

**The Role of *Lycopersicon esculentum* Ubiquitin-conjugating Enzymes *LeUBC1* and *SlUBC3*
in the Response to Stress Induced by Fungal Toxin Fumonisin B1**

By

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Abstract

Plants are constantly threatened by biotic stresses and, in order to overcome these stresses, they have developed an innate immune system. There is growing evidence that ubiquitination, a post-translational modification, plays a role in regulating the plant defense responses. The ubiquitination reaction involves three enzymes, an ubiquitin-activating enzyme, an ubiquitin-conjugating enzyme, and an ubiquitin ligase. The ubiquitination of a target protein leads to its degradation by a proteasome. The majority of studies have indicated the involvement of the ubiquitin ligase in plant defense regulation, but very little information is available regarding the role of ubiquitin-conjugating enzymes. The *Lycopersicon esculentum* (tomato) ubiquitin-conjugating enzymes, *LeUBC1* and *SlUBC3*, have been previously linked to stress responses. Here, we show through bioinformatics data that these two ubiquitin-conjugating enzymes have significant homology to ubiquitin-conjugating enzymes from other organisms and may perform similar function. Here, we report that the transcript level of *LeUBC1* and *SlUBC3* did not change in tomato leaves treated with fumonisin B1, a fungal toxin, and a proteasome inhibitor, Z-Leu-Leu-NVa-CHO. It has been reported that upon stress, particularly biotic stress, there is an accumulation of defense-related proteins such as glucanases, and a down regulation of metabolism-related enzymes such as glutamine synthetases. In my study, the activity of both β -1,3-glucanases and glutamine synthetase was significantly increased after 48 hours of treatment with H₂O, FB1, MG-115, and FB1+MG-115. Finally, *LeUBC1* and *SlUBC3* expression was successfully induced in *Escherichia coli* cells, paving the way for future purification and protein interaction analysis on these proteins.

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Abbreviations

ACRE: Avr9/Cf-9 rapidly elicited

ANOVA: Analysis of variance

APS: Ammonium persulfate

Avr: Avirulence

DEPC: Diethylpyrocarbonate

E1: Ubiquitin-activating enzyme

E2: Ubiquitin-conjugating enzyme

E3: Ubiquitin ligase

ERK: Extracellular signal-regulated kinases

ETI: Effector-triggered immunity

ERKI: ERK docking domain inhibitor 3-(2-Aminoethyl)-5-((4-ethoxyphenyl) methylene)-2, 4-thiazolidinedione hydrochloride

FB1: Fumonisin B1

GS: Glutamine synthetase

GS1: Cytosolic GS enzyme

GS2: Chloroplast GS enzyme

HECT: Homologous to E6-AP C terminus

HR: Hypersensitive response

Hog/p38: High osmolarity glycerol response or p38 kinases

IPTG: Isopropyl β -D-1-thiogalactopyranoside

JNK/SAPK: C-Jun amino(NH₂)-terminal kinases or stress-activated protein kinases

LB: Lysogeny broth

LeUBC1: Lycopersicon esculentum ubiquitin-conjugating 1

LRR: Leu-rich repeat

MAP-2 kinase: Microtubule-associated protein-2 kinase

MAPK: MAP kinase, mitogen-activated protein kinase

MAPKK: MAP kinase kinase
MAPKKK: MAP kinase kinase kinase
MG-115: Z-Leu-Leu-NVa-CHO
NB: Nucleotide binding
NCBI: National center for biotechnology information
PAMP: Pathogen associated molecular pattern
PBS: Phosphate-buffered saline
PR: Pathogenesis-related
PRR: Pattern recognition receptor
PTI: PAMP-triggered immunity
RACE: 5' rapid amplification of cDNA ends
RING: Really interesting new gene
ROS: Reactive oxygen species
SAR: Systematic acquired resistance
SC: Soluble cytoplasm
SCF: Skp1-Cullin-F-box
SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH3: Src homology 3 group
SIUBC3: Lycopersicon esculentum ubiquitin-conjugating 3
TCP: Total cell protein
TEMED: Tetramethylethylenediamine
 T_M : Melting temperature
Ub: Ubiquitin
UBA: Ubiquitin associated domain
UBC: Ubiquitin-conjugating enzyme
UBCd: Ubiquitin-conjugating domain
Y2H: Yeast two-hybrid

Chapter 1

Introduction

1.1 Plant innate immune system

Plants are under constant threat of infection by pathogens equipped with a variety of effector molecules that colonize their host. Plants have, in turn, developed an innate immune system that detects and protects against infection by pathogens and minimizes the impact of pathogens on plant growth and development. The first layer of the innate immune response is the detection of pathogen associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) in plant cell membranes. This is called PAMP-triggered immunity (PTI) and it prevents invasion of plants by pathogens [10]. The second layer of the innate immune system involves a host resistance (R) protein that recognizes pathogen-secreted effectors called avirulence (Avr) proteins and activate signal transduction cascades that result in defense responses including the hypersensitive response (HR) and the systematic acquired resistance (SAR) response [10]. This layer of the innate immune response is referred to as effector-triggered immunity (ETI). R proteins are classified into two classes: an intracellular and a membrane-anchored class. The intracellular class shares a central nucleotide binding (NB) site and C-terminal LRRs (leu-rich repeats) with variable repeat numbers. The other class is membrane-anchored by a single transmembrane helix, consists of variable repeat numbers of extracellular LRRs, and contains an intracellular Ser/Thr kinase module [5, 29].

