_i$, ADP, ATP, or PPP$_i$. The assay was conducted with 2µg of protein at 37°C for 30 – 40min. Following the reaction, 500µl of ddH$_2$O was added along with 250µl of Malachite green dye (1mg/ml Malachite green, 75mg/ml ammonium molybdate, 0.17% (v/v) Tween 20) in acidic solution (3M H$_2$SO$_4$). Samples were then incubated at 30°C for 10min and the absorbance at OD$_{630}$ was measured by a spectrophotometer.

For coupled activity assays, the reaction was carried out in a volume of 500µl containing 25mM glycine buffer pH 9, 2.5mM MgCl$_2$, and 0.5mM ADP ribose, thiamine pyrophosphate (ThPP), or NADH. The assay was conducted with 2µg of protein at 37°C for 30 – 40min. Following the reaction, the resultant organophosphate product was cleaved with calf intestinal alkaline phosphatase (CIAP, 1µl) for 30min at 37°C. Detection of free phosphates was carried out by the addition of 500µl of ddH$_2$O followed by 250µl of Malachite green dye. Samples were then incubated at 30°C for 10min and the absorbance at OD$_{630}$ was measured by a spectrophotometer.
2.16 High performance liquid chromatography (HPLC) analysis

A Zorbax SB-C18 (Agilent, 4.6x150mm, 3.5µm) column was used with an injection volume of 20µl. HPLC analysis was performed using isocratic 20% methanol, 150mM sodium acetate buffer pH 5.0 for 5 – 6min per sample.

2.17 Uridine kinase (UK) assay

Uridine monophosphate (UMP) formation was carried out in a reaction containing 100mM Tris buffer pH 8.0, 1mM ATP, 1mM uridine, 10mM MgCl₂, and 2µg of protein in a total volume of 500µl. The assay was conducted at 37°C for 30min and reaction products were detected by HPLC analysis.

2.18 Adenylate cyclase (AC) assay

3’,5’-cyclic adenosine monophosphate (cAMP) formation was carried out in a reaction containing 100mM Tris buffer pH 8.0, 1mM ATP, 10mM MgCl₂, and 2µg of protein in a total volume of 500µl. The assay was conducted at 37°C for 30min and reaction products were detected by HPLC analysis.
The Arabidopsis triphosphate tunnel metalloenzyme, 
\textit{AtTTM2}, is a negative regulator of the salicylic acid-
mediated feedback amplification loop for defense 
responses.

Modified from:

metalloenzyme, \textit{AtTTM2}, is a negative regulator of the SA-mediated amplification loop in 
3.1 ABSTRACT

The triphosphate tunnel metalloenzyme (TTM) superfamily represents a group of enzymes that possess a unique tunnel-shaped active site comprising eight or nine antiparallel β strands. While all TTMs share this unique catalytic tunnel, they appear to act on a variety of organophosphate substrates with the requirement of a divalent metal cofactor. Three TTM genes exist in Arabidopsis, which have been named AtTTM1, 2 and 3. Recent work on AtTTM3 revealed that it also possesses the unique catalytic tunnel along with strong tripolyphosphatase activity. While the structure and function of this Arabidopsis TTM was determined, the biological role of TTMs in plants remains to be elucidated. Here, we characterize the AtTTM2 knockout (KO) mutant, ttm2, and show that it exhibits an enhanced hypersensitive response, elevated pathogen resistance against both virulent and avirulent pathogens, and elevated accumulation of salicylic acid (SA) upon infection. In addition, stronger systemic acquired resistance was also observed. These enhanced defense responses are dependent on SA, PAD4, and NPR1. Despite their enhanced pathogen resistance, ttm2 plants did not display constitutively active defense responses, suggesting that AtTTM2 is not a conventional negative regulator, but a negative regulator of the amplification of defense responses. The transcriptional suppression of AtTTM2 by pathogen infection or treatment with flg22, SA, and its biological analog, BTH, further supports this notion. Such transcriptional regulation is conserved among TTM2 orthologs in the crop plants, soybean and canola, suggesting that TTM2 is involved in immunity in a wide variety of plant species. This indicates the possible usage of TTM2 KO mutants for agricultural application to generate pathogen-resistant crop plants.
3.2 INTRODUCTION

The TTM superfamily comprises a group of enzymes that are characterized by their ability to hydrolyze a range of organophosphate substrates. All members of this superfamily utilize triphosphate substrates and require a divalent cation cofactor for their activity, usually Mg$^{2+}$ or Mn$^{2+}$ (Bettendorff and Wins, 2013). This superfamily contains two previously characterized groups of proteins: RNA triphosphatases and CYTH domain proteins (Iyer and Aravind, 2002; Gong et al., 2006). The CYTH domain was named after its two founding members, the CyaB adenylate cyclase from Aeromonas hydrophila and the mammalian thiamine triphosphatase (Iyer and Aravind, 2002). Despite low overall amino acid sequence similarity, all TTM family members possess a tunnel structure composed of eight antiparallel β strands (β barrel) (Gong et al., 2006; Gallagher et al., 2006; Song et al., 2008; Moeder et al., 2013). The signature ExExK motif (where x is any amino acid) located in the β barrel has been shown to be important for catalytic activity (Lima et al., 1999; Gallagher et al., 2006).

The enzymatic and biological function of most TTM family members is unknown. However, they appear to act on nucleotide and organophosphate substrates (Bettendorff and Wins, 2013) and acquired divergent biological functions in different taxonomic lineages (Iyer and Aravind, 2002). Known functions include adenylate cyclase for CyaB from Aeromonas hydrophila and YpAC-IV from Yersinia pestis (Sismeiro et al., 1998; Gallagher et al., 2006), thiamine triphosphatase in mammals (Lakaye et al., 2004) and RNA triphosphatase in fungi, protozoa, and some viruses (Shuman, 2002). In some instances, the CYTH domain is fused to additional domains, such as a nucleotide kinase domain (Iyer and Aravind, 2002).

Plants possess two types of TTM proteins: one that comprises only the CYTH domain and another with a CYTH domain fused to a phosphate-binding (P-loop) kinase domain (Iyer and Aravind, 2002). Arabidopsis, as most other plant species, codes for three TTM genes. We termed them AtTTM1, 2 and 3. AtTTM3 possesses only a CYTH domain, while AtTTM1 and AtTTM2 encode a uridine kinase domain fused to the CYTH domain (Moeder et al., 2013). We previously analyzed AtTTM3 and found that it does not display adenylate cyclase activity despite its annotation, but acts on tripolyphosphate and with lower activity, nucleotide triphosphates, releasing inorganic phosphate (P$_i$), similar to the TTM proteins from Clostridium thermocellum (CthTTM) and Nitrosomonas.
Europaea (NeuTTM) (Keppetipola et al., 2007; Delvaux et al., 2011; Moeder et al.; 2013; Bettendorff and Wins, 2013). Additionally, a T-DNA insertion KO line of AtTTM3 displayed a delay in root growth as well as reduced length and number of lateral roots, suggesting a role for AtTTM3 in root development. Thus, the biological role of TTMs in plants remains unclear.

In order to gain insight into the biological function of AtTTM1 and AtTTM2, we surveyed public microarray data from the BAR (Toufighi et al., 2005; Winter et al., 2007) for any expression data that might provide clues. The expression of AtTTM2 was suppressed almost 2-fold after treatment with flg22, the well-studied pathogen-associated molecular pattern (PAMP) peptide, and after infection with various virulent and avirulent strains of Pseudomonas syringae. This data suggests the possible involvement of AtTTM2 in pathogen defense responses in plants.

The plant defense system has been studied extensively in the last two decades and two levels of resistance responses have been reported. The first line of defense is basal immunity, which is triggered by the recognition of molecules that are conserved among many pathogens (abovementioned PAMPs) and is thus referred to as PAMP-triggered immunity (PTI). Direct or indirect recognition of cognate effectors from the pathogen by resistance (R) genes leads to a stronger response to pathogen infection. This is known as effector-triggered immunity (ETI) (Jones and Dangl, 2006). The hypersensitive response (HR), which is characterized by apoptosis-like cell death at and around the site of pathogen entry is one common defense mechanism activated by R gene-mediated pathogen recognition. During HR development, an increase in salicylic acid (SA) and the accumulation of pathogenesis-related (PR) proteins are observed (Uknes et al., 1992; Jones and Dangl, 2006). Later, resistance against virulent pathogens can also be seen in uninoculated systemic leaves. This phenomenon is called systemic acquired resistance (SAR) and confers a long-lasting, broad-range resistance to subsequent infection (Durrant and Dong, 2004). Elevated SA levels and PR gene expression can also be detected in uninoculated leaves that exhibit SAR. Treatment with SA or synthetic SAR activators, such as benzothiadiazole (BTH), can also trigger SAR (Friedrich et al., 1996; Lawton et al., 1996). Recently, a number of metabolites that are involved in long-distance signaling have been identified, such as methyl salicylate (MeSA), dehydroabietinal (DA), azelaic acid (AzA), glycerol-3-phosphate (G3P), and the lysine catabolite pipcolic acid (Pip) (Shah and Zeier, 2013).
Each of these metabolites participates in a signaling network that has the ability to respond to and control the timely activation of SAR under various environmental conditions.

Over the last two decades, significant efforts have been made to identify components in the pathogen resistance signal transduction pathway. For instance, *isochorismate synthase1* (*ICS1*) has been revealed to play a critical role in the biosynthesis of pathogen-induced SA. *sid2/ics1* mutants fail to produce elevated levels of SA after pathogen infection and are thus, hypersensitive to pathogens (Nawrath et al., 1999; Wildermuth et al., 2001). *Nonexpresser of PR genes1* (*NPR1*) is a key regulator of SA-mediated resistance and *npr1* plants fail to respond to exogenously supplied SA (Cao et al., 1994). The lipase-like proteins, *enhanced disease susceptibility1* (*EDS1*) and *phytoalexin-deficient4* (*PAD4*), participate in both basal and R protein-mediated defense responses (Parker et al., 1996; Glazebrook et al., 1996; Falk et al., 1999; Jirage et al., 1999). EDS1 interacts with PAD4 and both are required for HR formation and the restriction of pathogen growth (Feys et al., 2001). Studies employing screens for mutants that exhibit constitutive activation of resistance responses also identified components in defense. They show heightened resistance, usually accompanied by elevated levels of SA and *PR* genes. These autoimmune mutants also frequently display spontaneous HR-like lesions and thus, are referred to as lesion mimic mutants (Moeder and Yoshioka, 2008).

Here, we demonstrate that *AtTTM2* acts as a negative regulator of plant immunity, likely at the positive amplification loop of defense responses. Knockout mutants for *AtTTM2* show enhanced pathogen resistance while overexpressers display enhanced susceptibility. The knockout mutants do not show constitutive activation of defense responses like most autoimmune mutants, but exhibit enhanced SAR upon treatment with pathogens or BTH, suggesting that they are in a primed state. Furthermore, the expression of *TTM2* orthologues in canola and soybean displays the same transcriptional downregulation after BTH treatment, suggesting that the biological function of *TTM2* in pathogen defense is conserved among agriculturally important crop plants.
3.3 RESULTS

3.3.1 AtTTM2 is downregulated after pathogen infection

The Arabidopsis Information Resource (TAIR) was initially surveyed for genes that are functionally annotated with a CYTH domain. Three genes were identified, At1g73980, At1g26190, and At2g11890 and subsequently named AtTTM1, 2, and 3 (Moeder et al., 2013). Two allelic homozygous T-DNA insertion knockout (KO) lines were obtained for AtTTM2 – Salk_145897 (ttm2-1) and Salk_114669 (ttm2-2). The T-DNA insertion positions were found to be located in exon 3 and intron 5 in ttm2-1 and ttm2-2, respectively (Fig. 3-2A). Reverse transcription (RT)-PCR analysis confirmed that both lines are indeed KO mutants (Fig. 3-2B). A morphological comparison showed no detectable difference in the size or shape of both ttm2 KO lines compared to wild type Columbia (Col) (Fig. 3-2C).

As mentioned, public microarray data revealed the downregulation of AtTTM2 during pathogen infection (Fig. 3-1). To confirm these results, quantitative real-time PCR (qPCR) was conducted on Col wild type plants that were infected with the avirulent Hpa isolate, Emwa1. We observed a more than 2-fold reduction in AtTTM2 transcript levels in infected cotyledons compared to mock treatment, indicating the involvement of AtTTM2 in pathogen defense (Fig. 3-3A). Interestingly, AtTTM2 was also downregulated in the uninfected systemic tissue of the same seedlings, indicating a role for AtTTM2 in SAR as well (Fig. 3-3B).
Figure 3-1. Visualization of the expression pattern of AtTTM2.

Data is based on publicly available AtGenExpress data from the Bio-Analytic Resource (BAR, Winter et al., 2007). Shown are relative gene expression values after treatment with PAMPs (flg22, HrpZ) or bacterial pathogens (virulent Pseudomonas syringae pv. tomato DC3000, avirulent Pseudomonas syringae pv. tomato DC3000 expressing AvrRpm1, and Pseudomonas syringae pv. phaseolicola).
Figure 3-2. T-DNA insertion line analysis.

(A) T-DNA insertion position in *ttm2-1* (SALK_145897) and *ttm2-2* (SALK_114669). Number above the triangle indicates the exact location of the T-DNA insertion. Filled boxes represent exons, grey represents untranslated regions, and lines represent introns. Arrows and corresponding numbers below the genomic map indicate primer positions.

(B) RT-PCR analysis for *AtTTM2* in Columbia wild type (Col), *ttm2-1* and *ttm2-2*. β-tubulin (*β-tub*) served as loading control. Primer positions are shown in (A) (*AtTTM2 = 1 + 2*). Primer sequences are listed in Table A1.

(C) Morphological phenotype of Col wild type, *ttm2-1* and *ttm2-2*. Photos show approximately 6 week-old plants. Scale bar = 1cm.
Figure 3-3. *AtTTM2* is downregulated after pathogen infection with *Hyaloperonospora arabidopsis* (Hpa).

(A) Quantitative real-time PCR (qPCR) analysis of *AtTTM2* expression in *Hpa* isolate, Emwa1-infected (Emwa1) or water-treated (H2O) cotyledons of 10 day-old Columbia wild type plants 7 days after infection.

(B) qPCR analysis of *AtTTM2* expression in uninfected true leaves of the same plants. Transcripts were normalized to *AtEF1a*. Each bar represents the mean of three independent experiments ± SE. Each sample is a pool of 16 seedlings. Asterisks indicate statistical significance (*p* < 0.01 (**), *p* < 0.05 (*), Student’s t-test). Primer sequences are listed in Table A1.
3.3.2 ttm2 exhibits enhanced resistance against *Hyaloperonospora arabidopsisidis* and *Pseudomonas syringae* pv. *tomato* DC3000 (AvrRps4)

Since *AtTTM2* is downregulated after pathogen infection, we asked whether *ttm2* mutants show alterations in defense-related phenotypes. Cotyledons of 7 to 10 day-old seedlings were infected with the avirulent *Hpa* isolate, Emwa1. It is notable that although the Emwa1 isolate is considered to have an incompatible interaction with the Col ecotype, the resistance in this ecotype is not perfect and initial layers of mesophyll cells may show the emergence of some hyphae (Fig. 3-4A, Hy). *ttm2* mutants, in addition to having fewer or no hyphae, also exhibited a greater manifestation of HR cell death on infected tissue compared to wild type, suggesting enhanced resistance (Fig. 3-4A, top panel). qPCR analysis also showed approximately 2-fold less *ITS2* (*internal transcribed spacer2*) transcript levels, a marker to quantify oomycete infection, indicating less growth of pathogens in *ttm2* plants (Fig. 3-4B; Quentin et al., 2009). We frequently observed the formation of micro-HR-like cell death in uninfected systemic leaves of wild type plants after avirulent infection on cotyledons similarly to the findings of Alvarez et al. (1998). Interestingly, *ttm2* plants also displayed significantly enhanced HR cell death on the uninfected systemic true leaves (Fig. 3-4A, bottom panel).

To determine whether this enhanced resistance was specific to ETI or whether it also affected PTI, infection with the virulent *Hpa* isolate, Emco5, was conducted. Trypan blue analysis revealed little to no hyphae on infected tissue of *ttm2* while in wild type plants, hyphal structures and oospore formation were clearly visible throughout the leaf (Fig. 3-4C). Interestingly, we also observed enhanced HR-like cell death along the veins of uninfected systemic leaves of *ttm2* seedlings (Fig. 3-4C, HR-like). Consistent with this observation, *ITS2* transcript levels in infected cotyledons of *ttm2* seedlings were also more than 2-fold lower compared to wild type (Fig. 3-4D).

To test whether *ttm2*-mediated enhanced resistance is specific to oomycete pathogens, we conducted infections with virulent *Pst* DC3000 and avirulent *Pst* DC3000 carrying *AvrRpt2* and *AvrRps4*. While *ttm2* plants did not show alterations to PTI or ETI against virulent *Pst* DC3000 or avirulent *Pst* DC3000 expressing *AvrRpt2*, we did observe enhanced resistance against avirulent *Pst* DC3000 expressing *AvrRps4* (Fig. 3-5). Taken together, these data indicate that *ttm2* plants exhibit enhanced resistance to oomycete pathogens and some bacterial pathogens.
SA has been shown to be a critical signaling molecule in pathogen defense. In line with the resistance phenotype, a significant increase in free SA and its conjugated form, salicylic acid glucoside (SAG), was observed in *ttm2* plants upon pathogen infection compared to wild type (Fig. 3-4E, F). Taken together, these data suggest that *AtTTM2* is likely involved in SA-mediated defense signaling.
Figure 3-4. *ttm2* exhibits enhanced resistance against *Hpa*.