Kinase cascades such as the mitogen-activated protein kinase (MAPK) pathways transfer information from sensors to cellular responses in PTI and ETI [23]. The MAPK was first discovered in 1986 from animal cells by Sturgill and Ray and named as microtubule-associated protein-2 kinase (MAP-2 kinase) [19]. Later it was discovered that this kinase is related to a set of proteins that are phosphorylated at a tyrosine residue in response to mitogens and it was renamed as MAPK. In 1990, it was classified as a serine/tyrosine kinase that belonged to a

multigene family. The first reported plant MAPK was from the pea plant in 1993 [19]. Currently, the cDNA clones of MAPKs are also reported from *Arabidopsis*, tomato, alfalfa, tobacco, and other plant species [19]. It is classified as one of the largest and most important category of kinases in plants. The MAPK cascade consists of phosphorylation events where MAP kinase kinase kinase (MAPKKK), the first component in this pathway, activates MAP kinase kinase (MAPKK) in response to a stimulus by phosphorylation of two serine and threonine residues. The MAPKK then phosphorylates threonine and tyrosine residues of MAP kinase (MAPK), activating it [19, 23]. Once the terminal kinase is activated, it can then phosphorylate specific downstream effector proteins, leading to the activation of cellular responses (Figure 1). There is significant evidence from studies on model systems including tobacco, *Arabidopsis*, and tomato indicating that MAPKs are components of defense pathways, playing roles in both basal defense and in interactions involving *R*-gene-mediated resistance [23]. Beyond protein phosphorylation, other post-translational modifications such as ubiquitination-mediated protein degradation is thought to be a regulator of these two layers of host defense [10].

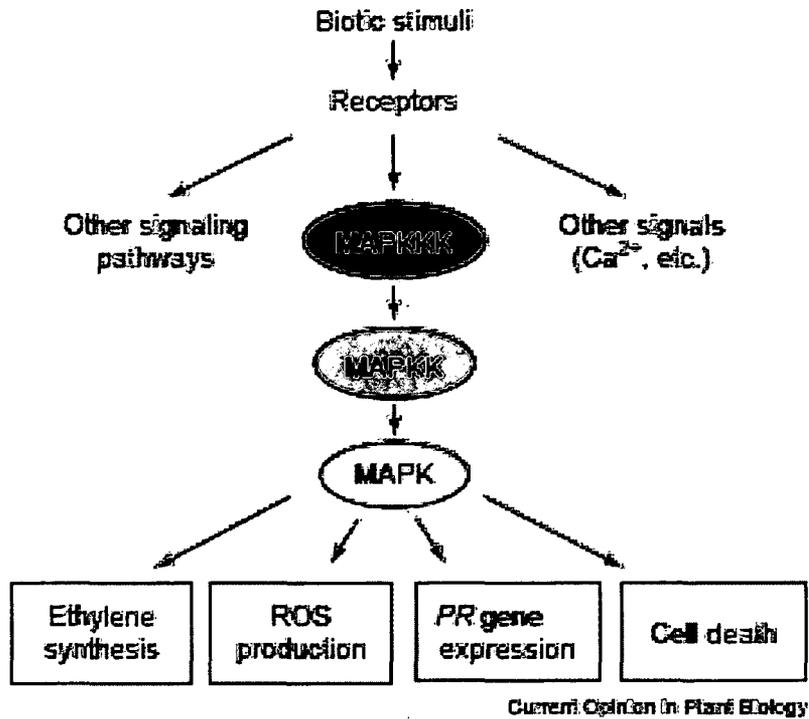


Figure 1. MAPK signaling pathways and the cellular responses they influence following the recognition of biotic stimuli i.e. pathogen infection [23]. ROS: reactive oxygen species; PR: pathogenesis-related. (adapted from Pedley and Martin, 2005)

1.2 Ubiquitination involvement in plant disease resistance

Regulation of critical cellular functions is required to maintain a stable intracellular environment. Ubiquitination is a process that regulates cellular functions mainly through selective degradation of regulatory proteins by proteasomes. The ubiquitination reaction involves three enzymes, an ubiquitin-activating enzyme (E1), an ubiquitin-conjugating enzyme (UBC), and an ubiquitin ligase (E3) (Figure 2A). E1 forms a thiol-ester bond between its conserved cysteine residue and ubiquitin's carboxyl group thereby activating its C-terminus. The ubiquitin moiety is then transferred to an UBC, again forming a thiol-ester bond with a conserved cysteine. Next, E3 catalyzes the formation of an isopeptide bond between the lysine residue of the activated ubiquitin and the target substrate. The ubiquitinated protein is degraded by proteasomes [14]. If not degraded by proteasomes, they are targeted for endocytosis and eventually proteolysis in the lysosome or cellular relocation [10]. Possible reaction consequences are determined by the subcellular localization of substrate and the number of substrate-attached ubiquitins (Figure 2B). Substrates whose fate is proteasomal degradation are attached to a polyubiquitin chain where single ubiquitins are linked by K48-G76 isopeptide bonds [18]. Those destined for nonproteolytic fate have K63-G76 isopeptide bonds linking their ubiquitin [18]. Target substrates are determined by the presence of structural motifs called ubiquitination signal and are recognized by the E3 enzymes [6].

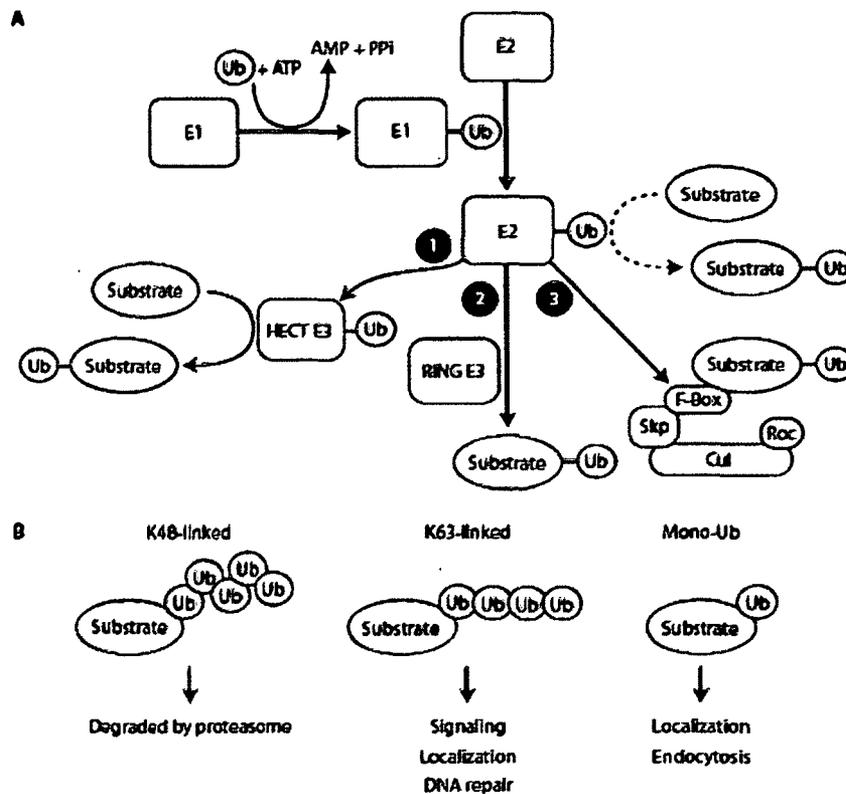


Figure 2. The ubiquitination pathway. (A) The ubiquitination cascade starts with the activation of ubiquitin (Ub) by the E1 enzyme. Then, ubiquitin is transferred to the UBC enzyme. A few UBCs can directly add ubiquitin to a substrate, but generally there is a third step where an E3 enzyme is involved. As shown the different classes of E3s function by either (1) receiving ubiquitin from the UBC and transferring it to the substrate as in HECT (homologous to E6-AP C terminus) E3s, or (2) acting as a scaffold to facilitate such transfer as in RING (really interesting new gene) and SCF (Skp1-Cullin-F-box) complex E3s. (B) Different types of ubiquitination lead to different outcomes. A K48-linked polyubiquitination chain leads to degradation by proteasome. A K63-linked polyubiquitination chain does not target a substrate for degradation by proteasome, rather it leads to substrate localization, and is involved in signaling events in DNA repair. Finally, monoubiquitination regulates substrate localization [18]. (adapted from Laine and Ronai, 2005)

In plants, there exists a small number of similar E1 isoforms with no apparent functional specificity. The UBC family is larger, and several UBC enzymes have specialized cellular functions. The diversity and number of proteins regulated by ubiquitination predicts the existence of a large number of E3s, because it is generally believed that substrate specificity of ubiquitination is determined by E3 [10]. E3 ubiquitin ligases can be subdivided into various groups based on the presence of HECT, F-box, RING, or U-box domains [18]. Ubiquitin protein ligases have been the most extensively studied component of the ubiquitination pathway in plant host defense, although other parts of ubiquitination such as the UBC are also believed to be important in the plant defense response [10]. A few studies have shown a role for UBC in host defense [5, 6, 19]. Generally, as shown in Figure 3, UBC proteins possess a conserved ubiquitin-conjugating domain (UBCd) of about 16 kDA between amino acids 4 and 136. UBCd contains an active site, FHPNINSNGSICLDIL (amino acids 74 to 89), in which the cysteine residue, Cys85 (underlined), is required for thiol ester formation since it is the acceptor site for ubiquitin [8, 9]. One of the first reports of UBC and E3 participating in an elicitor-induced defense response was presented [27]. The report shows that EL5, a rice gene that encodes a RING-H2 finger ubiquitin ligase, is responsive to N-acetylechitoooligosaccharide elicitor and works in cooperation with an elicitor-responsive ubiquitin-conjugating enzyme, *OsUBC5b* [27]. In addition, a particular subfamily of UBCs is involved in plant defense through the proteolytic degradation of abnormal proteins as result of stress [9]. The reported UBC genes of plant origin that are responsive to environmental stimuli include the tomato *LeUBC1* which shows significant similarity to yeast and bovine UBC1, and is up regulated by heat shock and cadmium. In *Saccharomyces cerevisiae* (yeast), *ScUBC4* and *ScUBC5* of the Ubc4/5 subfamily were shown to be up regulated by heat shock [9, 27]. The identification of E1 and UBC proteins that function

with a particular E3, and the characterization of the target protein of the E3 will provide insights on the downstream signaling mechanisms of E3 in plant defense responses [14]. To determine the general impact of ubiquitination on plant defense, it is necessary to identify E3s and their targets. The mode of action of E3 enzymes in plants is via the E3 ubiquitin ligase activity, and they may bind directly to their specific target proteins. This interaction is likely to lead to the ubiquitination of the target proteins followed by their proteasomal degradation. If the target protein is a negative regulator of defense, then degrading the protein has the effect of activating defense signaling [10]. An equivalent type of ubiquitination-mediated activation of signaling components such as kinases and transcription factors has been reported in yeast and mammals. Ubiquitination functions in basal defense and in *R* gene triggered resistance, although it remains unclear whether ubiquitin-dependent processes occur upstream of, coincident with, or downstream of R protein containing recognition complexes [29]. Evidence for the function of ubiquitination in *R* gene triggered resistance has been observed through the positive regulation of *R* gene-mediated resistance by Avr9/Cf-9 rapidly elicited (ACRE) genes, which code for several types of E3 ligases [10]. Cf-9 is an R protein that recognizes the avirulence factor Avr9, leading to a defense response including a few of the ACRE genes encoding ubiquitin E3 ligases such as the U-box gene ACRE74/CMPG1 and the F-box protein ACRE189/ACIF-1 [10].

In plants, several studies have demonstrated the importance of phosphorylation in regulating ubiquitination. This can take place at the level of ubiquitination components and at the substrate level [34]. Though not yet documented in plants, knowledge from other organisms and experimental systems suggests that ubiquitination components are regulated by MAPK phosphorylation [3]. For example, UBR5, an ubiquitin protein ligase that has a role in the DNA damage response is by regulated by phosphorylation and identified as a substrate of the MAP

kinase ERK2 [3]. There is also emerging evidence that ubiquitination plays a role in regulating MAPK through its role in the assembly of protein kinase complexes, subcellular localization, and the actual degradation of the kinase or its substrate [18].