(A) Infection phenotype of Columbia wild type (Col) and *ttm2* plants 10 days after infection with the avirulent *Hpa* isolate, Emwa1. Shown is Trypan blue staining of infected cotyledons (cot) and uninfected true leaves (TL). Hy, hyphae; HR, hypersensitive response.

(B) Quantitative real-time PCR (qPCR) analysis of the oomycete marker, *internal transcribed spacer2 (ITS2)*, in *Hpa* isolate, Emwa1-infected seedlings. Transcripts were normalized to *AtEF1a*. Each bar represents the mean of three technical replicates ± SE. Each sample is a pool of 16 seedlings.

(C) Infection phenotype of Col wild type and *ttm2* plants 12 days after infection with virulent *Hpa* isolate, Emco5. Shown is Trypan blue staining of infected cotyledons (cot) and uninfected true leaves (TL). Hy, hyphae; Oo, oospore; HR-like, hypersensitive response-like.

(D) qPCR analysis of *ITS2* in *Hpa* isolate, Emco5-infected seedlings. Transcripts were normalized to *AtEF1a*. Each bar represents the mean of three technical replicates ± SE. Each sample is a pool of 16 seedlings. Primer sequences are listed in Table A1.

(E-F) Free salicylic acid (SA; E) and conjugated salicylic acid (SAG; F) levels in *Hpa* isolate, Emwa1-infected cotyledons 5 days after infection. Each bar represents the mean of three replicates ± SE. Experiments were repeated three times with similar results. 10 day-old seedlings were used for all experiments. Scale bars = 250µm. Asterisks indicate statistical significance (p < 0.05, Student’s t-test).
Leaf bacteria (Log$_{10}$ CFU cm$^{-1}$)

A

DC3000

B

AvrRpt2

C

AvrRps4

Day 0  Day 3
Figure 3-5. Bacterial growth of *Pseudomonas syringae pv. tomato* (*Pst*).

(A-C) 4 week-old plants were infiltrated with $1 \times 10^5$ CFU·mL$^{-1}$ (A) *Pst* DC3000, (B) *Pst* DC3000 expressing *AvrRpt2*, or (C) *Pst* DC3000 expressing *AvrRps4*. Each bar represents the mean of three (*AvrRpt2, AvrRps4*) or five (DC3000) biological replicates ± SE. Asterisks indicate statistical significance (*p* < 0.05, Student’s t-test).
3.3.3  *ttm2* is not an autoimmune mutant

To date, various autoimmune mutants have been reported. They show enhanced resistance against various pathogens and often exhibit activation of resistance responses, such as accumulation of SA and constitutive *PR* gene expression, without pathogen infection. One well-studied class of autoimmune mutants, called lesion mimic mutants, additionally exhibits spontaneous cell death formation without pathogen infection (Moeder and Yoshioka, 2008). To test whether resistance responses are activated without pathogen infection in *ttm2*, Trypan blue analysis on uninfected *ttm2* seedlings was conducted and revealed no spontaneous cell death formation (Fig. 3-6A). Additionally, no elevated expression of the defense marker gene, *PR1*, was observed in *ttm2* seedlings without pathogen infection (Fig. 3-6B). These data suggest that *ttm2* is not a lesion mimic or conventional autoimmune mutant, but likely a priming mutant that exhibits enhanced resistance upon pathogen infection.
Figure 3-6. *ttm2* is not a lesion mimic mutant.

(A) Trypan blue staining of untreated Columbia wild type (Col), *ttm2-1*, and *ttm2-2* plants. cot, cotyledon; TL, true leaf. Scale bar = 250µm.

(B) RT-PCR analysis of *PR1* gene expression of untreated Col wild type, *ttm2-1*, *ttm2-2* plants and Col wild type plants treated with 100µM salicylic acid (SA). β-tubulin (β-tub) served as a loading control. 4 week-old plants were used for the analysis. Primer sequences are listed in Table A1.
3.3.4 *ttm2* exhibits enhanced SAR

The observation that *AtTTM2* was also downregulated in uninfected systemic leaves combined with the enhanced HR cell death in *ttm2* seedlings prompted us to investigate whether *ttm2* is also affected in its SAR response. To assess SAR, we first treated cotyledons of wild type and *ttm2* plants with either water (SAR-) or the avirulent *Hpa* isolate, Emwa1 (SAR+). We then performed challenge inoculation using the aggressive virulent *Hpa* isolate, Noco2, on the upper systemic leaves. We used very strong infection conditions, i.e. $1 \times 10^5$ conidiospores, of the aggressive isolate, Noco2, in order to see a clear difference between SAR-induced and non-induced groups. Thus, microscopic examination of stained leaves revealed comparable hyphae growth in water-treated plants (SAR-) for both wild type and *ttm2*. In contrast, Emwa1-treated *ttm2* plants (SAR+) revealed a stronger reduction in pathogen growth in systemic leaves compared to SAR+ wild type plants (Fig. 3-7A, B; $p < 0.001$, Fisher’s exact test). These data suggest that *ttm2* plants exhibit enhanced SAR.
A

Col  \( ttm2-1 \)  \( ttm2-2 \)

-SAR

+SAR

B

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Hyphae Index (%)
Figure 3-7. *ttm2* exhibits enhanced systemic acquired resistance (SAR).

(A) Primary infection of 10 day-old cotyledons of Columbia wild type (Col) and *ttm2* mutant plants was performed with the avirulent *Hpa* isolate, Emwa1 (+SAR) or H$_2$O (-SAR). Challenge infection was performed on systemic true leaves 7 days later with the virulent *Hpa* isolate, Noco2. Hyphae were visualized 10 days later by Trypan blue staining.

(B) Stained leaves were microscopically examined and assigned to different classes (see index panel). Data shown was taken from 50 plants. The experiment was repeated three times with similar results. Scale bar = 250µm. A statistically significant difference was observed between SAR+ Col wild type and *ttm2* lines (p < 0.0001, Fisher’s exact test).
3.3.5 The enhanced resistance phenotype of \textit{ttm2} requires \textit{PAD4}, \textit{ICS1}, and \textit{NPR1}

It has been shown that \textit{PAD4}, \textit{ICS1}, and \textit{NPR1} play key roles in SA-dependent defense responses (Glazebrook et al., 1996; Cao et al., 1997; Jirage et al., 1999; Wildermuth et al., 2001). To investigate whether \textit{ttm2}-mediated resistance requires these signaling components, we performed epistatic analyses by first isolating double mutants of \textit{ttm2-2} and \textit{pad4-1}, \textit{sid2-1}, or \textit{npr1-1} (Fig. 3-8). Col and Ws ecotypes are resistant and susceptible, respectively, to the \textit{Hpa} isolate, Emwa1. As expected, Col wild type exhibited resistance with some hyphae present on the infected tissue along with punctate areas of HR cell death in both infected tissue and uninfected systemic tissue (Fig. 3-9, Hy). On the other hand, Ws wild type exhibited susceptibility with massive hyphal growth and oospore formation in infected tissue and no visible signs of HR in the uninfected systemic leaves (Fig. 3-9, Oo). \textit{pad4-1}, \textit{sid2-1}, and \textit{npr1-1} single mutants also exhibited susceptibility with little or no visible HR, but a great presence of hyphae and in some cases, oospores, as expected (Fig. 3-9). All double mutants with \textit{ttm2} exhibited similar susceptibility as \textit{pad4-1}, \textit{sid2-1}, and \textit{npr1-1} single mutants (Fig. 3-9, 3-10). These data indicate that \textit{PAD4}, \textit{ICS1}, and \textit{NPR1} are all required for the enhanced resistance phenotype of \textit{ttm2}. 
### 244RP/LP  244RP/LBb1.3  CAPS Marker Analysis

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CAPS Marker Analysis is a method used to identify genetic markers in DNA. It involves the digestion of DNA with restriction enzymes followed by electrophoresis to reveal specific fragments. The images show the results of CAPS Marker Analysis on different genetic backgrounds and mutations, indicating the presence or absence of specific DNA fragments.
Figure 3-8. Isolation of double mutant lines for epistatic analysis.

(A) Isolation of the $ttm2$ $pad4$ double mutant using CAPS marker analysis with BsmFI. Wild type AtTTM2 was detected using 244RP/LP primers (1060bp). The presence of a T-DNA insertion in $AtTTM2$ was detected using 244RP/LBb1.3 primers (600bp). The restriction pattern of wild type Columbia (Col, 2100bp) and the homozygous $pad4$ mutant (2800bp) is shown. A 700bp band is also expected for wild type and is sometimes visible, but not shown.

(B) Isolation of the $ttm2$ $sid2$ double mutant using CAPS marker analysis with MfeI. Wild type AtTTM2 was detected using 244RP/LP primers (1060bp). The presence of a T-DNA insertion in $AtTTM2$ was detected using 244RP/LBb1.3 primers (600bp). The restriction pattern of wild type Col (730bp, 194bp) and the homozygous $sid2$ mutant (800bp, 194bp) is shown. A 70bp band is also expected for wild type and is sometimes visible, but not shown.

(C) Isolation of the $ttm2$ $npr1$ double mutant using CAPS marker analysis with NlaIII. Wild type AtTTM2 was detected using 244RP/LP primers (1060bp). The presence of a T-DNA insertion in $AtTTM2$ was detected using 244RP/LBb1.3 primers (600bp). The restriction pattern of wild type Col (200bp, 100bp) and the homozygous $npr1$ mutant (300bp) is shown. Vertical lines indicate splicing of images from the same gel. Primer sequences are listed in Table A1.
Figure 3-9. Involvement of *PAD4*, *NPR1*, and salicylic acid in *ttm2*-mediated resistance.

Infection phenotype of Columbia wild type (Col), Wassilewskija wild type (Ws), *pad4-1*, *sid2-1*, *npr1-1*, *ttm2*, and corresponding double mutants 10 days after infection with the avirulent *Hpa* isolate, Emwa1. Shown is Trypan blue staining of infected cotyledons (cot) and uninfected true leaves (TL). Experiments were repeated three times with similar results. 10 day-old seedlings were used for all experiments. Hy, hyphae; HR; hypersensitive response; Oo, oospore.
A

Infected Cot

npr1 ttm2-2
npr1
pad4 ttm2-2
pad4
sid2 ttm2-2
sid2
ttm2-2
Col

HR Index (%)

Ws

B

Uninfected TL

npr1 ttm2-2
npr1
pad4 ttm2-2
pad4
sid2 ttm2-2
sid2
ttm2-2
Col

HR Index (%)

Ws
**Figure 3-10. Epistatic analysis of ttm2.**

(A) Hypersensitive response (HR) index of cotyledons (cot) of Columbia wild type (Col), Wassilewskijia wild type (Ws), pad4-1, sid2-1, npr1-1, ttm2, and corresponding double mutants 10 days after infection with the avirulent Hpa isolate, Emwa1. Trypan blue-stained leaves were microscopically examined and assigned to different classes (see index panels).

(B) HR index of uninfected true leaves (TL) of the same plants. Data shown were taken from 12 plants. The experiment was repeated three times with similar results.
3.3.6 *AtTTM2* expression is suppressed by SA and PAMP treatment

Since pathogen infection downregulates the transcription of *AtTTM2*, the effect of SA on *AtTTM2* expression was tested. Col wild type plants were sprayed with 100µM SA and assessed 24h later for changes in expression levels. *AtTTM2* was downregulated by more than 2-fold after SA treatment (Fig. 3-11A). This downregulation was also observed after treatment with the SAR activator, BTH (200µM; Fig. 3-11B). This was correlated with an increase in *PR1* gene expression, which is a marker for the activation of defense responses (Fig. 3-11A, B).

Publicly available microarray data indicated that *AtTTM2* is also downregulated after treatment with the PAMP, flg22 (Fig. 3-1). Our qPCR analysis confirmed that *AtTTM2* is indeed downregulated as much as 70% 4h after treatment with the flg22 peptide (5µM; Fig. 3-11C). Since we observed the requirement of *PAD4*, *ICS1*, and *NPR1* for *ttm2*-mediated resistance, we tested whether they were also required for the transcriptional regulation of *AtTTM2*. Interestingly, flg22 treatment on *pad4*, *sid2*, and *npr1* plants led to the same level of *AtTTM2* downregulation as wild type plants (Fig. 3-11C). A similar result was seen after infection with *Pseudomonas syringae* pv. *maculicola* ES4326 (Fig. 3-11D). Taken together, these data suggest that *PAD4*, *ICS1*, and *NPR1* are not required for the transcriptional down-regulation of *AtTTM2*, but are required for the *ttm2*-mediated resistance phenotype.
**A**

AtTTM2

H₂O | SA
---|---
0.3 | 0.2

**B**

AtTTM2

H₂O | BTH
---|---
0.8 | 0.1

**C**

PR1

H₂O | flg22
---|---
2.0 | 1.5

**D**

Expression Units

Mock | Col | npr1 | pad4 | sid2
---|---|---|---|---
30 | 20 | 15 | 10 | 5

Psm ES4326
Figure 3-11. *AtTTM2* expression is suppressed by salicylic acid and flg22 treatment.

(A-B) Quantitative real-time PCR (qPCR) analysis of *AtTTM2* and *AtPR1* in 10 day-old Columbia wild type (Col) seedlings 24hr after treatment with 100µM salicylic acid (SA; A) and 48hr after treatment with 200µM benzothiadiazole (BTH; B) or water (H₂O).

(C) qPCR analysis of *AtTTM2* in 4 week-old Col wild type, *sid2*, *pad4*, and *npr1* plants 4hr after syringe-infiltration with 5µM flg22. All transcripts were normalized to *AtEF1a*. Each bar represents the mean of three technical replicates ± SE. Each sample is a pool of 16 seedlings (A, B) or 4 leaves (C). Primer sequences are listed in Table A1.

(D) Publically available microarray data from the Glazebrook Lab showing *AtTTM2* expression in Col wild type, *npr1*, *pad4*, and *sid2* plants 24hr after inoculation with MgCl₂ (Mock) or *Pseudomonas syringae* pv. *maculicola* ES4326 (*Psm* ES4326) (Wang et al., 2008; http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE11009).
3.3.7 Overexpression of \textit{AtTTM2} confers enhanced susceptibility to \textit{Hpa}

The observation that \textit{AtTTM2} is downregulated upon pathogen infection and SA/\textit{flg22} treatment combined with the fact that \textit{ttm2} plants display enhanced disease resistance strongly suggests that \textit{AtTTM2} is a negative regulator of disease resistance. Therefore, constitutive expression of \textit{AtTTM2} may lead to enhanced disease susceptibility. Thus, we created \textit{AtTTM2} overexresser lines where \textit{AtTTM2} expression is driven by the strong CaMV 35S promoter. To detect differences in disease outcome, we used relatively moderate infection conditions with the virulent \textit{Hpa} isolate, Emco5. We observed elevated expression of \textit{AtTTM2} in three independent transgenic lines even after pathogen infection (Fig. 3-12A). While only 60% of Col wild type plants and 30% of \textit{ttm2} plants exhibited heavy hyphal growth 10 days after infection, 100% of the plants of all transgenic lines tested showed strong infection (Fig. 3-12B, C). This was also confirmed quantitatively by measuring the expression of the oomycete marker, \textit{ITS2} (Fig. 3-12D). This data strongly suggests that downregulation of \textit{AtTTM2} is indeed required for normal levels of disease resistance.
**Figure 3-12. Overexpression of AtTTM2 causes enhanced susceptibility.**

(A) Quantitative real-time PCR (qPCR) analysis of AtTTM2 in Columbia wild type (Col) and two independent AtTTM2 overexpression lines (35S:AtTTM2 #2 and #5) 13 days after infection with the virulent Hpa isolate, Emco5. Transcripts were normalized to AtEF1a. Each bar represents the mean of three technical replicates ± SE. Each sample is a pool of 15 seedlings.

(B) Infection phenotype of Col wild type and 35S:AtTTM2 #2 and #5 seedlings after infection with the virulent Hpa isolate, Emco5. Shown is Trypan blue staining of infected cotyledons. Scale bar = 250µm.

(C) Stained leaves were microscopically examined and assigned to different classes (see index panel). Data shown was taken from 15 – 16 plants. The experiment was repeated three times with similar results. A statistically significant difference was observed between Col and AtTTM2 overexpression lines (p < 0.001, Fisher’s exact test).

(D) qPCR analysis of ITS2 in Col wild type and 35S:AtTTM2 #2 and #5 seedlings 10 days after infection with the virulent Hpa isolate, Emco5. Transcripts were normalized to AtEF1a. Each bar represents the mean of three technical replicates ± SE. Each sample is a pool of 15 seedlings. 10 day-old seedlings were used for all experiments. Primer sequences are listed in Table A1.
3.3.8 AtTTM2 function is likely conserved among different plant species

Data from Phytozome (Goodstein et al., 2012) indicated that AtTTM2 is highly conserved in a wide variety of plant species. This may indicate that these orthologs are also involved in pathogen defense responses. Similarities in the transcriptional expression pattern of AtTTM2 orthologs can serve as an indication of functional conservation. Thus, the expression of AtTTM2 orthologs of soybean (Glycine max) and canola (Brassica napus) was analyzed by qPCR after treatment with BTH. Interestingly, the AtTTM2 orthologs in B. napus (BnTTM2a, BnTTM2b) and in G. max (GmTTM2a/b; note that the two isoforms could not be distinguished due to high sequence identity) were similarly downregulated in response to BTH as their Arabidopsis ortholog (Fig. 3-13A, B). This data combined with the high sequence identity (BnTTM2a, 94%; BnTTM2b, 92%; GmTTM2a, 75%; GmTTM2b, 75%) suggests that the function of AtTTM2 as a negative regulator of defense responses is likely evolutionarily conserved in other plant species as well.
A

BnTTM2a

BnTTM2b

Relative Expression

B

GmTTM2a/b

GmPR1

Relative Expression

H₂O   BTH

H₂O   BTH

0  100  200  300  400  500  600  700

78
Figure 3-13. *AtTTM2* function is conserved in crop species.

(A) Quantitative real-time PCR (qPCR) analysis of *BnTTMa*, *BnTTM2b*, and *BnPR1* in canola (*Brassica napus* var. Westar, *Bn*) 48hr after treatment with 200µM benzothiadiazole (BTH) or water (H2O). Transcripts were normalized to *BnUBC21*. 