In our attempts to understand the components of the ubiquitination pathway and their involvement in plant defense, we examined the expression of *Lycopersicon esculentum* (tomato) ubiquitin-conjugating 1 (*LeUBC1*) enzyme and ubiquitin-conjugating 3 (*SIUBC3*) enzyme in plants treated with various stress inducing agents including fumonisin B1 (FB1), designed to mimic infection by pathogens. How these genes are involved in the response to pathogen infection, if they are involved, is examined in this thesis.

1.3 The history of *LeUBC1* and *SIUBC3*

Preliminary work on these two genes and their involvement in stress response lead to my research. The major study involving *LeUBC1* began with its isolation from a ZAP-cDNA library, which was constructed from poly (A⁺) mRNA of tomato *Lycopersicon esculentum* (tomato) cells grown in suspension culture for 3 days. The initial discovery of this gene was by immunoscreening and isolating a positive clone, called A7, out of 1 x 10⁶ recombinant λ-phages (Figure 3) [9]. Comparison of the nucleotide and the deduced amino acid sequence of A7 with sequences of data bases (EMBL, Heidelberg, Germany) using TFASTA algorithm revealed strong homology to UBCs. As a result of this homology the clone A7 was named *LeUBC1*, and the corresponding protein was named *LeE2_{21K}* [9]. Northern blot analysis of *LeUBC1* mRNA accumulation revealed a strong accumulation of *LeUBC1* transcript in tomato cells stressed by cadmium and heat shock. After *LeUBC1* was overexpressed in *E.coli*, separation of the

recombinant protein by SDS-PAGE revealed a molecular mass that closely corresponds to that predicted from the deduced amino acid sequence [9].

The identification of *S/U*BC3 was through a study that described the interaction of β Cl with the host ubiquitin proteasome pathway through its binding to a tomato UBC enzyme, referred to as *S/U*BC3 [8]. β Cl is a gene that encodes a pathogenicity protein important for symptom expression found in DNA β associated with *Begomovirus* spp. Yeast two-hybrid (Y2H) screen of tomato cDNA library for proteins that interact with β Cl encoded by DNA β was performed. Five β Cl interactor sequences from tomato were identified as β Cl-S1, β Cl-S2, β Cl-S3, β Cl-S4, and β Cl-S5. Based on alignment with the plant UBC sequences, the clone β Cl-S1 lacked 31 amino acids at the N-terminus. As a result, the missing 5' cDNA sequence of clone β Cl-S1 was amplified from tomato leaf total RNA using 5' rapid amplification of cDNA ends (RACE) to obtain a full-length clone. The 148-amino acid protein of the full length β Cl-S1 clone was found to be identical to a tomato UBC (accession P35135). Therefore, the clone β Cl-S1 was designated as *S/U*BC3 and a member of the tomato UBC gene family deposited in Genbank [8].

Linker
[GGCTGCMGG] AA

TTCCGCCACGAGGGGCGACTTTTGCATRAACCRAAATTGGAATCAAAATGGAGAGGCAAAAAA

1	ATG	GTG	GAC	TTG	GCT	AGG	GTT	CAA	AAG	GAG	CTC	CAT	GAA	TGC	AAC	AGA	
1	M	V	D	L	A	R	V	Q	K	E	L	H	E	C	N	R	
49	GAT	GTT	CAG	GTT	TCT	GGA	ATT	AAT	GTT	ACC	CTT	AAA	GGT	GAC	AGT	CTC	
17	D	V	Q	V	S	G	I	N	V	T	L	K	G	D	S	L	
97	ACT	CAC	TTG	ATT	GGT	ACA	ATC	CCT	GGT	CCT	GTT	GGT	ACT	CCT	TAC	GAA	
33	T	H	L	I	G	T	I	D	G	P	V	G	T	P	Y	E	
145	GGC	GGT	ACT	TTC	AAG	ATC	GAT	ATC	ACT	CIT	ACT	GAT	GGC	TAC	CCA	TTT	
49	G	G	T	F	K	I	D	I	T	L	T	D	G	Y	P	P	
193	GAG	CCT	CCA	AAA	ATG	AAA	TTC	GCC	ACA	AAA	GTT	TGG	CAT	CCC	AAC	ATA	
65	E	P	P	K	M	K	P	A	T	K	V	W	<u>H</u>	<u>P</u>	<u>N</u>	<u>I</u>	
241	AGT	AGT	CAA	AGT	GGA	GCA	ATA	TGC	CTA	GAC	ATC	CTG	AAG	GAC	CAG	TGG	
81	<u>S</u>	<u>S</u>	<u>Q</u>	<u>S</u>	<u>G</u>	<u>A</u>	<u>I</u>	<u>G</u>	<u>L</u>	<u>D</u>	<u>I</u>	<u>L</u>	<u>K</u>	<u>D</u>	<u>Q</u>	<u>H</u>	
289	AGC	CCA	GCA	CTA	ACT	CTC	AAG	ACA	GCT	CTC	CTT	TCT	ATA	CAA	GCA	TTA	
97	S	P	A	E	T	L	K	T	A	L	L	S	I	Q	A	L	
337	CTT	TCT	GCT	CCT	GAA	CCT	GAT	GAT	CCA	CAA	GAT	GCA	GTT	GTT	GCA	CAG	
113	L	S	A	P	E	P	D	D	P	Q	D	A	V	V	A	Q	
385	CAG	TAT	CTT	ACA	GAA	CAT	CAG	ACC	TTT	GTC	GGC	ACA	GCT	CGT	TAC	TGG	
129	Q	Y	L	R	E	H	Q	T	F	V	G	T	A	R	Y	W	
433	ACT	GAG	ACT	TTT	GCA	AAA	ACA	TCC	ACA	CTT	GCT	GCA	GAC	GAC	AAG	ATA	
145	T	E	T	F	A	K	T	S	T	L	A	A	D	D	K	I	
481	CAA	AAG	CTT	GTG	GAA	ATG	GGC	TTT	CCT	GAA	GCT	CAA	GTG	AGG	AGT	ACT	
161	Q	K	L	V	E	M	G	F	F	E	A	Q	V	R	S	T	
529	TTG	GAA	GCA	AAT	GGT	TGG	GAT	GAA	AAC	ATG	GCT	CTT	GAA	AAG	CTG	TTG	
177	L	E	A	N	G	W	D	E	N	M	A	L	E	K	L	L	
577	TCC	AGC	TAA														
193	S	S	Ter														
586	AACCC	TTCTACTGCAACTCATATTTTGATAAGACAATTATATCCTTCCAGCAAAAGCTGATGA															
649	CTAGA	ATAGAGTCACTCGGTTATACTGTGCTTGGCAATCTTGTCTCTCTTTATGGTT															
712	TGCTG	TTGACATCTCTTCATATCCGTGGAAGATTCTGATGTTATTTTACAATATCAAGCAAA															
775	TTGCATATGAATCATGGGAGGAAGTGGACTTTCCGGGTGAAAAA																
838	AAAAA	AAAAA	AAAAA	AAAAA	AAAAA	AAAAA	AAAAA	AAAAA	AAAAA	AAAAA	AAAAA	AAAAA	AAAAA	AAAAA	AAAAA	AAAAA	(CTCGAGGGGG)