(B) qPCR analysis of *GmTTM2a/b* and *GmPR1* in soybean (*Glycine max* var. Harasoy, *Gm*) 48hr after treatment with 200µM BTH or water (H2O) (Note: primers could not distinguish between the two soybean paralogs due to high sequence homology). Transcripts were normalized to *GmEF1b*. All bars represent the mean of three technical replicates ± SE. 5 week-old plants were used for all experiments. Primer sequences are listed in Table A1.
3.4 DISCUSSION

In order to understand the biological function of the triphosphate tunnel metalloenzyme, \textit{AtTTM2}, we have characterized the \textit{AtTTM2} KO mutants, \textit{ttm2-1} and \textit{ttm2-2}. Both lines displayed enhanced resistance against both virulent and avirulent pathogens, as they exhibited lower growth of both types of pathogens combined with an enhancement of HR cell death. In addition, SAR was also enhanced in these mutants. The enhanced resistance was dependent on the well-known defense signaling components, SA, \textit{PAD4} and \textit{NPR1}, which indicates that \textit{AtTTM2} is involved in the \textit{bona fide} defense signaling pathway and is likely a negative regulator. Transcriptional suppression of \textit{AtTTM2} after pathogen infection, PAMP recognition, or SA/BTH treatment further supports this notion. Interestingly, the enhanced pathogen resistance is only observed upon pathogen infection - no significant auto-activation of defense responses, such as spontaneous cell death formation and elevated levels of basal SA or \textit{PR1} gene expression were observed. This differentiates \textit{AtTTM2} mutants from the majority of conventional autoimmune mutants (Moeder and Yoshioka, 2008).

A similar phenomenon was reported in the Arabidopsis mutants \textit{enhanced disease resistance (edr) 1 and 2} (Frye and Innes, 1998; Tang et al., 2005a). \textit{EDR1} and 2 encode a CTR1 family MAPKKK and an unknown protein with a PH, a START, and a DUF1336 domain, respectively (Frye et al., 2001; Tang et al., 2005a, 2005b; Vorwerk et al., 2007). Both mutants were identified in the same screen for decreased susceptibility against \textit{Pseudomonas syringae} DC3000 without constitutive \textit{PR} gene expression and also show enhanced resistance against other pathogens such as \textit{Erysiphe cichoracearum}. Interestingly, both mutants display stronger and faster defense responses upon pathogen infection; however, no obvious auto-activation of defense was observed, just like for \textit{ttm2}. These phenotypes were suppressed in mutants with defects in the SA signal transduction pathway (e.g., \textit{sid2, pad4, npr1, eds1}), but not by those with defects in the ethylene/jasmonate pathway, suggesting that they are hypersensitive to or have a lower threshold in activating the SA pathway (Frye et al., 2001; Tang et al., 2005; Vorwerk et al., 2007). The precise molecular mechanisms of these mutants are not yet clear; however, the reported phenotypes are remarkably similar to those of \textit{ttm2}. The only outstanding difference between \textit{ttm2} and \textit{edr2} is the enhanced SAR phenotype in \textit{ttm2}. As shown, \textit{ttm2} displayed strong enhancement of SAR, including HR cell death,
in uninfected systemic leaves, but *edr2*-mediated enhancement of resistance does not occur in uninfected systemic leaves. This indicates that although the mutant phenotypes are similar, the molecular mechanism behind the phenomena is fundamentally different.

In terms of SAR, *AGD2-LIKE DEFENSE RESPONSE PROTEIN1* (*ALD1*) was suggested to be involved in both local and systemic resistance (Song et al., 2004). *ald1* mutant plants have increased susceptibility to avirulent pathogens and cannot activate SAR properly. The *ALD1* aminotransferase is involved in the biosynthesis of pipecolic acid, which serves as a long distance signal during SAR (Návarová et al., 2012). *ALD1* is transcriptionally induced by pathogen infection as well as BTH treatment in both inoculated and systemic tissues and has been hypothesized to be involved in the SA-mediated feedback amplification loop for defense responses. Considering that *ttm2* also does not show constitutive activation of resistance and displays a SAR phenotype, *AtTTM2* may act by fine-tuning the amplification of defense responses in both inoculated and uninoculated leaves. Indeed, an SA-mediated feedback amplification loop has been suggested for a long time (Shah, 2003). For instance, *EDS1* and *PAD4*, which are important defense signaling components, are both regulators and effectors of SA signaling, strongly suggesting the existence of a SA-mediated feedback amplification loop (Dong, 2004). Likewise, *ACCELERATED CELL DEATH6* (*ACD6*), which is believed to work upstream of SA biosynthesis, is transcriptionally induced by BTH (Lu et al., 2003).

Thus, it can be hypothesized that recognition of pathogen infection suppresses the expression of *AtTTM2*, which acts as a negative regulator of the amplification loop, to facilitate a quick and strong resistance response. At a later time point, SA accumulation induced by pathogen infection further suppresses the expression of *AtTTM2* to boost the positive feedback amplification loop of defense responses. Transcriptional down-regulation of *AtTTM2* can already be seen 4h after treatment with flg22 and 24h after infection with *Pseudomonas syringae* (Fig. 3-1; Fig. 3-11C). Interestingly, *AtTTM2* downregulation was also observed in flg22-treated as well as *Pseudomonas syringae*-infected *sid2*, *npr1* and *pad4* mutant plants (Fig. 3-11C, D), indicating that the downregulation is triggered upstream of *PAD4*. SA/BTH treatment causes *AtTTM2* down-regulation either through an additional mechanism or through feedback via the SA amplification loop (Fig. 3-14). In this scenario, *AtTTM2* plays a role to prevent accidental activation of defense responses through the positive feedback amplification loop in the
absence of pathogens. Thus, *ttm2* exhibits a primed mutant phenotype: it can induce resistance responses faster and stronger than wild type plants, but no constitutive activation of defense responses is observed. While a SA-mediated feedback amplification loop has been discussed for quite some time (Shah, 2003), only a few studies have identified components of this feedback loop (Song et al., 2004; Raffaele et al., 2006; Roberts et al., 2013). The molecular mechanism of *AtTTM2* will further our understanding of the SA-mediated feedback amplification loop.
Figure 3-14. *AtTTM2* is a negative regulator of the salicylic acid (SA)-mediated amplification loop for defense.

Pathogen recognition suppresses the transcription of *AtTTM2* to quickly amplify defense responses. At a later time point, production of SA further leads to continuous transcriptional suppression of *AtTTM2*, further amplifying the feedback loop. The knockout mutants of *AtTTM2*, thus, behave like in a “primed” state and show enhanced resistance upon pathogen recognition. The mutant phenotype requires the known defense signaling components *PAD4*, *ICS1*, and *NPR1*. 
The ttm2-mediated resistance we observed against Hpa prompted us to test whether this disease resistance was also effective against other pathogens. We observed enhanced resistance against bacterial Pst DC3000 expressing AvrRps4. R protein-mediated resistance against this avirulent strain of Pst is mediated by RESISTANCE AGAINST P. SYRINGAE4 (RPS4), which is a TIR-NBS-LRR R protein (Hinsch and Staskawicz, 1996; Gassmann et al., 1999). The enhanced resistance against the avirulent Hpa isolate, Emwa1, is also mediated by a TIR-NBS-LRR R protein in the Col ecotype, called RESISTANCE TO P. PARASITICA4 (RPP4) (van der Biezen et al., 2002). Interestingly, we did not observe enhanced resistance to Pst DC3000 expressing AvrRpt2, which is recognized by the CC-NBS-LRR R protein, RPS2. This suggests that there might be a discrimination of signals mediated by each NBS-LRR class in ttm2 plants. However, according to our model, ttm2-mediated resistance requires PAD4 and ICS1 and the transcriptional downregulation of AtTTM2 occurs by SA application. Since SA accumulates downstream of both branches of resistance mediated by TIR-NBS-LRR or CC-NBS-LRR signals, it is unlikely that this is the case (Nawrath and Métraux, 1999). Surprisingly, we also did not detect a statistically significant difference in the growth of virulent Pst DC3000. Since we did observe reduced susceptibility to the virulent Hpa isolate, Emco5, it might be possible that our experimental conditions may not have been ideal to reveal such differences with Pst DC3000. Thus, future work should be done with other virulent strains of P. syringae as well as other avirulent Pst strains recognized by CC-NBS-LRR R proteins to determine how ttm2 responds to pathogen attack by bacteria.

Genomic sequence analyses indicated that all three TTM family members are conserved among most plant species, indicating distinct functions of all three TTMs in plants. Interestingly, transcriptional suppression of TTM2 by BTH was observed in soybean and canola, as in Arabidopsis, strongly indicating that the orthologs of TTM2 in these crop plants likely also work as negative regulators of defense responses. This raises the possibility that KO crop mutants for TTM2 will also show enhanced resistance similar to Arabidopsis ttm2 plants, providing a useful tool in agricultural biotechnology to generate pathogen-resistant crop plants.
Involvement of *Arabidopsis triphosphate tunnel metalloenzyme1* (*AtTTM1*) in leaf senescence indicates biological diversification of the TTM family in plants

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4.1 ABSTRACT

The triphosphate tunnel metalloenzyme (TTM) superfamily comprises a group of enzymes that hydrolyze organophosphate substrates. Members of this family have diverse biological functions in different organisms. Arabidopsis encodes three TTM genes, \textit{AtTTM1}, 2 and 3. \textit{AtTTM3} has previously been reported to be involved in root development whereas \textit{AtTTM2} is a negative regulator of pathogen defense. Here, we show that \textit{AtTTM1} and \textit{AtTTM2} are diversified in their biological functions since \textit{AtTTM1} is transcriptionally upregulated during leaf senescence, and knockout mutants of \textit{AtTTM1} exhibited delayed dark-induced senescence. The double mutant of \textit{AtTTM1} and \textit{AtTTM2} displayed the same degree of delayed senescence and disease resistance as the corresponding single mutants, and did not show synergistic effects, further suggesting that they play distinct roles in two independent processes. However, promoter swap analyses revealed that they can functionally complement each other. Taken together, \textit{AtTTM1} has distinct biological roles that are different from those of \textit{AtTTM2} and the transcriptional regulation of the two genes governs their specific biological functions.
4.2 INTRODUCTION

The triphosphate tunnel metalloenzyme (TTM) superfamily comprises two groups of enzymes, RNA triphosphatases and CYTH phosphatases (CyaB adenylate cyclase, thiamine triphosphatase) that possess common characteristics in their catalytic sites (Iyer and Aravind, 2002; Gong et al., 2006). Members of this superfamily are able to hydrolyze a variety of triphosphate substrates, giving them important 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). Most TTMs possess a unique tunnel structure composed of eight antiparallel β strands forming a β barrel and a characteristic ExExK motif (where x is any amino acid), which is important for catalytic activity (Iyer and Aravind, 2002; Lima et al., 1999; Gallagher et al., 2006). In addition, TTMs also share the requirement of a divalent metal cation cofactor, usually Mg\(^{2+}\) or Mn\(^{2+}\) (Bettendorff and Wins, 2013; Moeder et al., 2013).

While the catalytic activity of some TTMs has been elucidated, the biological function of most TTMs is unknown. However, it appears they have acquired the ability to act on a divergent range of nucleotide and organophosphate substrates (Iyer and Aravind, 2002; Bettendorff and Wins, 2013). Known functions of TTMs include fungal and protozoan RNA triphosphatases (Cet1; Lima et al., 1999; Gong et al., 2006) adenylate cyclase (CyaB from Aeromonas hydrophila and YpAC-IV from Yersinia pestis; Sismeiro et al., 1998; Gallagher et al., 2006) and mammalian thiamine triphosphatases (Lakaye et al., 2004; Song et al., 2008). More recently, tripolyphosphatase activity was discovered for CthTTM from Clostridium thermocellum and NeuTTM from Nitrosomonas europaea, highlighting the functional diversity of this superfamily (Keppetipola et al., 2007; Delvaux et al., 2011). In some instances, TTM proteins can also possess additional domains, adding further complexity to their range of functions.

Plant genomes contain two types of TTMs: one with a singular CYTH domain and one with a CYTH domain fused to a P-loop kinase domain (Iyer and Aravind, 2002). In Arabidopsis, there are three TTM family members, AtTTM1, 2 and 3: AtTTM1 and 2 belong to the latter type, possessing a uridine kinase domain in addition to the CYTH domain while AtTTM3 possesses the former type, comprising a single CYTH domain (Fig.1A). Previously, we demonstrated that AtTTM3 exhibits tripolyphosphatase activity and may play a role in root development (Moeder et al., 2013) whereas AtTTM2 is a
negative regulator of pathogen defense responses (Chapter 3; Ung et al., 2014). While AtTTM1 and AtTTM2 possess the same domain arrangement, publicly available microarray data suggests distinct roles for these TTM genes where AtTTM1 is likely involved in leaf senescence.

Leaf senescence is an active and highly regulated process where nutrients are remobilized to other growing tissues of the plant. Individual cells within a leaf undergo metabolic changes in order to dismantle each component before programmed cell death (PCD) occurs and sink-source relationships begin to transition. The initiation of leaf senescence naturally occurs by aging, but can also be induced by a range of external factors such as drought, darkness, detachment, and hormones (ABA, ethylene), resulting in the visible loss of chlorophyll or yellowing since the chloroplasts are the first organelles to be disassembled (Weaver et al., 1998; Breeze et al., 2011). Leaf senescence is generally believed to be a special form of PCD, which shares some, but not all of the characteristics of PCD (van Doorn and Woltering, 2004).

To better understand the molecular events that occur during leaf senescence, changes in gene expression have been monitored (Lohman et al., 1994; Weaver et al., 1998; Breeze et al., 2011). Through cDNA library screens, a class of genes that are reliably upregulated during leaf senescence were identified, termed senescence-associated genes (SAGs) (Lohman et al., 1994; Weaver et al., 1998). By the same approach, senescence-downregulated genes (SDGs) were also identified. Of the SAGs, two have emerged as reliable markers of senescence due to their senescence-specific expression. SAG12, which encodes a papain-like cysteine protease, had no detectable expression in young leaves, but was significantly induced in senescing leaves after visible yellowing had occurred (Lohman et al., 1994; Weaver et al., 1998; Noh and Amasino, 1999). Due to its specificity, it is arguably the best marker of senescence to date. SAG13 encodes a putative tropinone reductase, which is involved in alkaloid biosynthesis, and was also undetectable in younger leaves (Lohman et al., 1994; Weaver et al., 1998). While still senescence-specific, SAG13 was strongly induced shortly before visible yellowing had occurred. Thus, it is a good marker of early senescence. While screening for SDGs, a gene involved in photosynthesis emerged as a reliable reference for the rate of reduction of transcript levels during senescence called chlorophyll A/B binding protein (CAB). CAB transcript levels declined at approximately the same rate as the SDGs under study, which were shown to sharply decrease during
later stages of senescence (Lohman et al., 1994). Therefore, CAB is also a reliable marker for the onset of senescence.

Here, we show that \textit{AtTTM1} plays a role in dark-induced leaf senescence. \textit{AtTTM1} shares high sequence homology with \textit{AtTTM2} at the amino acid level and it can functionally complement the knockout phenotype of \textit{AtTTM2} and vice versa. In spite of the pyrophosphatase activity that is common to both, these two genes do not appear to be involved in the same biological processes. Rather, it is their different gene expression patterns that dictate their distinct biological roles.
4.3 RESULTS

4.3.1 ttm1 plants do not show altered disease resistance

The biological roles of Arabidopsis TTM family members have been previously reported where AtTTM3 potentially plays a role in root development whereas AtTTM2 is a negative regulator of pathogen defense responses (Moeder et al., 2013; Ung et al., 2014; Chapter 3). Since AtTTM1 and AtTTM2 share the same domain arrangement as well as almost 66% amino acid sequence identity, we first analyzed the role of AtTTM1 in pathogen defense responses. Two allelic T-DNA insertion knockout lines of AtTTM1, ttm1-1 and ttm1-2, were isolated for phenotypic characterization (Fig. 4-1). Both mutants exhibited no significant morphological difference to their wild type, Columbia (Col), just like ttm2 mutants (Fig. 4-1C). Following infection with the avirulent Hpa isolate, Emwa1, ttm2 mutants developed substantially more HR cell death compared to wild type, which is an indication of strong defense activation, as expected (Fig. 4-2A, B; p < 0.0001, Fisher’s exact test). On the other hand, ttm1 did not show enhanced HR cell death. Rather, slightly less HR was detected (p = 0.02, Fisher’s exact test); however the significance of this result remains to be determined. Infection with the virulent Hpa isolate, Emco5, revealed that ttm2 plants displayed significantly less growth of the pathogen while ttm1 mutants showed no significant difference from wild type plants (Fig. 4-2C, D). These results suggest that AtTTM1, unlike AtTTM2, is likely not involved in pathogen defense responses.

It has been shown that AtTTM2 is a negative regulator of the SA-dependent amplification loop for pathogen defense responses and is also transcriptionally suppressed approximately 2-fold upon pathogen infection and treatments with the pathogen-associated molecular pattern (PAMP), flg22, the defense hormone salicylic acid (SA) or the biological analog of SA, benzothiadiazole (BTH; Chapter 3; Ung et al., 2014). Therefore, we first monitored the transcript levels of AtTTM1 after pathogen infection. We had previously shown that AtTTM2 expression was suppressed upon infection with the avirulent Hpa isolate, Emwa1 (Fig. 3-3; Ung et al., 2014). In contrast, AtTTM1 expression did not change in both cotyledon and true leaf tissues upon pathogen infection, indicating a fundamental difference in the transcriptional regulation between AtTTM1 and AtTTM2 (Fig. 4-3A, B). These data suggest that AtTTM1 does not
play the same role as \textit{AtTTM2} in pathogen defense and therefore, is likely involved in a different biological process than \textit{AtTTM2}. 
Figure 4-1. T-DNA insertion line analysis.

(A) T-DNA insertion position in *ttm1-1* (SALK_079237) and *ttm1-2* (GABI_672E02). Number above the triangle indicates the exact location of the T-DNA insertion. Filled boxes represent exons, grey represents untranslated regions, and blank spaces represent introns. Arrows and corresponding numbers below the genomic map indicate primer positions.

(B) RT-PCR analysis for *AtTTM1* in Columbia wild type (Col), *ttm1-1* and *ttm1-2*. β-tubulin (*β-tub*) served as loading control. Primer positions are shown in (A) (*AtTTM1 = 1 + 2*). Primer sequences are listed in Table A1.

(C) Morphological phenotype of Col wild type, *ttm1-1* and *ttm1-2*. Photos show approximately 6 week-old plants. Scale bar = 1cm.
Figure 4-2. *ttm1* shows no alteration in its immune response to pathogen infection.

(A) Infection phenotype of Columbia wild type (Col) and *ttm1* plants 10 days after infection with avirulent *Hpa* isolate, Emwa1. Shown is Trypan blue staining of infected cotyledons (cot) and uninfected true leaves (TL) revealing hypersensitive response (HR) cell death. Scale bar = 250µm.

(B) Stained leaves were microscopically examined and assigned to different classes (see index panel). Data shown was taken from 42 – 47 plants. The experiment was repeated four times with similar results. Significantly more HR was observed in *ttm2* plants (p < 0.0001, Fisher’s exact test) whereas significantly less HR was observed in *ttm1* plants (p = 0.02, Fisher’s exact test).

(C) Infection phenotype of Col and *ttm1* plants 12 days after infection with virulent *Hpa* isolate, Emco5. Shown is Trypan blue staining of infected cotyledons revealing hyphae (Hy) and oospores (Oo). Scale bar = 250µm.

(D) Stained leaves were microscopically examined and assigned to different classes (see index panel). Data shown were taken from 30 plants. The experiment was repeated four times with similar results. A statistically significant difference was seen between Col and *ttm2*, but not *ttm1* plants (*ttm1*, p = 0.69; *ttm2*, p < 0.0001; Fisher’s exact test).
Figure 4-3. The transcriptional response of \textit{AtTTM1} does not alter in response to pathogen infection.

(A) Quantitative real-time PCR (qPCR) analysis of \textit{AtTTM1} expression in \textit{Hpa} isolate, Emwa1-infected (Emwa1) or water-treated (H$_2$O) cotyledons of Columbia wild type (Col) plants 7 days after infection. Each bar represents the mean of three independent experiments ± SE. Each sample is a pool of 16 seedlings.

(B) qPCR analysis of \textit{AtTTM1} expression in uninfected true leaves of the same plants. Each bar represents the mean of three independent experiments ± SE. Each sample is a pool of 16 seedlings. 10 day-old seedlings were used for all experiments. Primer sequences are listed in Table A1.
4.3.2 *AtTTM1* and *AtTTM2* are differentially expressed across various tissues

In order to gain insight into the biological role of *AtTTM1*, we examined publically available microarray data from the BAR (Schmid et al., 2005; Toufighi et al., 2005; Winter et al., 2007). While *AtTTM2* was predominantly expressed in the shoot apices of inflorescences, *AtTTM1* appeared to be ubiquitously expressed in all tissues with strong expression detected in senescent leaves (Fig. 4-4A). To validate this, we generated transgenic plants carrying the GUS reporter gene (*uidA*) under the promoters of either *AtTTM1* or *AtTTM2*. Transgenic plants were monitored across various tissues over the course of 9 weeks where GUS activity driven by the *AtTTM1* promoter was ubiquitously detected across all tissues sampled (Fig. 4-4B-A, B, D) with increasing activity in older leaves (Fig. 4-4B-C). In contrast, GUS activity driven by the *AtTTM2* promoter was mostly detected in the shoot apices of vegetative rosettes (Fig. 4-4B-E, F, G, H, I) as well as stigmata and pollen (Fig. 4-4B-J). Considering the identical domain arrangement and the high amino acid sequence identity between AtTTM1 and AtTTM2, these data suggest that the transcriptional regulation of the two genes governs their specific biological functions.
Figure 4-4. AtTTM1 and AtTTM2 are differentially expressed across various tissues.

(A) Tissue-specific expression profile from the Bio-Analytic Resource (BAR, Schmid et al., 2005; Toufighi et al., 2005; Winter et al., 2007). Each bar represents the mean of three biological replicates ± SD.

(B) Activities of the AtTTM1 (A-D) and AtTTM2 (E-J) promoters were histochemically analyzed using promoter-GUS (uidA) reporter constructs introduced into Columbia wild type plants. GUS-staining patterns of cotyledon-stage seedlings (A, E, F), 3 week-old seedlings (B, G, H), and 9 week-old rosette leaves (C, I) and flowers (D, J) are shown. Scale bar = 250μm (A, D, E, G, H, J) or 1mm (B, C, G, I).

(C) Quantitative real-time PCR analysis of AtTTM1 and AtTTM2 expression detached leaves of 4 – 5 week old Columbia wild type plants after dark treatment. Each bar represents the mean of three technical replicates ± SE. Primer sequences are listed in Table A1.
4.3.3 *AtTTM1* is transcriptionally upregulated in senescent leaves

Since *AtTTM1* transcript was found to be relatively abundant in senescent leaves, we hypothesized that *AtTTM1* is involved in leaf senescence and assessed transcriptional changes during senescence using the well-established dark-induced senescence assay (Riefler et al., 2006). Indeed, we could validate the microarray data by monitoring the expression of *AtTTM1* in detached leaves over the course of 7 days in darkness. Where *AtTTM1* transcript levels were already over 3-fold increased after one day in darkness and continued to rise to over 8-fold after 7 days in darkness, *AtTTM2* transcript levels increased after one day, but did not continue to rise over the course of the experiment (Fig. 4-4C). Interestingly, analysis of public expression datasets also revealed that *AtTTM1* is coexpressed with several senescence-related genes, such as *CLPX* (CLP protease regulatory subunit X), *γVPE* (vacuolar processing enzyme/cysteine protease), *SAG12* (cysteine protease), and a cysteine protease superfamily protein (At1g02305; Table 4-1; Expression Angler, BAR, Toufighi et al., 2005). Taken together, these data suggest that *AtTTM1* plays a role in leaf senescence.
### Table 4-1. Genes that are co-expressed with *AtTTM1*.

<table>
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<td>248255_atl</td>
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</table>

Data was obtained from the NASCArray 392 set (Expression Angler, BAR, Toufighi et al., 2007). Genes that are upregulated during leaf senescence are highlighted in yellow.
4.3.4  ttm1 displays delayed dark-induced leaf senescence

To further assess the role of AtTTM1 in leaf senescence, we monitored the senescence progression of detached leaves of Col wild type and ttm1 plants induced by dark treatment. Strikingly, ttm1 leaves consistently displayed less chlorophyll loss during the course of the experiment (Fig. 4-5A). This difference was observed as early as 5 days after dark treatment (not shown), but was most dramatic 7 days after. Measurement of the total chlorophyll content confirmed this observation quantitatively (Fig. 4-5B).

To correlate the loss of chlorophyll in ttm1 leaves with a senescence response, the expression of several senescence markers were monitored. CAB6 and SAG13 are known to be downregulated and upregulated, respectively, during the transition from vegetative growth to senescence whereas SAG12 is senescence-specific and is strongly induced when this process is activated (Lohman et al., 1994). In ttm1 leaves, the transcriptional downregulation of CAB6 was delayed by two days compared to wild type over the course of 7 days in darkness (Fig. 4-5C). In addition, SAG13 transcript levels were visibly lower in ttm1 leaves compared to Col wild type (Fig. 4-5C). Furthermore, SAG12 expression was starkly induced at 5 days after darkness in Col wild type leaves, but did not express in ttm1 leaves until 7 days (Fig. 4-5C).

To confirm that the difference in chlorophyll retention was indeed due to the absence of AtTTM1, a genomic fragment comprising the promoter region and the AtTTM1 gene was introduced into the ttm1-1 knockout mutant background and two independent transgenic lines were analyzed (Fig. 4-6A, B). Complementation of the ttm1-1 mutant plants with wild type AtTTM1 rescued the delayed senescence phenotype of ttm1-1, returning chlorophyll retention to wild type levels (Fig. 4-6C, D).

Since ttm1 leaves displayed a delayed senescence response, we postulated that overexpression of AtTTM1 might quicken senescence. Therefore, we created AtTTM1 overexpression lines where the expression of AtTTM1 is driven by the strong CaMV 35S promoter (Fig. 4-7A). Interestingly, two independent lines displayed the same degree of chlorophyll retention as Col wild type plants (Fig. 4-7B, C). This suggests that elevated levels of AtTTM1 expression do not lead to premature senescence. Taken together, these data suggest that AtTTM1 is a positive regulator of dark-induced senescence.
Figure 4-5. *ttm1* displays delayed dark-induced leaf senescence.

(A) Leaves of Columbia wild type (Col), *ttm1-1*, and *ttm1-2* plants were detached and floated for 7 days on water in the dark. Scale bar = 1cm.

(B) Total chlorophyll content was measured at 0 and 7 days after dark treatment. Each bar represents the mean of three biological replicates ± SE (n = 3). An asterisk denotes significance at p < 0.01 (Student’s t-test). The experiment was repeated three times with similar results.

(C) RT-PCR analysis of senescence markers, *CAB6*, *SAG12*, and *SAG13* in detached leaves of Col wild type and *ttm1-1* plants after dark treatment. *β-tubulin* (*β-tub*) served as loading control. Primer sequences are listed in Table A1.
A. Genomic AtTTM1

B. 5' AtTTM1

\[
\begin{array}{c}
\text{Col} \\
\text{ttm1-1} \\
\text{ttm1/TTM1}
\end{array}
\]

C. Day 0

\[
\begin{array}{c}
\text{Col} \\
\text{ttm1-1} \\
\frac{ttm1-1/TTM1}{#1} \\
\frac{ttm1-1/TTM1}{#4}
\end{array}
\]

Day 7

\[
\begin{array}{c}
\text{Col} \\
\text{ttm1-1} \\
\frac{ttm1-1/TTM1}{#1} \\
\frac{ttm1-1/TTM1}{#4}
\end{array}
\]

D. Chlorophyll Content (μg gFW⁻¹)

\[
\begin{array}{c}
\text{Col} \\
\text{ttm1-1} \\
\text{ttm1-1/TTM1 #1} \\
\text{ttm1-1/TTM1 #4}
\end{array}
\]

- Day 0
- Day 7

\[\text{Day 0} \quad \text{Day 7}\]

- a
- b
Figure 4-6. Isolation and characterization of *ttm1-1/TTM1* complementation lines.

(A) Genotyping of *ttm1-1/TTM1* using genomic DNA. Genomic *AtTTM1* under its native promoter was introduced into *ttm1-1* plants. Genomic *AtTTM1* was detected with 229RP/LP primers. T-DNA was detected with 229RP/LBb1.3 primers. Columbia wild type (Col) was used as a control. Vertical lines indicate splicing of images from the same gel.

(B) RT-PCR analysis of *ttm1-1/TTM1* using cDNA. 5′ *AtTTM1* was detected with 980RT-F/R primers. Full-length *AtTTM1* was detected with 980RT-F/732RT-R primers. β-tubulin (*β-tub*) served as loading control.

(C) Leaves of Col wild type, *ttm1-1*, and two independent *ttm1-1/TTM1* complementation lines were detached and floated for 7 days on water in the dark. Scale bar = 1cm. Primer sequences are listed in Table A1.

(D) Total chlorophyll content was measured 0 and 7 days after dark treatment. Each bar represents the mean of three biological replicates ± SE (n = 3). Statistical significance between groups was determined at p < 0.0001 (Student's t-test). The experiment was repeated twice with similar results.
A

<table>
<thead>
<tr>
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<tr>
<td></td>
<td>Col</td>
</tr>
<tr>
<td>AtTTM1</td>
<td></td>
</tr>
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B

<table>
<thead>
<tr>
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</tr>
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<tr>
<td></td>
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<td>AtTTM1</td>
<td></td>
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<tr>
<td>β-tub</td>
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</tr>
</tbody>
</table>

C

Chlorophyll Content (μg gFW⁻¹)

- Day 0
- Day 7

Col | ttm1-1 | #2 | #11 |

35S:AtTTM1
**Figure 4-7. Overexpression of AtTTM1 does not affect dark-induced senescence.**

(A) RT-PCR analysis of AtTTM1 expression in Columbia wild type (Col) and two 35S:AtTTM1 overexpression lines. β-tubulin (β-tub) served as loading control. Primer sequences are listed in Table A1.

(B) Leaves of Col wild type, ttm1-1, and two independent overexpression lines were detached and floated for 7 days on water in the dark. Shown are samples after 7 days. Scale bar = 1cm.

(C) Total chlorophyll content was measured at 0 and 7 days after dark treatment. Each bar represents the mean of three biological replicates ± SE. Statistical significance between groups was determined at p < 0.05 (Student’s t-test). The experiment was repeated three times with similar results.
4.3.5  *ttm2* does not show a senescence phenotype and the *ttm1 ttm2* double mutant does not exhibit additive effects

Transcriptional analysis of *AtTTM2* upon dark-induced senescence treatment suggested that *AtTTM2* is not involved in senescence and its biological role is different from that of *AtTTM1*. Combined with an absence of a disease resistance phenotype in *ttm1* plants, this indicates that *AtTTM1* and *AtTTM2* are not biologically redundant. To further address this point, we generated a double knockout line by cross-pollination of *ttm1-1* with *ttm2-1* (Fig. 4-8). *ttm1 ttm2* plants do not exhibit any significant morphological differences compared to wild type, indicating that the loss of both TTM superfamily members is not detrimental to plant growth and development (Fig. 4-8A).

Next, we analyzed the phenotypes of *ttm2* and *ttm1 ttm2*. After 7 days in the dark, detached leaves of *ttm2* plants exhibited the same degree of chlorophyll retention as wild type (Fig. 4-9A, B). In contrast, the double mutant displayed the same chlorophyll retention as *ttm1* after 7 days in the dark (Fig. 4-9A,B). Furthermore, pathogen infection using the virulent *Hpa* isolate, Emco5, was also performed on *ttm1*, *ttm2*, and *ttm1 ttm2*. The double mutant displayed a phenotype similar to *ttm2*, while *ttm1* behaved more similarly to Col wild type plants (Fig. 4-9C, D). Taken together, these observations further support the notion that *AtTTM1* and *AtTTM2* are involved in independent biological processes.
Figure 4-8. Isolation of the *ttm1 ttm2* double mutant.

(A) Morphological phenotype of 5 week-old Col wild type (Col), *ttm1-1*, *ttm1-2*, and *ttm1 ttm2* (*ttm1-1 x ttm2-1*) plants. Scale bar = 1 cm.

(B) Genotyping of *ttm1 ttm2* using genomic DNA. Genomic *AtTTM1* or *AtTTM2* were detected with 229RP/LP primers (Lanes 1 and 3) or with 897RP/LP primers (Lanes 2 and 4). The T-DNA insertion within *AtTTM1* or *AtTTM2* was detected with 229RP/LBb1-F primers (Lanes 1 and 3) or with 897RP/LBb1-F primers (Lanes 2 and 4).

(C) RT-PCR analysis of *ttm1 ttm2* using cDNA. *AtTTM1* was detected with 980RT-F/R primers. *AtTTM2* was detected with 190RT-F/R primers. *β-tubulin* (*β-tub*) served as loading control. Vertical lines indicate splicing of images from the same gel. Primer sequences are listed in Table A1.
Figure 4-9. The *ttm1 ttm2* double mutant does not exhibit additive effects.

(A) Leaves of Columbia wild type (Col), *ttm1-1*, *ttm1-2*, and *ttm1 ttm2* (*ttm1-1* x *ttm2-1*) plants were detached and floated for 7 days on water in the dark. Scale bar = 500µm.

(B) Total chlorophyll content was measured at 0 and 7 days after dark treatment. Each bar represents the mean of three biological replicates ± SE. Statistical significance between groups was determined at p < 0.01 (Student’s t-test). The experiment was repeated three times with similar results.

(C) Infection phenotype of Col wild type, *ttm1-1*, *ttm1-2*, and *ttm1 ttm2* plants 11 days after infection with the virulent *Hpa* isolate, Emco5. Shown is Trypan blue staining of infected cotyledons revealing hyphae (Hy) and oospores (Oo). Scale bar = 250µm.