Linker

Figure 3. The structure of clone A7. (A) Nucleotide sequence and the derived amino acid sequence of clone A7. Nucleotides and amino acid residues are numbered on the left. By sequence comparison the clone was identified as an UBC homologue of tomato, called *LeUBC1* (LeE2_{21K}). The underlined corresponds to the highly conserved region of the active site region of UBCs. The active site cysteine residue for the binding of ubiquitin and a possible phosphorylation site of protein kinase, TAR, are in bold.[9]. (adapted from Feussner *et al.* 1997)

1.4 Fumonisin B1 mycotoxin

Fusarium is a large genus of fungal species that can be plant-pathogenic, causing diseases in several agriculturally important crops including corn, wheat, and other cereals, and it can also be harmful for humans and other animals [12, 20]. These fungi produce biologically active secondary metabolites (mycotoxins) that can be detrimental to plants, and are associated with cancer and other diseases in humans and domesticated animals [20]. *Fusarium* can infect plants at all developmental stages, from germinating seeds to vegetative tissues, depending on the host plant and the *Fusarium* species involved. Since most *Fusarium* species have specific mycotoxin profiles, early and accurate identification of the *Fusarium* species occurring in the plants, at every step of their growth, is critical to predict the potential toxicological risk to which plants are exposed and to prevent toxins entering the food chain [20]. The fumonisin B1 (FB1) is one of the mycotoxins produced by the pathogen *Fusarium verticillioides* (formerly *F. moniliforme*) that kills plant cells to allow the fungus to feed on them. Pure fumonisins at low concentrations have been shown to cause necrosis and other symptoms in maize seedlings, tomato seedlings, and other plants [6]. FB1 triggers programmed cell death in different plant species including *Arabidopsis* and tomato [31]. Cell death is caused by FB1 blockage of sphingolipid metabolism and accumulation of long chain sphingoid bases via competitive inhibition of N-acetyltransferase (ceramide synthase). FB1 is used for research on host pathogen interactions for its importance in human epidemiology, and also its ease of use since it can be infiltrated directly into plant leaves to mimic fungal induced cell death [1, 26, 28]. HR-like physiological indicators have been noted in FB1 infiltrated plants, including the accumulation of reactive oxygen species (ROS) and the expression of defense-related genes including pathogenesis-related (PR) genes such as glucanases [31]. FB1-induced cell death is now been utilized as the basis of model systems to

investigate cell death in a pathogen-free condition. In my study, the effect of FB1 was combined with Z-Leu-Leu-NVa-CHO (MG-115), a proteasome inhibitor, which would prevent the proteasome from degrading proteins targeted by ubiquitination during the FB1 treatment to see if *LeUBC1* and *S/UBC3* would lead to down regulation or negative feedback for UBC expression (Figure 4).