(D) Stained leaves were microscopically examined and assigned to different classes (see index panel). Shown is data taken from 29 – 36 plants. The experiment was repeated four times with similar results. A statistically significant difference was detected between Col versus *ttm2* and *ttm1 ttm2*, but not *ttm1* plants (*ttm1*, p = 0.28; *ttm2*, p < 0.0001; *ttm1 ttm2*, p < 0.0001; Fisher’s exact test). A statistically significant difference was also observed between *ttm1 ttm2* and *ttm1*, but not *ttm2* plants (*ttm1*, p < 0.0001; *ttm2*, p = 0.09; Fisher’s exact test).
4.3.6 AtTTM1 and AtTTM2 can functionally complement each other

AtTTM1 and AtTTM2 exhibit distinct expression patterns and knockout mutant phenotypes. Furthermore, the double mutant did not display enhanced phenotypes for either pathogen resistance or delayed senescence (Fig. 4-9). However, the two genes display over 92% amino acid sequence similarity and all known catalytic amino acid residues are conserved between them. Thus, we asked whether the biological function of the two genes is solely conferred by their expression patterns. To address this question, functional complementation was conducted using four promoter swap constructs: the AtTTM1 promoter followed by either the coding sequence (CDS) of AtTTM1 or AtTTM2 and the AtTTM2 promoter followed by the CDS of either AtTTM1 or AtTTM2. Three independent transgenic lines each were analyzed. The AtTTM1 promoter lines were subjected to the dark-induced senescence assay and revealed that both AtTTM1 as well as AtTTM2 CDS can complement the chlorophyll retention phenotype of ttm1 (Fig. 4-10A). To confirm that the promoter construct behaved as expected, quantitative real-time PCR was used to confirm the upregulation of both AtTTM1 and AtTTM2 under the control of the AtTTM1 promoter after 7 days in dark treatment (Fig. 4-10B).

In the reverse experiment, the AtTTM2 promoter lines were subjected to virulent Hpa infection. Fig. 4-10C shows that expression of either AtTTM2 or AtTTM1 under the AtTTM2 promoter can rescue the reduced susceptibility phenotype of ttm2 to the same degree. Quantitative real-time PCR analysis revealed that both AtTTM1 and AtTTM2 under the control of the AtTTM2 promoter were similarly downregulated after treatment with BTH, as previously shown (Fig. 4-10D; Fig. 3-11B; Ung et al., 2014). Taken together, these data strongly suggest that the enzymatic function of AtTTM1 and AtTTM2 is identical or at least very similar. This suggests that the different expression pattern of these two genes is critical for their specific biological functions.
Figure 4-10. *AtTTM1* and *AtTTM2* can complement each other.

(A) Total chlorophyll content was measured in Columbia wild type (Col), *ttm1-1*, and three independent *ttm1-1* complementation lines expressing *AtTTM1* under its native promoter (*pTTM1:TTM1*) or *AtTTM2* under the *TTM1* promoter (*pTTM1:TTM2*) 0 and 7 days after dark treatment. Each bar represents the mean of three biological replicates ± SE. Statistical significance between groups was determined at p < 0.05 (Student’s t-test). The experiment was repeated four times with similar results.

(B) Quantitative real-time PCR (qPCR) analysis of *AtTTM1* expression in detached leaves of 4 – 5 week-old transgenic plants 0 and 7 days after dark treatment. Transcripts were normalized to *AtEF1a*. Each bar represents the mean of three technical replicates ± SE. Each sample is a pool of 3 leaves.

(C) Trypan blue-stained leaves of Col wild type, *ttm2-1*, and three independent *ttm2-1* complementation lines expressing *AtTTM2* under its native promoter (*pTTM2:TTM2*) or *AtTTM1* under the *TTM2* promoter (*pTTM2:TTM1*) were microscopically examined 12 days after infection with the virulent *Hpa* isolate, Emco5, and assigned to different classes. Shown are data taken from 16 plants. The experiment was repeated four times with similar results.

(D) qPCR analysis of *AtTTM2* expression in seedlings 48hr after treatment with 200µM benzo thiadiazole (BTH) or water (H₂O). Transcripts were normalized to *AtEF1a*. Each bar represents the mean of three technical replicates ± SE. Each sample is a pool of 12 – 14 plants. Primer sequences are listed in Table A1.
4.4 DISCUSSION

The TTM superfamily is characterized by an active site situated within a tunnel composed of antiparallel β-sheets. Furthermore, members of this superfamily act on triphosphate substrates with a strict dependency on a metal cation cofactor. TTM proteins are present in all living organisms where they have taken up a range of different functions, owing to the ability of the tunnel to accommodate different triphosphate substrates (Bettendorff and Wins, 2013).

Plants are unique in two ways: first, they usually possess three TTM genes while other organisms usually only encode one type. Secondly, AtTTM3 and its orthologs comprise only a CYTH domain while AtTTM1 and 2 and their orthologs possess an additional N-terminal uridine kinase domain. This fusion of a uridine kinase and a CYTH domain is only seen in plants and members of the slime mold family (Mycetozoa), such as Dictyostelium discoideum (Iyer and Aravind, 2002). Interestingly, D. discoideum also encodes two TTM genes, udkC and udkD (uridine-cytidine kinase; http://dictybase.org/).

We previously characterized AtTTM3 and showed that it encodes a triplyphosphatase with potential function in root development (Moeder et al., 2013) and AtTTM2 as a negative regulator of the SA amplification loop during pathogen resistance responses (Chapter 3; Ung et al., 2014). Knockout lines of AtTTM1, which shows 65% identity and 92% similarity to AtTTM2 at the amino acid level, did not display enhanced disease resistance, but rather, showed a delayed senescence phenotype, as was expected based on our analysis of publically available microarray data and co-expressed genes. When senescence was induced by dark treatment of detached leaves, ttm1 could retain chlorophyll longer than Col wild type. Furthermore, they displayed delayed induction of the senescence marker genes, SAG12 and SAG13, suggesting that AtTTM1 plays a role during the senescence process.

An obstacle in analyzing natural senescence is that the individual cells of a leaf are at different stages of senescence. Therefore, inducers such as dark treatment of detached leaves have been used to induce a more synchronized senescence response (Weaver et al., 1998; Lin et al., 2004; Buchanan-Wollaston et al., 2005). Buchanan-Wollaston et al. (2005) compared the transcriptional response of natural senescence with dark-induced senescence. They found that 827 genes were upregulated in senescent leaves compared to nonsenescent leaves. Of these genes, approximately
53% were also upregulated in dark-induced senescent leaves. Many of these genes either have putative or determined roles in macromolecule degradation, carbohydrate metabolism, membrane transport, secondary metabolism, and autophagy. This suggests that there are clear molecular differences between natural and dark-induced senescence; however, the overlapping genes may constitute a core senescence pathway of components required for the execution of senescence (van der Graaff et al., 2006). *AtTTM1* is upregulated in both data sets, indicating that it likely plays a specific role in the senescence program. Interestingly, *AtTTM1* was also upregulated in another dataset, which identified genes that are induced during starvation-induced senescence of suspension cells (Swidzinski et al., 2002). These cells also exhibited clear symptoms of programmed cell death (PCD). *AtTTM1* is part of a group of 229 genes that are upregulated in all three types of senescence suggesting that *AtTTM1* is part of a core senescence pathway of components required for the execution of senescence (van der Graaff et al., 2006).

The molecular mechanism underlying the delayed chlorophyll loss in *ttm1* plants is still unclear. However, an obvious function of AtTTM1 could be in pyrimidine catabolism through its uridine kinase domain. Stasolla et al. (2004) suggested that decreased salvage of uracil and uridine and increased salvage of thymidine represents a metabolic switch for the induction of PCD. While the link between senescence and PCD is still unclear, transcriptional profiling studies have shown the induction of PCD-related genes, such as two Arabidopsis metacaspases (*MC1* and *MC2*) and an autophagy gene (*ATG7*), as early as 27 days after sowing and approximately 5 days before the first signs of leaf yellowing occur (Breeze et al., 2011). Thus, future enzymatic studies with AtTTM1 will shed light on the importance of its reaction product in promoting leaf senescence.

Both AtTTM1 and AtTTM2 possess an N-terminal uridine kinase domain adjacent to a CYTH domain. Furthermore, amino acid sequence analysis not only reveals a high sequence identity (approximately 67%) and similarity (over 92%), but the known catalytic residues of each domain are also conserved between AtTTM1 and AtTTM2. This suggests that the enzymatic function of these proteins is either very similar or identical. To date, several TTM superfamily members have been biochemically characterized. For instance, fungal and protozoan TTMs have been shown to function as RNA triphosphatases (Cet1) whereas mammalian TTMs exhibit thiamine triphosphatase activity (Lima et al., 1999; Lakaye et al., 2004; Gong et al., 2006; Song et al., 2008).
Furthermore, TTM s from *Aeromonas hydrophila* and *Yersinia pestis* display adenylate cyclase activity and triplyphosphatase activity was recently shown for *Clostridium thermocellum* (CthTTM), *Nitrosomonas europaea* (NeuTTM), and *Arabidopsis thaliana* (AtTTM3; Keppetipola et al., 2007; Delvaux et al., 2011; Moeder et al., 2013). Despite the substrate diversity of TTM s, every member studied to date exhibits activity on a triphosphate (Bettendorff and Wins, 2013). Thus, it is important to analyze whether AtTTM1 and AtTTM2 also display activity on a triphosphate substrate or whether they have developed a different substrate preference. Their enzymatic activity will be discussed in Chapter 5.

*AtTTM1* and *AtTTM2* knockout lines displayed distinct phenotypes in senescence and disease resistance, respectively. Considering the high homology in their catalytic region (98% and 91% similarity in the uridine kinase and CYTH domains, respectively), it was of question whether they would act on the same substrate. Therefore, we tested whether *AtTTM1*, under control of the *AtTTM2* promoter, could complement the *ttm2* mutant phenotype and vice versa. In both cases, one paralog could complement the other mutant paralog phenotype and vice versa, suggesting that the substrate may be similar or identical for both proteins. This suggests that the spatial and temporal differences in the expression of *AtTTM1* and *AtTTM2* determine the different functions during senescence and disease resistance. Therefore, the identification of the *in vivo* substrate of AtTTM1 and AtTTM2 will be of utmost importance in order to elucidate the molecular mechanisms underlying the involvement of AtTTM1 and AtTTM2 in these processes.
Biochemical characterization of AtTTM1 and AtTTM2

Majority of this work has been previously submitted as:

Ung H, Moeder W, and Yoshioka K. (2014) Involvement of Arabidopsis triphosphate tunnel metalloenzyme1 (AtTTM1) in leaf senescence indicates biological diversification of the TTM family in plants. Submitted to Plant Cell.
5.1 ABSTRACT

As previously mentioned, Arabidopsis possesses three TTM genes, *AtTTM1*, *AtTTM2*, and *AtTTM3*. It was demonstrated that each of these members plays distinct biological roles: *AtTTM1* is involved in promoting leaf senescence; *AtTTM2* is a negative regulator of pathogen defense responses; and *AtTTM3* appears to play a role in root development. All three *AtTTM* members are annotated with a CYTH domain. *AtTTM3* comprises a sole CYTH domain while *AtTTM1* and *AtTTM2* are composed of a P-loop kinase domain adjacent to the CYTH domain. The function of *AtTTM3* was previously determined, revealing tripolyphosphatase activity in the presence of Mg$^{2+}$. Additionally, *AtTTM3* exhibits canonical TTM characteristics, possessing a catalytic tunnel comprising eight antiparallel $\beta$ strands and requiring several conserved residues for activity. In this unpublished chapter, I show that *AtTTM1* and *AtTTM2* share high sequence homology with over 92% sequence similarity and approximately 67% identity. Interestingly, sequence analysis with other CYTH domain proteins reveals that *AtTTM1* and *AtTTM2* lack the majority of conserved catalytic residues in the CYTH domain. However, we demonstrate that both *AtTTM1* and *AtTTM2* possess *in vitro* Mg$^{2+}$-dependent phosphatase activity with strong affinity for pyrophosphate and a weaker activity for ATP and ADP. While *AtTTM1*, *AtTTM2*, and *AtTTM3* are annotated with adenylate cyclase function, we show that this is not the case. Thus, the unique substrate specificity observed in *AtTTM1* and *AtTTM2* compared to other studied TTMs could have arisen from the loss of these conserved residues in the CYTH domain or perhaps, the presence of an additional domain.
The TTM superfamily comprises two previously existing classes of enzymes: RNA triphosphatases and the CYTH phosphatases (Iyer and Aravind, 2002; Gong et al., 2006). RNA triphosphatases play an important role in mRNA capping. The 5’ 7-methylguanosine cap of eukaryotic mRNA is formed by three reactions, the first of which involves the action of RNA triphosphatase in the hydrolysis of the 5’ triphosphate end of nascent pre-mRNA to a diphosphate. The prototypical member of this class of enzymes is Cet1 from \textit{Saccharomyces cerevisiae}. Crystal structure analysis revealed that the active site of Cet1 resembles a tunnel comprising eight antiparallel $\beta$ strands and biochemical analysis showed the requirement of a divalent metal ion for activity (Lima et al., 1999). Interestingly, CYTH phosphatases also share these structural properties as well as the requirement for a metal cofactor (Iyer and Aravind, 2002). Thus, the RNA triphosphatases and CYTH phosphatase families were merged to form a new superfamily, called the tunnel triphosphate metalloenzymes (TTMs; Gong et al., 2006).

CYTH phosphatases exhibit phosphohydrolase activity on a variety of organophosphate substrates. The canonical members of this subgroup of the TTM superfamily are an adenylate cyclase from \textit{Aeromonas hydrophila} called CyaB and mammalian thiamine triphosphatase (Sismeiro et al., 1998; Lakaye et al., 2004; Song et al., 2008). CyaB exhibits unusual hyperthermophilic properties in addition to high sequence homology to proteins from hyperthermophilic bacteria (Sismeiro et al., 1998). When a large-scale database search for other related proteins was conducted, mammalian thiamine triphosphatase was identified as a closely related protein based on sequence analysis (Iyer and Aravind, 2002). Position-specific iterative BLAST (psi-BLAST) searches using CyaB and thiamine triphosphatase as template sequences revealed a larger group of proteins that possess similar sequence characteristics. For instance, the ExExK motif (where x is any amino acid) was identified as a consensus sequence among all CYTH domain proteins and later determined to be catalytically important for activity (Lima et al., 1999; Iyer and Aravind, 2002; Gallagher et al., 2006). Additionally, a conserved set of 5 basic residues and 6 acidic residues was also identified to be involved in the binding of the phosphate moieties and coordination of the two divalent metal cofactors, respectively (Iyer and Aravind, 2002). Interestingly, sequence analysis of AtTTM1 and AtTTM2 reveals that not only do they possess a
TYILK sequence in place of the ExExK motif, but also only 1 out of 6 acidic residues and 4 out of 5 basic residues are conserved. While the CYTH domain is commonly the sole domain in CYTH domain proteins, it can also be found fused to additional domains, such as a P-loop kinase domain. This fusion is only found in CYTH domain proteins from plants and slime molds, such as *Dictyostelium discoideum*. In both cases, the CYTH domain lacks the majority of the conserved acidic and basic residues.

The P-loop kinase domain found in *AtTTM1* and *AtTTM2* is typically found in proteins that belong to the P-loop kinase family (Leipe et al., 2003). At the sequence level, P-loop kinases can be identified by the presence of several conserved motifs: Walker A (GxxxxGK[ST], where x is any residue), Walker B (hhhhD, where h is any hydrophobic residue), and a lid module (Rx₂₃R, where x is any residue). Studies using the *Mus musculus* bifunctional ATP sulfurylase/adenosine 5'-phosphosulfate (APS) kinase enzyme revealed the catalytic importance of the Walker A motif where mutation of the last three residues (G, K, T) abrogated APS kinase activity almost completely (Deyrup et al., 1998). The aspartate in the Walker B motif is thought to coordinate the binding of a metal cofactor whereas the lid module motif helps to stabilize the interactions between the protein and the substrate (Leipe et al., 2003).

*AtTTM3* differs from *AtTTM1* and *AtTTM2* since it only comprises a CYTH domain. It possesses an intact ExExK motif along with all conserved acidic and basic residues. Thus, it was purported to have adenylate cyclase activity or some other conserved CYTH domain-related function. However, it was shown that *AtTTM3* is not an adenylate cyclase, but rather, displayed Mg²⁺-dependent triphosphatase (PPPase) activity and a low level of ATPase activity in the presence of several metal cation cofactors, including Mg²⁺, Co²⁺, and Mn²⁺ (Moeder et al., 2013). Several key residues were shown to be important for NTPase and PPPase activity, such as E2, R52, and E171. The equivalent residue of R52 in the TTM protein from *Plasmodium falciparum* (Prt1), R197, was shown to be required for enzymatic activity (Gong et al., 2006). Furthermore, crystallographic analysis with *S. cerevisiae* Cet1 revealed that the equivalent residue, R393, coordinates the binding of a sulfate ion (Lima et al., 1999). Thus, it was hypothesized that this arginine in *AtTTM3* is required for catalysis through phosphate binding (Moeder et al., 2013). Furthermore, crystallographic analysis of *AtTTM3* revealed the characteristic catalytic tunnel with inwardly facing acidic residues that are likely involved in catalysis, which includes E2, R52, and E171. The biochemical
properties exhibited by AtTTM3 closely resemble that of previously studied TTM from Clostridium thermocellum (CthTTM) and Nitrosomonas europaea (NeuTTM) (Keppetipola et al., 2007; Delvaux et al., 2011).

Here, we report that AtTTM1 and 2 possess pyrophosphatase activity in the presence of Mg^{2+}. Furthermore, preliminary activity assays suggest that they do not function as adenylate cyclases or uridine kinases, despite being annotated with such functions.
5.3 RESULTS

5.3.1 AtTTM1 and AtTTM2 share many common properties

*Arabidopsis thaliana* possesses three *TTM* genes (Moeder et al., 2013). AtTTM3 comprises only a CYTH domain, but possesses an intact catalytic ExExK (where x is any residue) motif (EVEVK) as well as all conserved acidic (6 D/E) and basic (5 K/R) residues (Iyer and Aravind, 2002; Moeder et al., 2013). Interestingly, AtTTM1 and AtTTM2 possess a CYTH domain adjacent to an N-terminal P-loop kinase domain, which is annotated as a uridine kinase (UK) domain (Fig. 5-1A). Both proteins possess approximately 66% overall sequence identity, which increases within individual domains to 81.4% (UK domain) and 72.6% (CYTH domain; Table 5-1). In addition, AtTTM1 and AtTTM2 possess C-terminal coiled coil and transmembrane domains (Fig. 5-1A). Thus, AtTTM1 and AtTTM2 differ dramatically from AtTTM3.

AtTTM1 and AtTTM2, however, share many common properties. For instance, key residues of the CYTH domain, which are fully conserved in AtTTM3, are mostly missing in AtTTM1 and AtTTM2. These include the catalytic motif, ExExK, as well as 5 out of 6 acidic and 1 out of 5 basic residues that are required for metal cofactor and substrate binding (Fig. 5-1B; Iyer and Aravind, 2002). Furthermore, the specific variations that are found within each motif of the P-loop kinase domain are also identical. These modifications include a glycine residue that resides just outside of the Walker A motif, a glutamate and glycine residue that are found in place of the aspartate residue in the Walker B, and an additional [RK]xx (where x is any residue) motif can be found adjacent to the lid module motif (Fig 5-1B; Leipe et al., 2003). This suggests that AtTTM1 and AtTTM2 have either identical or very similar enzymatic properties.

Many other plant species also possess both TTM1 and TTM2 orthologs. In addition to falling into separate TTM1 and TTM2 clades, these orthologs also share high sequence homology with AtTTM1 and AtTTM2 (Fig. 5-2; Table 5-2). The orthologs in canola, soy, cucumber, and orange display complete conservation of the Walker A, Walker B, lid module, and TYILK sequences as well as the key acidic and basic residue replacements that are found in the CYTH domains of AtTTM1 and AtTTM2. This suggests that the function of AtTTM1 and AtTTM2 may be conserved in these plant orthologs. Indeed, we recently reported that the *AtTTM2* orthologs in canola and soy are...
transcriptionally downregulated in response to SA and BTH treatment similar to the response of AtTTM2 (Fig. 3-13A, B; Ung et al., 2014).
### Table 5-1. Sequence homology of AtTTM1 and AtTTM2.

<table>
<thead>
<tr>
<th>Domain</th>
<th>AtTTM1 (aa)</th>
<th>AtTTM2 (aa)</th>
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<th>% Similarity</th>
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<td>65 - 237</td>
<td>PRINTS</td>
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<td>CYTH</td>
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<td>252 - 408</td>
<td>Pfam</td>
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<td>91.1</td>
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</tbody>
</table>

UK, uridine kinase; CYTH, CyaB, thiamine triphosphatase; aa, amino acid
TAIR, http://arabidopsis.org
PRINTS, http://www.bioinf.manchester.ac.uk/dbbrowser/PRINTS/index.php
Pfam, http://pfam.xfam.org
Figure 5-1. AtTTM1 and AtTTM2 share high sequence homology.

(A) Domain annotations of AtTTM1 and AtTTM2, referenced from The Arabidopsis Information Resource (TAIR). UK, uridine kinase; CYTH, CyaB thiamine triphosphatase; CC, coiled-coil; TM, transmembrane.

(B) Amino acid sequence alignment of TTM1 and TTM2 consensus motifs. Asterisks (*) indicate identical match, colons (:) indicate a highly similar match, and dots (.) indicate a similar match based on shared biochemical properties. Consensus sequences are underlined. Structural features are bolded. Red, conserved acidic residue positions; blue, conserved basic residue positions.
Figure 5-2. *AtTTM1* and *AtTTM2* are conserved across other plant species.

Evolutionary relationship of *TTM1* and *TTM2* family members inferred using the Neighbour-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The tree is drawn to scale and the evolutionary distances were computed using the p-distance method (units = number of amino acid differences per site). The analysis involved 16 amino acid sequences. There were a total of 200 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).
Table 5-2. Sequence homology of AtTTM1 and AtTTM2 orthologs.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Ortholog</th>
<th>AtTTM1 % Identity</th>
<th>AtTTM2 % Identity</th>
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</thead>
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<td>-</td>
<td>65.7</td>
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<td>AtTTM1</td>
<td>74.7</td>
<td>70.9</td>
</tr>
</tbody>
</table>

At, *Arabidopsis thaliana*; Glyma, *Glycine max* (soybean); Bra, *Brassica rapa* (canola); Cucsa, *Cucumis sativus* (cucumber); Csi, *Citrus sinensis* (orange)
5.3.2 AtTTM1 and AtTTM2 possess pyrophosphatase (PPase) activity

Since AtTTM3 displayed strong tripolyphosphatase activity, we assessed the enzymatic properties of AtTTM1 and AtTTM2 on several organophosphate substrates. To facilitate solubility, the C-terminal transmembrane (TM) domain (from S621 and S648, respectively) of AtTTM1 and AtTTM2 was removed from the coding sequence and truncated AtTTM1 (ΔTM) and AtTTM2 (ΔTM) were both expressed as N-terminal glutathione-S-transferase (GST) fusion proteins in E. coli BL21 codon plus cells, resulting in a molecular weight of approximately 97kDa and 101kDa, respectively (Fig. 5-3A). Purification by affinity chromatography using glutathione agarose yielded modest levels of soluble AtTTM1 and AtTTM2, which could be detected by Western blot using an anti-GST antibody (Fig. 5-3B).

While AtTTM3 showed strong activity with tripolyphosphate (PPP), weaker activity with ATP and no activity with pyrophosphate (PP) (Moeder et al., 2013), AtTTM1 and AtTTM2 surprisingly displayed strongest activity with PP, weaker activity with ATP and ADP, and almost none with PPP (Fig. 5-4A). Since AtTTM1 and AtTTM2 were both expressed as GST fusion proteins, protein extracted from E. coli expressing the GST tag alone confirmed that the observed activities were not due to contaminating bacterial proteins (Fig. 5-4A, GST). The pyrophosphatase activity of AtTTM1 and AtTTM2 was further characterized by testing a pH range of 6 – 10 in the presence of Mg²⁺ and by testing a range of cofactors buffered at pH 9. Interestingly, while pyrophosphatase activity was optimal from pH 8 – 9 for both TTM1 and 2, relatively strong activity was still observed between pH 6 – 10 (Fig. 5-4B). A number of divalent metal cofactors were tested at pH 9 and revealed a strong preference for Mg²⁺ (Fig. 5-4C).
Figure 5-3. Protein expression and purification of AtTTM1 and AtTTM2.

(A) Expression and purification of AtTTM1 (left) and AtTTM2 (right). N-terminal GST fusions were expressed in *E. coli* BL21 codon plus cells and purified by affinity chromatography using glutathione agarose. Each lane was loaded with 20μl of the specified fraction and analyzed by SDS-PAGE followed by Coomassie Brilliant Blue R-250 staining. Molecular weight markers are shown in kiloDaltons (kDa). P, pellet; S, soluble; FT, flow-through; W, wash.

(B) Western blot using a nitrocellulose membrane probed with mouse anti-GST antibody followed by horseradish peroxidase (HRP)-conjugated anti-mouse antibody. Glutathione-S-transferase (GST), 25kDa; AtTTM1 (left), 97kDa; AtTTM2 (right), 101kDa.
**Figure 5-4. AtTTM1 and AtTTM2 exhibit phosphatase activity.**

(A) Substrate specificity of AtTTM1 and AtTTM2. Phosphate release was tested with pyrophosphate (PP_i), adenosine triphosphate (ATP), tripolyphosphate (PPP_i), and adenosine diphosphate (ADP). Reactions were performed at 37°C at pH 9.0 in the presence of 2.5mM Mg^{2+}, 0.5mM substrate (except 0.03mM ADP), and 2μg of protein. GST, glutathione-S-transferase (tag).

(B) Pyrophosphatase (PPase) activity of AtTTM1 and AtTTM2 as a function of pH. Reactions were performed at 37°C in the presence of 0.5mM Mg^{2+}, 0.5mM pyrophosphate, and 2μg of protein.

(C) Divalent cation specificity of AtTTM1 and AtTTM2 PPase activity. Reactions were performed at 37°C at pH 9.0 in the presence of 0.5mM cation cofactor, 0.5mM pyrophosphate, and 2μg of protein. Phosphate release was detected by the Malachite green assay. All bars represent the mean of three technical replicates ± SE.
5.3.3 AtTTM1 and AtTTM2 exhibit high affinity for pyrophosphate

Kinetic analysis was also conducted for AtTTM1 and AtTTM2 to determine the affinity for pyrophosphate in the presence of Mg\(^{2+}\). The $K_m$ values were determined to be $16.7 \pm 4.7\, \mu M$ ($V_{\text{max}} = 284 \pm 19\, \text{nmol}\cdot\text{min}^{-1}\cdot\mu\text{g}^{-1}$) and $17 \pm 2.9\, \mu M$ ($V_{\text{max}} = 365 \pm 15\, \text{nmol}\cdot\text{min}^{-1}\cdot\mu\text{g}^{-1}$) for AtTTM1 and AtTTM2, respectively (Fig. 5-5). Although the substrate specificities are different between AtTTM1 and AtTTM2 versus AtTTM3, the $K_m$ observed for AtTTM1 and AtTTM2 for pyrophosphate is within the same order of magnitude as the $K_m$ reported for AtTTM3, which was $42.9 \pm 8\, \mu M$ (Moeder et al., 2013).

In order to determine the specificity of pyrophosphate as a substrate for AtTTM1 and AtTTM2, other diphosphate molecules were tested. Adenosine diphosphate (ADP) ribose possesses a diphosphate bond situated between adenosine and ribose moieties (Fig. 5-6A). AtTTM1 and AtTTM2 activity is possible when the resulting exposed phosphates of the cleaved diphosphate bridge are further digested by an organic phosphatase enzyme, such as calf intestine alkaline phosphatase (CIAP), which would release free phosphate. The specific action of CIAP on exposed phosphate moieties can be seen in the presence of ADP, but not on ADP ribose where the diphosphate moiety is bonded to adenosine and ribose (Fig. 5-6B). Very little phosphate release was detected in the presence of AtTTM1 or AtTTM2 even with CIAP cleavage, indicating that ADP ribose is not a substrate of AtTTM1 or AtTTM2 (Fig. 5-6C). Next, we tested thiamine pyrophosphate (ThPP), which possesses an exposed pyrophosphate moiety. However, free phosphate release by the action of AtTTM1 and AtTTM2 was also very low (Fig. 5-6D). Lastly, reduced nicotinamide adenine dinucleotide (NADH) was tested, which also possesses a contained diphosphate moiety and requires the action of CIAP for detection of free phosphates (Fig. 5-6E). In the presence of CIAP, AtTTM1 and AtTTM2 did not exhibit any phosphate release. Thus, so far, inorganic pyrophosphate is the only identified in vitro substrate.
**Figure 5-5.** AtTTM1 and AtTTM2 exhibit strong pyrophosphatase (PPase) activity.

PPase activity of AtTTM1 and AtTTM2 as a function of pyrophosphate (PP_i) concentration. Reactions were performed at 37°C at pH 9.0 in the presence of 0.5mM Mg^{2+} and 1μg of protein. Phosphate release was detected by the Malachite green assay. Data points represent the mean of three technical replicates ± SE. Kinetic parameters for AtTTM1 (K_m = 16.71 ± 4.71μM, V_max = 284.09 ± 19.47nmol·min^{-1}·μg^{-1}) and AtTTM2 (K_m = 17.00 ± 2.92μM, V_max = 365.83 ± 15.05nmol·min^{-1}·μg^{-1}) were obtained by GraFit (Erithacus Software Ltd.).
Figure 5-6. AtTTM1 and AtTTM2 have specific pyrophosphatase activity.

(A) Chemical composition of various diphosphate molecules. ADP, adenine diphosphate; ADP ribose, adenine diphosphate ribose; ThPP, thiamine pyrophosphate; NADH, reduced nicotinamide adenine dinucleotide. All images were retrieved from the Wikipedia Commons.

(B) Phosphatase activity of calf intestine alkaline phosphatase (CIAP). Phosphate release was tested with ADP and ADP ribose.

(C-E) Phosphatase activity of AtTTM1 and AtTTM2 on (C) ADP ribose, (D) ThPP, and (E) NADH. All reactions were performed at 37°C at pH 9.0 in the presence of 2.5mM Mg²⁺, 0.5mM substrate, and 2μg of protein. All reactions with CIAP were performed with 20 units of CIAP. Phosphate release was detected by the Malachite green assay. All bars represent the mean of three technical replicates ± SE. GST, glutathione-S-transferase (tag).
5.3.4 Selected mutations in \textit{AtTTM1} do not abolish PPase activity

Several studies have identified key residues required for activity for both the P-loop kinase domain and the CYTH domain. For example, the \textit{Mus musculus} bifunctional enzyme, ATP sulfurylase/APS kinase, contains a P-loop in the APS kinase portion of the protein. Through a series of substitutions in the Walker A motif (GLSGAGKT), it was determined that the last three residues (G, K, T) were each essential for APS kinase activity, but did not affect ATP sulfurylase activity (Deyrup et al., 1998). Likewise, several key residues have been identified to be necessary for the enzymatic activity of TTM proteins. For instance, substitution of 10 of the inwardly facing arginine and glutamate side chains of the catalytic tunnel of Prt1 determined that 8 of the 10 are required for catalysis (Gong et al., 2006). Prt1 R197 was one of these residues and is the equivalent of Cet1 R393, which was shown to coordinate a sulfate ion through crystallographic analysis (Lima et al., 1999). The same residue in AtTTM3, R52, was also shown to be required for both PPPase and ATPase activity (Moeder et al., 2013). Thus, it is likely that this arginine residue is required for catalysis through its role in phosphate binding.

To examine the contribution of the UK and the CYTH domains to the PPase activity of AtTTM1, three mutations were created: 1) the conserved lysine in the P-loop Walker A motif was substituted with an alanine (K78A); 2) the CYTH ExExK motif was reconstituted by substitution of TYILK with EYELK; and 3) the AtTTM1 equivalent of the conserved arginine (Prt1 R197, Cet1 R393, AtTTM3 R52) was substituted with an alanine (R299A). These mutations were introduced into the truncated version of AtTTM1 (ΔTM), sequenced, and expressed as N-terminal glutathione-S-transferase (GST) fusion proteins. Purification by affinity chromatography using glutathione agarose yielded variable concentrations of soluble AtTTM1 protein (Fig. 5-7A). Out of 6 protein extractions from independent transformants for the K78A mutation (K1 through K6), protein could be detected from 4, as indicated by the presence of the 101kDa band (Fig. 5-7A, B). Out of the protein extractions that yielded detectable levels of target protein, K2 and K5 showed very little to no activity while K1 and K6 did show activity levels resembling that of wild type AtTTM1. Thus, it is likely that the K78 position is not required for PPase activity; however, this should be confirmed with an optimized protein extraction protocol.
For the TYILK substitution to EYELK (referred to as the “ExExK” mutation), protein samples from 4 independent transformants (E1 through E4) were analyzed. All but one protein sample (E1, E2, E4) showed comparable activity to wild type AtTTM1, which could be correlated with the presence of visible protein bands (Fig. 5-7A, B). Similar to K2 and K5, E2 was visible by SDS-PAGE, but only relatively weak PPase activity could be detected. However, considering the activity of the other 3 protein samples, it is likely that this mutation does not abolish PPase activity. The R299A mutation also did not abolish PPase activity, as phosphate release comparable to wild type AtTTM1 could be detected from both protein samples from independent transformants, which was also correlated with the presence of visible protein bands by SDS-PAGE (Fig. 5-7A, B).
Figure 5-7. Selected mutations in AtTTM1 do not abolish PPase activity.

(A) Expression and purification of wild type and mutant forms of AtTTM1 (K78A, ExExK, R299A). N-terminal GST fusions were expressed in E. coli BL21 codon plus cells and purified by affinity chromatography using glutathione agarose. Each lane was loaded with 3μg of total protein and analyzed by SDS-PAGE followed by Coomassie Brilliant Blue R-250 staining. Molecular weight markers are shown in kilodaltons (kDa). P, pellet; S, soluble; FT, flow-through; W, wash. Glutathione-S-transferase (GST), 25kDa; AtTTM1, 97kDa.

(B) Phosphatase activity of wild type and mutant forms of AtTTM1 on pyrophosphate (PPi) and adenosine triphosphate (ATP). All reactions were performed at 37°C at pH 9.0 in the presence of 2.5mM Mg2+, 0.5mM sodium pyrophosphate or ATP, and 2μg of protein. Phosphate release was detected by the Malachite green assay. Each bar represents the mean of three technical replicates ± SE. Vertical lines represent independent experiments. K, K78A; E, ExExK; R, R299A. Numbers indicate independent transformants.
5.3.5 AtTTM1 and AtTTM2 do not possess uridine kinase or adenylate cyclase activity

AtTTM1 and AtTTM2 possess an N-terminal region comprising a UK domain adjacent to a CYTH domain. This UK domain is likely annotated as such due to the distinct modifications of the consensus sequences that are found in the motifs within the P-loop kinase domain (Leipe et al., 2003). Thus, AtTTM1 and AtTTM2 could potentially act on uridine (forming uridine monophosphate; UMP) or ATP (forming cyclic-adenosine monophosphate; cAMP) on the basis of the presence of each domain. We first assessed the UK activity of AtTTM1 and AtTTM2 on uridine. At 37°C and pH 8 in the presence of Mg\(^{2+}\), we could not detect UMP formation by HPLC analysis despite clear resolution of signal peaks for ATP, ADP, uridine, and UMP standards (Fig. 5-8A). However, recombinantly expressed Arabidopsis uridine kinase-like1 (AtUKL1, At5g40870) could form UMP under the same conditions (Fig. 5-8A-C). Next, we tested the AC activity of AtTTM1 and AtTTM2 using ATP as a substrate. At 37°C and pH 8 in the presence of Mg\(^{2+}\), we could not detect cAMP formation by HPLC analysis despite clear resolution of signal peaks for ATP and cAMP standards (Fig. 5-9). Thus, it is likely that AtTTM1 and AtTTM2 do not possess UK or AC activity, at least under these experimental conditions.
Figure 5-8. AtTTM1 and AtTTM2 do not exhibit uridine kinase (UK) activity.