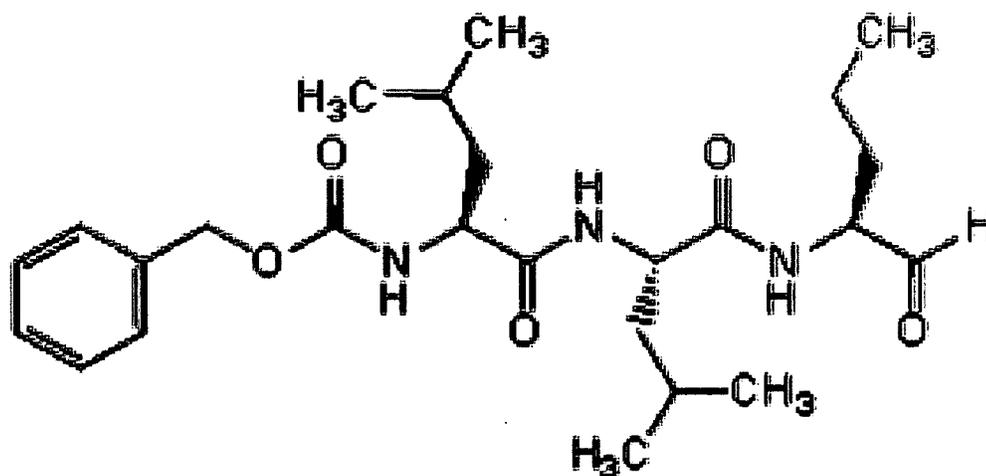
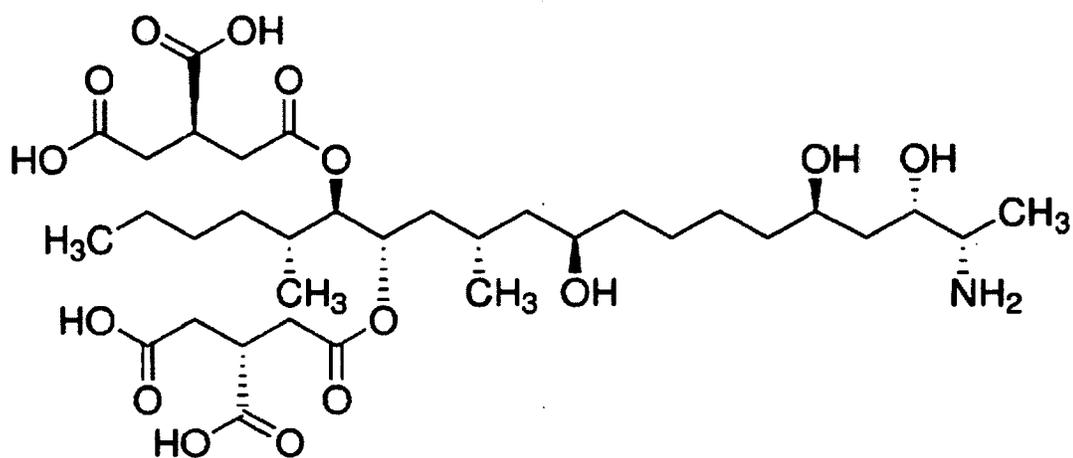


Figure 4. Chemical structures of FB1 (upper) and MG-115 (lower). (adapted from Sigma Aldrich and EMD Millipore)

1.5 ERK docking domain inhibitor 3-(2-Aminoethyl)-5-((4-ethoxyphenyl) methylene)-2, 4-thiazolidinedione hydrochloride (ERKI)

It was previously stated in the section *1.2 Ubiquitination involvement in plant disease resistance* that phosphorylation regulates ubiquitination in plants. Previous work in our lab has indicated that constitutive activation of tMEK2 (a tomato MAPKK) enhanced phosphorylation of an ubiquitin-conjugating enzyme under heat stress (Xing lab, unpublished). Ubiquitination components have also been shown to be regulated by MAPK phosphorylation in other organisms. In yeast and animals, the MAPKs belong to three major groups, the extracellular signal-regulated kinases (ERK), the c-Jun amino(NH₂)-terminal kinases or stress-activated protein kinases (JNK/SAPK) and high osmolarity glycerol response or p38 kinases (Hog/p38) [19]. The majority of plant MAPKs described so far contain a TEY (Thr-Glu-Tyr) sequence in the 'T loop' or 'activation domain' and are homologous to the ERK/MAP kinases group of mammals and yeast [19]. Commercially available specific inhibitors of MAP kinase pathways are potentially useful tools in the analysis of various steps in the pathway through which MAPKs phosphorylate ubiquitination proteins. For example, ERKI is a specific inhibitor of ERK MAP kinases. FB1-treated tomato plants were treated with ERKI to see if MAP kinases in plants would have an effect on the response of *LeUBC1* and *S/UBC3* to an infection scenario.

1.6 β -1,3-glucanase and glutamine synthetase isoforms

Several reports concerning pathogen infections have shown that pathogen attacks lead to major metabolic changes such as accumulation of defense-responsive proteins as well as down regulation of photosynthesis- and metabolism-related proteins [2]. The accumulation of PR proteins upon infection with viroids, viruses, bacteria or fungi has been documented in several

plant species [13]. β -1,3-glucanase, a PR protein, plays a role in defense against pathogen attacks by catalyzing the degradation of β -1,3-glucan in fungal cell wall, and oligosaccharides with elicitor activity are released that further enhance the host defense response [16]. The activity of β -1,3-glucanase in tomato leaves is used as a marker of plant defense response. Glutamine synthetase (GS) one of the metabolism-related proteins that controls nitrogen management in plants catalyzes the first reaction in the ammonium assimilation pathway, producing glutamine that serves as a nitrogen donor in the biosynthesis of amino acids, nucleotides and other nitrogen containing compounds such as chlorophylls [24, 25]. Green tissues of plants contain two different forms of GS, a cytosolic enzyme (GS1) and a chloroplast located isoenzyme (GS2). The isoform GS2 is localized in the photosynthetic cells, and is involved in the assimilation of ammonium obtained from nitrate reduction and photorespiration. The cytosolic isoform of glutamine synthetase (GS1) is involved in nitrogen remobilization and responds to pathogen attacks [24, 25]. The major sources of nitrogen for plants are the soil and the atmosphere. In higher plants, inorganic nitrogen must be reduced to ammonia before it can be assimilated. Ammonium assimilation is catalyzed by GS and other enzymes and produces nitrogen-carrying molecules such as glutamine, glutamate, asparagines, and aspartate. These molecules are the building blocks for the synthesis of a variety of essential plant constituents. Nitrogen supply can affect disease development. Nitrogen provides plants with the building blocks that are required for growth and to resist or recover from disease injury [30]. Plants with nitrogen deficiency tend to be weaker, grow slower, and age faster. As a result, they become more susceptible to pathogen attacks. Thus, the activity of ammonium assimilating factors like GS is important for the ability of plants to resist and recover from disease. For example it has been reported that a lack of nitrogen increases the susceptibility of tomato to infection by certain fungi that belong to the

genus *Fusarium* [30]. Also, it has been shown that pathogen infection and fungal elicitors cause an alteration in the expression of metabolic enzymes like GS in an attempt to better combat the pathogen through upregulation of defense proteins [22]. In my study, glucanase and glutamine synthetase assays have been performed to determine the relative activity of the enzyme under the influence of FB1 and MG-115.