(A) Reactions were performed at 37°C at pH 8.0 in the presence of 10mM Mg\(^{2+}\), 1mM ATP, 1mM uridine, and 2\(\mu\)g of protein. High performance liquid chromatography (HPLC) analysis was conducted to resolve the reaction products in isocratic solvent (20% methanol, 150mM sodium acetate buffer pH 5.0). UMP, uridine monophosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate.

(B) Expression and purification of AtUKL1 (At5g40870). N-terminal 6xHis fusions were expressed in *E. coli* BL21 codon plus cells and purified by affinity chromatography using Ni-NTA agarose. Each lane was loaded with 20\(\mu\)l of the specified fraction and analyzed by SDS-PAGE followed by Coomassie Brilliant Blue R-250 staining. Molecular weight markers are shown in kiloDaltons (kDa). P, pellet; S, soluble; FT, flow-through; W, wash.

(C) Western blot using a nitrocellulose membrane probed with mouse anti-His antibody followed by horseradish peroxidase (HRP)-conjugated anti-mouse antibody. AtUKL1, 55kDa.
Figure 5-9. AtTTM1 and AtTTM2 do not exhibit adenylate cyclase (AC) activity. Reactions were performed at 37°C at pH 8.0 in the presence of 10mM Mg$^{2+}$, 1mM ATP, and 2μg of protein. High performance liquid chromatography (HPLC) analysis was conducted to resolve the reaction products in isocratic solvent (20% methanol, 150mM sodium acetate buffer pH 5.0). ATP, adenosine triphosphate; ADP, adenosine diphosphate; cAMP, 3',5'-cyclic adenosine monophosphate.
5.4 DISCUSSION

The TTM superfamily in *Arabidopsis thaliana* comprises three members, AtTTM1, AtTTM2, and AtTTM3. AtTTM1 and AtTTM2 possess a rare combination of two domains, namely the P-loop kinase domain and the CYTH domain. The full-length primary sequences of AtTTM1 and AtTTM2 share almost 67% identity and over 92% similarity. A closer look at the individual domains shows that this high sequence homology can be attributed to the UK domain (81% identity) and to the CYTH domain (~73%). Thus, it was predicted that both AtTTM1 and AtTTM2 would have similar substrate preferences. We observed some ATPase activity in the presence of Mg\(^{2+}\), but much stronger activity with pyrophosphate in the presence of Mg\(^{2+}\) (K\(_m\) = 16.71 \(\mu\)M and 17.00 \(\mu\)M, respectively). Biochemical analyses of several TTM proteins show substrate preferences for ATP, PPPi, thiamine triphosphate, or phosphate-terminated RNA, which are all triphosphate molecules. For instance, AtTTM3 exhibits ATPase activity in the presence of a range of metal cofactors, such as Mn\(^{2+}\), Co\(^{2+}\), Mg\(^{2+}\), and Zn\(^{2+}\), but exhibits strong triphosphatase activity only in the presence of Mg\(^{2+}\) (Moeder et al., 2013). PPPase activity is also exhibited by NeuTTM from *N. europaea* as well as CthTTM from *C. thermocellum* (Keppetipola et al., 2007; Delvaux et al., 2011). On the other hand, RNA triphosphatases like Cet1 and Prt1 exhibit phosphatase activity on the 5' triphosphate end of nascent pre-mRNA (Lima et al., 1999; Gong et al., 2006). AtTTM1 and AtTTM2 are the first TTM proteins that have been reported to possess activity for PPPi where TTM proteins have only been reported to exclusively catalyze the hydrolysis of triphosphate compounds (Bettendorff and Wins, 2013). AtTTM1 and AtTTM2 are both missing the catalytic motif, ExExK, as well as the majority of the acidic residues (5 out of 6) required for metal binding. However, pyrophosphatase activity can still be detected only in the presence of Mg\(^{2+}\). Thus, it is possible that AtTTM1 and AtTTM2 have acquired novel substrate specificity through this deviated version of the CYTH domain.

In addition to the CYTH domain, AtTTM1 and AtTTM2 also have an N-terminal P-loop kinase domain that is adjacent to the CYTH domain. This rare combination of domains is only found in CYTH domain homologs that have lost the conserved residues, and so far, is only found in plant homologs and slime molds, such as *Dictyostelium discoideum* (Iyer and Aravind, 2002). The P-loop kinase domain found in AtTTM1 and AtTTM2 is also found in proteins that are part of a larger family of kinases called the P-
loop kinases (Leipe et al., 2003). This domain comprises the Walker A (GxxxxGK[ST], where x is any residue), Walker B (hhhhD, where h is any hydrophobic residue), and lid module (Rx_{2-3}R, where x is any residue) motifs (Leipe et al., 2003). Substrate binding is coordinated by the Walker A motif, metal cofactor binding by the Walker B motif, and the lid module helps to stabilize these interactions.

The P-loop kinase domain is found in 41 different protein families, which possess distinct modifications of these motifs. On the basis of these modifications, AtTTM1 and AtTTM2 belong to the UCPP protein family, which encompasses proteins with uridine/cytidine kinase, phosphoribulokinase, pantothenate kinase, fructokinase, and 2-phosphoglycerate kinase activities (Leipe et al., 2003). This protein family is exemplified by a modified hhhhEG (where h is any hydrophobic residue) Walker B motif, [RK]xxRxxxR (where x is any residue) lid module, and a small residue (G, A, or S) just outside of the Walker A motif. Further modifications are present for each of these protein classes. For instance, bacterial pantothenate kinases, such as *E. coli* CoA, lack the first conserved arginine within the lid module motif (Leipe et al., 2003). CoA homologs are not found in eukaryotes, which possess an unrelated enzyme with pantothenate kinase function. Fructokinases from bacteria, plants, and fungi are tightly conserved and possess distinct Walker A (GPPGxGKST, where x is any residue) and Walker B (hhhEGN, where h is any hydrophobic residue) motifs. The 2-phosphoglycerate kinases represent the most divergent class of the UCPP protein family with an additional ATP-cone domain at their N termini, which is thought to function in allosteric regulation. Furthermore, there is an extended hhhhEGxH (where h is any hydrophobic residue and x is any residue) Walker B signature that is also shared with the phosphoribulokinases. Interestingly, the uridine kinase class does not appear to possess any of these modifications. While AtTTM1 and AtTTM2 possess the general motif modifications of the UCPP protein family, they do not possess any of the signature modifications for these other protein classes. Thus, it seemed most likely that AtTTM1 and AtTTM2 possessed uridine kinase or some closely related activity based on sequence analysis. However, we could not detect uridine kinase activity for AtTTM1 or AtTTM2 while the same conditions could form UMP in the presence of AtUKL1. Since the P-loop kinase domain is fully conserved in AtTTM1 and AtTTM2, it is possible that uridine kinase function could not be detected due to potential differences in the biochemical properties between AtTTM1 and AtTTM2 to AtUKL1. Alternatively, the substrate could be a uridine derivative or related
compound. In addition to testing uridine, we tested the possibility of AtTTM1 and AtTTM2 to produce cAMP from ATP, since many CYTH domain proteins are annotated with adenylate cyclase function. Expectedly, we did not observe AC activity, which we also had not observed for AtTTM3.

It still remains to be elucidated whether AtTTM1 and AtTTM2 act on inorganic pyrophosphate in a biological context or whether the true substrate is a different phosphate species altogether. It appears there are only two types of enzymes that can utilize inorganic pyrophosphate: soluble inorganic pyrophosphatases (iPPases) and pyrophosphatase-energized vacuolar membrane proton pumps (V-PPase) (Schulze et al., 2004; Martinoia et al., 2007). In Arabidopsis, there are 6 iPPases encoded by the AtPPa genes 1 through 6. AtTTM1 and AtTTM2 bear no primary sequence resemblance to this protein family nor do they possess the active site consensus sequence, DxDPxD (where x is any residue) (Schulze et al., 2004). Interestingly, AtTTM1 and AtTTM2 possess a higher affinity for pyrophosphate than AtPPa6, which was reported at $K_m = 0.6\text{mM}$ in the presence of $\text{Mg}^{2+}$. These $K_m$ values appear to be physiologically relevant since the cytosolic concentration of $\text{PPI}$ in plant cells is approximately 200$\mu\text{M}$ (Maeshima, 2000). In addition, AtPPa6 is capable of being imported into the chloroplasts where many chemical reactions produce $\text{PPI}$ as a byproduct of metabolism. Inorganic pyrophosphatases are thought to play a role in maintaining the direction of biochemical reactions by the removal of $\text{PPI}$. While AtTTM1 and AtTTM2 do not possess any organellar transit sequences, they do possess a C-terminal transmembrane domain. Interestingly, prediction tools indicate that both AtTTM1 and AtTTM2 are likely anchored to an organellar membrane such that the C terminus is exposed to the cytosol and the remainder of the protein is located within the organelle. Thus, AtTTM1 and AtTTM2 may play a similar role in $\text{PPI}$ removal within these compartments. Since AtTTM1 and AtTTM2 do not share any sequence homology with the AtPPa proteins, they would constitute a novel type of pyrophosphatase if inorganic pyrophosphate is the biological substrate of these proteins.

The other type of pyrophosphatase is the pyrophosphate-energized vacuolar membrane proton pump. In Arabidopsis, there are 3 genes that encode for two types of V-PPases: AtAVP1 (type I), AtAVP2;1 (VHP2;1, type II), and AtAVP2;2 (VHP2;2, type II). A mutant of AtAVP1, fugu5, exhibits elongated cotyledons that can be rescued by sucrose or glucose (Ferjani et al., 2011). Expression of the $S.\text{cerevisiae}$ inorganic
pyrophosphatase driven by the AVP1 promoter in Arabidopsis could rescue the elongated cotyledon phenotype as well as other fugu5 phenotypes, indicating that these phenotypes are caused by elevated PPi levels. Thus, it would be interesting to generate the same transgenic plants using the AtTTM1 or AtTTM2 promoters to determine whether the loss of pyrophosphatase activity is the cause of the ttm1 and ttm2 phenotypes. This would help to provide a mechanistic link between the pyrophosphatase activity and the biological roles in which they play.

In addition to determining the biological importance of pyrophosphate, we also attempted to determine the enzymatic contribution of each domain. Since the missing conserved residues in the CYTH domains of AtTTM1 and AtTTM2 would theoretically only allow phosphate binding, but not metal cofactor binding, it is possible that this domain provides allosteric regulation to the intact P-loop kinase domain. Alternatively, it is possible that AtTTM1 and AtTTM2 could be bifunctional enzymes with P-loop kinase activity as well as pyrophosphatase activity, which could be derived from the deviated version of the CYTH domain present in these proteins. In an attempt to determine the contributions of each domain to the pyrophosphatase activity of AtTTM1, we created targeted mutations in the UK (K78A) and CYTH domains (TYILK → ExExK and R299A). Since the P-loop kinase domain encodes for potential kinase activity, we did not expect a mutation in the Walker A motif to abolish pyrophosphatase activity. We observed PPase activity for 2 out of 4 samples with detectable levels of protein. However, the fact that very little to no activity was observed for the other 2 samples indicates that further improvements could be made to the protein extraction protocol in use. Protein extraction was buffered at pH 7.5 while the isoelectric point of AtTTM1 is reported to be 7.2. The proximity of the buffer pH to the isoelectric point of AtTTM1 may have favoured the formation of protein aggregates, rendering the proteins inactive, but still detectable by SDS-PAGE. This might explain the case where activity could not be detected even in the presence of a visible band by SDS-PAGE. Of course, it is possible that K2 and K5 exemplify the detrimental mutation of K78A to activity. However, the presence of activity for other protein samples show that this is likely not the case. While it is unlikely that the P-loop kinase domain is responsible for PPase activity, further improvements to the extraction protocol should be made and used to retest the contribution of K78.

We also attempted to recapitulate the ExExK motif in AtTTM1 by substituting the TYILK sequence with EYELK. This mutation was hypothesized to change the substrate
specificity from PP\textsubscript{i} to ATP. Alternatively, while it is unknown what role the deviated catalytic motif (TYILK) plays in governing PP\textsubscript{ase} activity, it was possible that disruption could abrogate the PP\textsubscript{ase} activity. However, we observed PP\textsubscript{ase} activity and weak ATP\textsubscript{ase} activity, similar to wild type At\textit{TTM}1. Thus, the alteration of this motif did not affect the activity of At\textit{TTM}1.

Lastly, we mutated a specific arginine that was found to be essential for the catalytic activity of a number of TTMs, including Cet1, Prt1, and At\textit{TTM}3 (Lima et al., 1999; Gong et al., 2006; Moeder et al., 2013). This arginine (At\textit{TTM}1 R299) was hypothesized to be important for positioning the $\gamma$-phosphate group since the equivalent in Cet1, R393, was shown to be important for sulfate binding during crystallization (Lima et al., 1999). Therefore, we expected the abrogation of PP\textsubscript{ase} activity with this mutation. Surprisingly, PP\textsubscript{ase} activity was still retained in these mutants. These results suggest the possibility that the residue deviations found in the CYTH domain of At\textit{TTM}1 constitute a novel reaction mechanism for the catalysis of PP\textsubscript{i}. Thus, it would be valuable to ascertain the roles of other essential catalytic residues in the PP\textsubscript{ase} activity of At\textit{TTM}1 in order to test this hypothesis.

While many works have established signature properties of TTM members in other organisms, the work presented in this chapter is one of the first accounts of the biochemical properties of TTMs in plants. Many other plant species also possess one or two copies of \textit{AtTTM}1 and \textit{AtTTM}2 orthologs in their genomes, suggesting the biological importance of both At\textit{TTM}1 and At\textit{TTM}2. Sequence alignment revealed astonishingly high sequence conservation between the uridine kinase and CYTH domains of At\textit{TTM}1 and At\textit{TTM}2 with their orthologs, including the missing conserved CYTH residues. This suggests that there must be some functional importance associated with the deviated CYTH domains of At\textit{TTM}1 and At\textit{TTM}2. Interestingly, the transcriptional downregulation of \textit{AtTTM}2 after SA and BTH treatment on Arabidopsis can be recapitulated in canola and soybean (Fig. 3-13A, B). Thus, it is likely that the biological role of \textit{AtTTM}2 is conserved in these orthologs. Future studies to determine the significance of the conservation of \textit{TTM}1 and \textit{TTM}2 orthologs as distinct partners in different clades will reveal interesting insights into the biological functions of TTMs in plants.
CHAPTER 6

DISCUSSION AND FUTURE DIRECTIONS
The findings presented in this thesis have revealed novel insights into the biological importance of the TTM superfamily. Prior to this study, several structure-function analyses established the signature biochemical properties of TTM proteins, showcasing the versatility of the TTM tunnel in accommodating various triphosphate substrates. However, the biological importance of TTMs was never adequately established. For instance, studies showing the adenylate cyclase activities of CyaB and YpAC-IV, the thiamine triphosphatase activities of the mammalian TTMs, and the tripolyphosphatase activities of CthTTM and NeuTTM pioneered our current understanding of the biochemical properties of TTMs (Sismeiro et al., 1998; Gallagher et al., 2006; Keppetipola et al., 2007; Delvaux et al., 2011). However, the relevance of these enzymatic functions to the biology was neither well described or known. In fact, the only studies clearly establishing the functional importance of TTMs in a biological context are those concerning the RNA triphosphatase, Cet1, from S. cerevisiae (Ho et al., 1998; Lima et al., 1999; Shuman 2002). Thus, this thesis not only describes the first characterization of TTM superfamily members in plants, but our findings also represent one of the first accounts of the importance of TTM proteins in a biological context. The impact and contributions of these results as well as future perspectives are further discussed below.
6.1 AtTTM2 as a negative regulator of pathogen defense responses

We uncovered a biological role for AtTTM2 as a negative regulator of defense responses. Both SA and its biological analog, BTH, trigger the transcriptional downregulation of AtTTM2 and SA is required for the enhanced defense responses seen in ttm2 plants. Thus, we hypothesized that AtTTM2 is involved in the SA amplification loop as a negative regulator that becomes transcriptionally suppressed upon pathogen infection (Fig. 3-14). In order to maintain defense responses during the course of infection, AtTTM2 is further transcriptionally downregulated by ICS1-mediated SA production. Effectively, these signaling events lead to the establishment of defense responses and SAR. Interestingly, while the loss of AtTTM2 confers enhanced resistance, it does not lead to constitutive defense signaling. ttm2 plants do not exhibit defense responses, spontaneous cell death formation, or PR gene expression without pathogen infection. Collectively, these phenotypic characteristics have not been observed in any other mutant class, making ttm2 a unique defense mutant.

The enhanced disease resistance (edr) class of defense mutants closely resembles the phenotypes of ttm2, but do not exhibit enhanced SAR (Frye and Innes, 1998; Tang et al., 2005a). This suggests a fundamental difference between the molecular mechanisms of edr mutants and ttm2. EDR1 encodes a Raf-like MAPKKK and possesses an N-terminal regulatory domain that is capable of interacting with MKK4 and MKK5 (Zhao et al., 2014). While the mechanism is still unknown, it was determined that this interaction reduces MKK4 or MKK5 protein levels in vivo. Furthermore, the absence of both EDR1 and MKK4 or MKK5 led to enhanced susceptibility and MPK3 and MPK6 activity. Thus, it was proposed that EDR1 negatively regulates defense responses by interacting with and affecting the protein stability of MKK4 or MKK5, thereby shunting defense signaling upstream of MPK3 and MPK6 (Zhao et al., 2014). It will be interesting to analyze the activity of MPK3 and MPK6 in ttm2 plants to depict the differences between ttm2 and edr1.