1.7 *LeUBC1*, *SUBC3*, β -1,3-glucanase, and glutamine synthetase in this thesis

This thesis describes the genes *LeUBC1* and *SUBC3*, as well as glucanase and glutamine synthetase, and what their role might be in plant disease resistance. A bioinformatics approach was used to study the two UBC genes and their possible functions as indicated by their homology to other UBCs with known functions. To demonstrate the role of *LeUBC1* and *SUBC3* in plant disease resistance, their expression was studied at the transcriptional level in response to FB1 treatment in tomato plants as well as in response to MG-115 and ERKI. Since they are expected to be a component of plant defense, it was hypothesized that *LeUBC1* and *SUBC3* transcript levels will be up regulated in response to FB1. In contrast, their transcript levels are expected to down regulate in response to MG-115 due to a negative feedback mechanism imposed by the accumulation of polyubiquitinated proteins. It was also hypothesized that *LeUBC1* and *SUBC3* expression will be downregulated in response to ERK-type MAP kinase inhibitor, ERKI, since the MAP kinases are believed to activate ubiquitination proteins. Finally, enzyme assays were used to measure the relative activity of glucanase and glutamine synthetase in response to the treatments mentioned above. It was hypothesized that the overall relative activity of glucanase would increase and glutamine synthetase would decrease in tomato leaves treated with FB1.

1.8 Objectives

My objectives in this study include the following:

- 1) Examine whether *LeUBC1* and *S/UBC3* are involved in defense response at transcriptional level;
- 2) Examine whether the regulation of these two genes involves MAP kinase pathways;
- 3) Examine whether a reprogramming of metabolism occurs during a defense response;
- 4) Express *LeUBC1* and *S/UBC3* proteins for protein-protein interaction study by pull-down experiments.

Chapter 2

Materials and Methods

2.1 Plant materials and treatments

Tomato seeds (cv. Bonny Best, from Ritchie Feed&Seed, Ottawa) were sterilized in 70% ethanol for 2 min, then in a solution containing 30% (V/V) bleach and 0.005% (V/V) Triton X-100 for 8 min. The seeds were then rinsed 10 times with autoclaved water. The sterilized tomato seeds were sowed in autoclaved soil and put in a growth chamber (ECONAIR Technologies Inc., Canada) for ~4 weeks with 16 hours of light period and 8 hours of darkness and at 22-24 °C temperature. All experiments were performed using detached leaflets from 4 to 6-week-old tomato plants. Before the different experiments, tomato leaves were detached and treated with water, 5µM FB1, 400nM MG-115, and 250µM ERKI. The concentrations applied were based on previous publications from this lab [26]. All treatments involved a 0 hour and 48 hour time period. Water was used as a control, and MG-115 is a proteasome inhibitor. Vacuum infiltration of liquids into leaves was used. Leaves were kept under liquid while reducing the pressure allowing intercellular air to escape from the stomata. Then air was released into the desiccator to press the solution into intercellular space. After vacuum infiltration the 0 hour leaves were frozen by liquid nitrogen and stored in a -80 °C freezer, while the 48 hour leaves were incubated in Petri dishes containing the treatments in the growth chamber. After the 48 hour incubation, the leaves were also frozen in liquid nitrogen and stored in the -80 °C freezer.

2.2 RNA extraction and cDNA synthesis

The mortars and pestles used for RNA extraction were treated with 0.1% (V/V) diethylpyrocarbonate (DEPC) overnight and autoclaved. DNase- and RNase-free pipette tips and microcentrifuge tubes were also autoclaved. Approximately 0.1 g-0.5 g of frozen tomato leaf tissues were ground to powder under liquid nitrogen using a pre-chilled mortar and pestle. To

each powder sample, 1 mL of Trizol™ reagent (Life Technologies, USA) was added and samples were immediately homogenized to become one layer, and were then incubated at room temperature for 5 min. To this, 200 µL of chloroform was added and the samples were shaken thoroughly for 15 sec, and were then incubated at room temperature for 3 min. Samples were centrifuged at 12,000 x g for 15 min at 4 °C. After centrifugation, 500 µL of 2-propanol was added to the isolated aqueous phase that contained the RNA, incubated at room temperature for 10 min, and centrifuged at 12,000 x g for 10 min at 4 °C. The resulting pellet was washed with 500 µL 75 % ethanol by inverting and centrifugation at 7,500 x g for 5 min at 4 °C. The pellet was air dried for 10 min and was then dissolved by the addition of 25 µL of DEPC-treated water. RNA quality and concentrations were assessed using NanoDrop spectrophotometer ND-1000 (Thermo Fisher Scientific, USA). RNA quality was assessed based on the ratio of absorbencies at 260 and 280 nm. The RNA samples were stored at -80 °C until used for cDNA synthesis.

cDNA was synthesized using first strand cDNA synthesis components (Life Technologies, USA). In 0.2 mL or 0.5 mL tube, the following were added: 1 µL oligo (dT)₂₀, 1 µg-5 µg RNA, 2 µL dNTP mixture (10 mM each), and DEPC-treated water to make a final volume of 12 µL. The sample was incubated at 65 °C for 5 minutes then placed on ice. The following ingredients were added to the sample: 4 µL 5 x cDNA synthesis buffer (Life Technologies, USA) (vortex sample immediately for 5 seconds), 1 µL 0.1M DTT, 1 µL DEPC-treated water, 1 µL 40 U/µL RNase OUT, and 0.5 µL 15 U/µL cloned AMV RT. The sample was then incubated at 48 °C for 48 minutes, followed by incubation at 85 °C for 5 minutes. cDNA concentrations were estimated using NanoDrop spectrophotometer ND-1000. The cDNA samples were diluted to a final concentration of ~500 ng/µL and all samples were stored at -20 °C until analysis.