Our findings support a role for AtTTM2 as a negative regulator of the SA amplification loop. However, the molecular mechanism by which this occurs can be further explored. Sequence analysis of AtTTM2 reveals a predicted coiled coil region at the C terminus, which could be involved in protein-protein interactions. This indicates the potential for AtTTM2 interactors or the dimerization of AtTTM2. Interestingly, AtTTM2
possesses no known or predicted interactors in BAR, IntAct, BioGrid, or STRING
databases (accessed through the Arabidopsis Interactions Viewer, BAR, Geisler-Lee et
al., 2007). Thus, yeast two hybrid or co-immunoprecipitation studies can be employed to
determine possible interactors for AtTTM2 or whether it dimerizes. The results of this
analysis would not only further define the molecular mechanism of AtTTM2 in defense
responses, but would also contribute to our overall understanding of the SA amplification
loop, which has only been discussed by a few studies (Shah 2003; Dong 2004; Song et
al., 2004; Raffaele et al., 2006).

6.2 The molecular mechanism behind the role of AtTTM1 in leaf senescence

While we did not observe alterations in defense responses for ttm1 plants, we did
find a role for AtTTM1 in leaf senescence. ttm1 plants exhibit enhanced chlorophyll
retention during dark-induced senescence, which is correlated with the delayed
expression of senescence marker genes. Taken together with the transcriptional
upregulation of AtTTM1 in response to dark treatment, we proposed that AtTTM1 is a
positive regulator of dark-induced senescence. Although it is clear that the loss of
AtTTM1 delays leaf senescence, it is still unknown how this occurs. Interestingly,
AtTTM1 was reported to be upregulated in leaves undergoing both dark-induced and
natural senescence, indicating the possibility of a broader role for AtTTM1 in senescence
(Buchanan-Wollaston et al., 2005). In other words, AtTTM1 might participate in a
common signaling pathway that is central to the senescence responses induced by
various treatments rather than playing a specific role in dark-induced senescence.

Transcriptional studies have also shed light on the role of phytohormones in leaf
senescence. For instance, ethylene and ABA application can lead to leaf yellowing that
is concomitant with the elevated expression of several SAGs (Weaver et al., 1998;
Quirino et al., 2000). Furthermore, transcriptional profiling of leaves indicates a
downregulation of cytokinin signaling genes that is concomitant with the upregulation of
JA and ABA signaling genes (Breeze et al., 2011). The JA-related genes included those
involved in its biosynthesis as well as signaling. Interestingly, the ABA-related genes that
were upregulated included those involved in dehydration responses, such as ERD1,
ERD14, RAB18, and ABF2, suggesting a role for water loss during senescence
responses (Breeze et al., 2011). These transcriptional changes occurred several days
before the first signs of leaf yellowing occurred, indicating the importance of the concerted actions of different phytohormones in early senescence signaling.

One study that utilized ABA to induce senescence reported chlorophyll loss to occur as early as 5 days after continuous treatment with 50µM ABA in a light/dark cycle (Jia et al., 2013). To examine the role of ABA in *ttm1*-mediated delayed senescence responses, I have conducted a similar experiment with detached leaves of *ttm1* plants. Interestingly, preliminary data showed significantly delayed chlorophyll loss in the leaves of *ttm1* plants as early as 4 days in 50µM ABA solution (Fig. A1).

Since ABA influences the progression of senescence, these results indicate involvement of ABA in *AtTTM1*-mediated senescence signaling. A similar senescence phenotype induced by dark treatment and ABA has been reported in the *oresara* mutants, *ore1*, *ore3*, and *ore9*, which also exhibit delayed senescence after ethylene and methyl jasmonate (MeJA) treatment (Kim et al., 2011). Because of their responses to various inducers of senescence, it was proposed that the responses of the *oresara* mutants indicate the existence of a common point of convergence among the specific pathways that are activated by these various inducers. Indeed, ORE1/NAC2 is a transcription factor that was induced in the transcriptional profiling studies of Buchanan-Wollaston et al. (2005), indicating a potential role in the transcriptional activation of genes during leaf senescence. ORE3/EIN2 plays diverse roles in ethylene, ABA, and auxin signaling and ORE9 encodes an F-box protein involved in proteasome-mediated protein degradation (Woo et al., 2001; Kim et al., 2011). It is possible that *AtTTM1* also participates in a pathway that is central to those activated by different inducers of senescence. It will be interesting to test the effects of ethylene and JA on *ttm1* in the future.

Interestingly, 74% enrichment of the *AtTTM1* phosphopeptide, LSLDDDTVS SPK (where S437 is the ABA-induced phosphosite), was detected in plants treated with 50µM ABA for 15 minutes compared to control plants (Kline et al., 2010). This suggests that *AtTTM1* itself is regulated by phosphorylation. Combined with the reduced ABA responsiveness of *ttm1* leaves in the dark-induced senescence assay, it is possible that *AtTTM1* is regulated through ABA-mediated phosphorylation. In this scenario, rising ABA levels in early-senescing leaves would lead to the phosphorylation of *AtTTM1*, which would regulate its function and promote senescence. Therefore, loss of *AtTTM1* would
equate to the loss of an ABA-mediated signal, which promotes senescence. This can explain why ttm1 leaves retain more chlorophyll in the presence of ABA. Further analyses to determine the insensitivity of ttm1 to ABA should be done.

If ttm1 leaves are indeed less responsive to ABA treatment, then the next step would be to determine the protein kinase that phosphorylates AtTTM1. The transcriptional profiling study by Buchanan-Wollaston et al. (2005) showed the upregulation of several classes of kinases during leaf senescence. Among these kinases were MKK9 and MPK7, which were both upregulated over 6-fold. Interestingly, public microarray data also show that both MKK9 and MPK7 are responsive to ABA with transcript levels increasing over 4-fold after 3 hours in the presence of 10µM ABA (BAR; Toufighi et al., 2005; Winter et al., 2007). Therefore, in-gel kinase assays could be performed to determine whether MPK7, activated by MKK9, can phosphorylate AtTTM1 in the presence of ABA. This experiment would provide some insight into the mechanism behind the role of AtTTM1 in promoting leaf senescence.

6.3 Autophagy: the connection between pathogen defense and senescence

Autophagy is a degradative process that directs misfolded or aggregated proteins to the lysosome for proteolysis via the formation of vesicular compartments called autophagosomes (Coll et al., 2014). Thus, autophagy was traditionally regarded as a pro-survival housekeeping system important in times of nutrient stress. However, recent work has highlighted the complexity of autophagy by showing its importance in both pro-death and pro-survival roles (Hofius et al., 2009; Coll et al., 2014). For instance, loss-of-function mutations of autophagy genes involved in autophagosome formation, ATG5 and ATG18a, led to a suppression of HR that is normally mounted against Pst expressing AvrRpm1 (Coll et al., 2014). Loss of the same autophagy genes also led to early senescence phenotypes, which was shown to be correlated with the hyperaccumulation of SA. These data suggest a dual role for autophagy where it plays a pro-death role in infected cells, but a pro-survival role in senescing tissue. It is still not known how this complex regulation occurs – whether the process of autophagy is similar in both cases, but is differentially regulated by specific defense or senescence signals or whether the process of autophagy itself is different in both scenarios still remains to be determined.
*AtTTM1* and *AtTTM2* can functionally complement the loss of each other, which indicates that their enzymatic functions should be highly similar or identical. This suggests that they should participate in a common mechanism important for both pathogen defense and senescence. Whether this mechanism involves the favouring of reaction equilibriums in certain directions through the hydrolysis of pyrophosphate remains to be determined. However, it is possible that TTM function could be important for autophagy regulation. Our data support a role for *AtTTM2* as a negative regulator of defense responses and *AtTTM1* in promoting leaf senescence. Thus, it is possible that TTM function could be important for the negative regulation of autophagy responses. In this scenario, the negative regulation of autophagy by *AtTTM1* would promote senescence. Thus, the delayed senescence phenotype of *ttm1* could be caused by the loss of negative regulation on the pro-survival role of autophagy. In the case of *AtTTM2*, its negative regulatory role on defense responses could also affect autophagy in order to maintain control over timely defense activation and HR. Thus, the enhanced defense responses of *ttm2* could be explained by the loss of negative regulation on the pro-death role of autophagy. Interestingly, it has been reported that autophagy is important for HR formation in response to *Pst* expressing *AvrRps4* or *AvrRpm1*, but not *AvrRpt2* (Hofius et al., 2009). We observed enhanced resistance of *ttm2* against *Pst* expressing *AvrRps4*, but not *AvrRpt2* (Fig. 3-5). Therefore, observation of the response of *ttm2* to *Pst* expressing *AvrRpm1* would be interesting to conduct in the future. Furthermore, observation of autophagosome formation in senescing *ttm1* and infected *ttm2* plants using confocal microscopy could yield some interesting results.

6.4 Further characterization of the PPase activity of *AtTTM1* and *AtTTM2*

Our promoter swap studies and biochemical analyses indicate that *AtTTM1* and *AtTTM2* possess similar or identical biochemical functions. Supporting this result, biochemical analyses showed that both *AtTTM1* and *AtTTM2* possess a strong affinity for pyrophosphate. However, it is not clear at this stage whether this *in vitro* activity is related to *in vivo* function. Thus, what also remains a question is whether the PPase activity we observed for *AtTTM1* and *AtTTM2* is mechanistically connected to the phenotypes observed in *ttm1* and *ttm2*. This possibility can be tested in complementation studies by expressing the yeast inorganic pyrophosphatase, IPP1,
under the control of either the *AtTTM1* or *AtTTM2* promoter (Pérez-Castiñeira et al., 2002). Successful complementation would indicate that pyrophosphatase is indeed required for the *in vivo* function of these proteins. This will also be the first step in narrowing down the *in vivo* substrates.

Another aspect we must consider is the implications of the missing catalytic residues of the CYTH domains of AtTTM1 and AtTTM2. Does this mean that AtTTM1 and AtTTM2 have lost the catalytic activity of the CYTH domain or possibly acquired a novel function for this domain? Could the changes be related to substrate specificity? And if the CYTH domain has lost its function, from where does the observed PPase activity originate? To address these points, we should first test whether the PPase activity of AtTTM1 and AtTTM2 does indeed originate from the CYTH domain. Currently, we are attempting to express only the CYTH domains of AtTTM1 and AtTTM2 to test PPase activity. Should PPase activity still be detected, this would indicate that the CYTH domain alone is responsible for this activity. This would further suggest that while key catalytic residues are missing in the CYTH domains of AtTTM1 and AtTTM2, there is some other mechanism for substrate and metal binding that is different from that of other CYTH domain proteins, such as AtTTM3.

The fact that the CYTH/TTM tunnel can accommodate a number of triphosphate substrates in light of the high degree of conservation of key catalytic residues amongst the TTM superfamily indicates that this catalytic tunnel is remarkably versatile in substrate docking (Sismeiro et al., 1998; Song et al., 2008). Since the CYTH domains of AtTTM1 and AtTTM2 still possess 5 out of 11 catalytic residues, it might be possible that these proteins have retained phosphatase activity, but act on some other phosphate substrate. Thus, two possibilities exist: 1) loss of CYTH-mediated activity due to loss of metal cofactor binding and thus, only P-loop kinase activity remains. In this scenario, PPase activity might be an *in vitro* artefact and the CYTH domain may provide allosteric regulation to the kinase domain or may serve to position the substrate for the kinase domain; or 2) novel substrate specificity for the CYTH domain (bifunctional enzyme with kinase activity and novel PPase activity). Expression of the CYTH domains of AtTTM1 or AtTTM2 alone and detection of PPase activity could discern whether the loss of these catalytic residues have conferred it a different function from that of the typical triphosphatase activities. Since every TTM characterized to date exhibits metal-dependent triphosphatase activity, validation of the PPase activity would make AtTTM1
and AtTTM2 the first TTM members to possess metal-dependent diphosphatase activity, thereby expanding the range of TTM substrates. In that case, it might be interesting to test the activity of the Dictyostelium TTM proteins, which have the same domain structure and catalytic residue deviations as AtTTM1 and AtTTM2.

### 6.5 Determining the biological substrate of AtTTM1 and AtTTM2

Should the first hypothesis prevail, the true biological substrate would not be pyrophosphate. In this case, efforts to elucidate the biological substrate could be initially focused on a small molecule screen using recombinantly expressed AtTTM1 or AtTTM2. A thermal shift assay (differential scanning fluorimetry, DSF) could be employed along with the commercially available SYPRO Orange protein dye (Life Technologies) to detect changes in protein stability upon ligand binding. This method has been used to screen for protein ligands, small molecule inhibitors of proteins, and to characterize interactions between specific proteins and their ligands (Soon et al., 2012). The thermal shift assay utilizes a quantitative real-time PCR method to gradually increase the temperature of a small-volume sample containing protein and a ligand. As the temperature increases, proteins that are stabilized by ligand binding will denature more slowly than samples containing unbound protein; thus, exhibiting a higher melting temperature. Due to the rapidness of this analysis, many small molecules can be screened for AtTTM1 or AtTTM2 and further validated by specific enzymatic assays. In addition, molecular docking simulations or three-dimensional modeling of AtTTM1 and AtTTM2 against the crystal structure of AtTTM3 could provide some clues for a substrate and determine which residues might be important for catalysis. Efforts to elucidate a biological substrate will be paramount in explaining the molecular mechanism underlying the biological roles of AtTTM1 and AtTTM2.

### 6.6 Concluding Remarks

TTM genes have been uncovered in virtually every organism. The work presented in this thesis and that of Moeder et al. (2013) are the first accounts of TTM research in plants. Many other plant species also possess orthologs of all three Arabidopsis *TTM* genes. For instance, *Brassica napus* (canola), *Glycine max* (soybean),
*Solanum lycopersicum* (tomato), and *Medicago truncatula* (barrel clover) (Phytozome, Goodstein et al., 2012). *AtTTM3* orthologs possess a single CYTH domain and the active site is conserved as EVEVK, similarly to *AtTTM3*. Alignment of *AtTTM1* and *AtTTM2* orthologs reveals a high degree of identity with the same domain arrangement. The high degree of conservation of all three Arabidopsis *TTM* genes suggests the importance of TTM function in plants. Furthermore, conservation of the deviated CYTH domains of *AtTTM1* and *AtTTM2* in other plant species also highlights the importance of this diverged form of the CYTH domain and raises the question of its biological significance.

The reverse genetics approach taken in this thesis yielded very valuable insights into the roles of TTMs in plants. We uncovered three TTMs in Arabidopsis on the basis of the presence of a CYTH domain, *AtTTM1*, *AtTTM2*, and *AtTTM3*. For the first time, we show the biological importance of the TTM superfamily through the characterization of T-DNA insertion knockout lines. We discovered that *AtTTM1* plays a role in promoting leaf senescence and *AtTTM2* is involved in the SA amplification loop for defense responses as a negative regulator. Importantly, we also determined that these two TTM members can functionally complement each other. Taken together with the high degree of identity between *AtTTM1* and *AtTTM2* and our finding of PPase activity for both proteins, this indicates that their distinct biological roles are governed by their expression patterns. This implies that there is a common mechanism required for both pathogen defense and senescence programs involving TTM members. Efforts to further understand the implications of this will be challenging, but extremely rewarding. Ultimately, this thesis provides a framework for our understanding of both the biochemical properties and the biological roles of TTMs in plants.
REFERENCES


## Table A1. List of primers.

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Figure A1. *ttm1* exhibits enhanced chlorophyll retention in response to ABA-induced senescence.

(A) Leaves of Columbia wild type (Col) and *ttm1-1* plants were detached and floated for 7 days on water or 50μM ABA solution in a 16hr light/dark cycle. Scale bar = 1cm.

(B) Total chlorophyll content was measured at 0 and 4 days after ABA treatment. Each bar represents the mean of three biological replicates ± SE (n = 3). An asterisk denotes significance at p < 0.01 (Student’s t-test).
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Figure A2-1. The ‘Open Series’ pORE binary vector.

The pORE-O1 vector was used to generate the ttm1/TTM1 construct (Xhol/PstI) for plant complementation experiments. Image was obtained from the pORE binary vector manual (Coutu et al., 2007).
Figure A2-2. The ‘Reporter Series’ pORE binary vector.

The pORE-R2 vector was used to generate constructs for promoter-GUS analyses (XbaI/EcoRI for both pTTM1 and pTTM2) and promoter swap experiments (NotI/SpeI for all constructs). Image was obtained from the pORE binary vector manual (Coutu et al., 2007).
Figure A2-3. The pBI121 binary vector.

The pBI121 vector (Clontech) was used to generate overexpression constructs (Xmal/Sacl) by replacing the \( \beta \)-glucuronidase (GUS, uidA) gene with AtTTM1 or AtTTM2. Image was obtained from SnapGene (http://www.snapgene.com/resources).
**pGEX-6P-1**

The pGEX-6P-1 expression vector was used to generate constructs (SalI/NotI) for protein expression in *E. coli*. Image was obtained and modified from the pGEX vector manual (GE Healthcare Life Sciences).

**Figure A2-4. The pGEX-6P-1 expression vector.**

The pGEX-6P-1 expression vector was used to generate constructs (SalI/NotI) for protein expression in *E. coli*. Image was obtained and modified from the pGEX vector manual (GE Healthcare Life Sciences).
Figure A2-5. The pET15b expression vector.

The pET15b expression vector was used to generate the AtUKL1 expression construct (NdeI/BamHI). Image was obtained from the pET15b vector manual (Novagen).