Total RNA and cDNA were prepared from tomato leaves exposed to the following treatments: 0 hour water, 48 hour water, 0 hour FB1, 48 hour FB1, 0 hour FB1+MG-115, 48 hour FB1+MG-115, 0 hour FB1+ERKI, and 48 hour FB1+ERKI.

2.3 Reverse transcriptase (RT-PCR)

Each PCR mixture contained 10 x PCR buffer (BioShop, Canada), 25 mM MgCl₂, dNTP mixture (10 mM each), 10 μM forward primer, 10 μM reverse primer, *Taq* polymerase, diluted cDNA template, and DEPC-treated water for a total volume of 20 μL. The PCR primers for each gene, the optimal melting temperature (T_M), the amplified PCR product size, and the corresponding PCR protocol are shown in Table 1.

RT-PCR for transcript level analysis. This step was done to measure the relative mRNA levels by comparing the intensity of the tested gene band against the actin band.

RT-PCR for subcloning. This step was done to obtain *LeUBC1* and *SlUBC3* cDNA fragments that were subcloned into pET14b vector for the purpose of bacterial gene expression. The RT-PCR conditions for the subcloning of *LeUBC1* and *SlUBC3* into the pET 14b vector were the same as the conditions in Table 1. The PCR mixture contained *Pfx* polymerase (high fidelity) instead of *Taq* polymerase.

The PCR products were separated on 0.8% agarose gels, stained with 0.5 mg/mL ethidium bromide (EtBr) and visualized using the AlphaImagerTM 2200 (Alpha Innotech Corporation, USA).

2.4 Protein extraction for enzyme assays

Leaf tissue for total protein extraction was harvested from Petri dishes, frozen in liquid nitrogen, and stored in a -80 °C freezer until required. Using a pre-chilled mortar and pestle 0.5 g -1 g leaves were ground and protein was extracted in 1mL of extraction buffer (pH 8.0) containing 200 mM Tris, 0.25 mM EDTA, 5 mM DTT, and 1 mM PMSF. The homogenate was then centrifuged at 12,000 x g at 4 °C for 20 min. The resultant supernatant containing the protein was transferred into a 1.5 mL microcentrifuge tube and was stored at -20 °C until used for glutamine synthetase and β -1,3-glucanase assays [16]. Protein concentration was estimated using the Bradford method using bovine serum albumin (BSA) (BioRad Laboratories Inc., USA) as standard. The dye reagent was prepared by diluting 10 mL Dye Reagent Concentrate (BioRad Laboratories Inc., USA) with 40 mL double distilled water and was filtered through a Whatman #1 filter to remove residues. The standard and the sample solutions were prepared by pipetting 10 μ L of each and 200 μ L of the dye reagent into separate microplate wells. After incubation at room temperature for approximately 5 min, the absorbance was measured at 595 nm using a microplate reader. Protein concentrations were then calculated based on the BSA standard curve. The protein concentration values of samples were adjusted to the same value in mg/mL by dilution.

2.5 Enzyme assays

Glutamine synthetase assay. GS relative activity was measured based on the method described by Bernard et al. [4]. Total protein extraction (100 μ L) was added to 380 μ L of assay mix which consisted of 100 mM triethanolamine (TEA), 80 mM glutamate, 6 mM hydroxylamine HCl, 20 mM MgSO₄, 4 mM EDTA at pH 7.6. The reaction was started by the addition of 20 μ L of 0.2 M adenosine triphosphate (ATP) at pH 7.6. After 10 min of incubation at 30 °C, then the reaction

was stopped by the addition of 500 μL of ferric chloride reagent which consisted of 0.24 M trichloroacetic acid (TCA), 0.1 M ferric chloride, and 1.0 M HCl. Samples were then centrifuged at 10,000 g for 5 min and absorbance read at 505 nm.

β -1,3-glucanase assay. β -1,3-glucanase relative activity was measured based on the method described by Joosten and De Wit [15]. β -1,3-glucanase activity was assayed by measuring the rate of reducing sugar production with laminarin (Sigma-Aldrich, USA) as the substrate. The reaction mixture consisted of 0.4 mL of citric acid-phosphate buffer (pH 5.6) containing 1 mg/mL laminarin and 0.1 mL of total protein extraction. After 15 min of incubation at 37 °C, 0.5 mL of the alkaline copper reagent was added and the mixture was heated at 100 °C for 10 min.

The alkaline copper reagent was prepared by adding 12 g sodium potassium tartrate and 24 g anhydrous sodium carbonate in 250 mL distilled water. A solution of 4 g copper sulfate pentahydrate and 16 g sodium hydrogen carbonate in 200 mL distilled water was added. Separately, a solution of 180 g anhydrous sodium sulfate in 500 mL of boiling distilled water was prepared. The two solutions were combined and diluted to 1 liter. The solution can be stored in a glass bottle up to 1 year at room temperature.

After cooling on ice, 0.5 mL of the arsenomolybdate reagent was added, followed by 3.0 mL of water after development of the blue color. The arsenomolybdate reagent was prepared by adding 25 g ammonium molybdate in 450 mL distilled water. While stirring, 21 mL concentrated sulfuric acid and 25 mL of distilled water containing 3 g disodium hydrogen arsenate heptahydrate was added. Stirring continued 24 hours at 37 °C, then the solution was stored in a 1 liter aluminum foiled glass bottle up to 6 months at room temperature. The absorbance was measured 660 nm.

2.6 Subcloning of *LeUBC1* and *SIUBC3* into pET14b vector

The desired cDNA was amplified using *pfu* polymerase to generate blunt end PCR products. The PCR products were cloned into TOPO vector and then transformed in MachT1 cells as described in the Zero Blunt TOPO PCR Cloning Kit for Sequencing (Life Technologies, USA). The plasmids of positive colonies were isolated as described in the user protocol of Wizard Plus SV Minipreps DNA Purification System (Promega, USA). Prior to ligation, the recombinant TOPO plasmids and a pET14b vector were digested with XhoI and NdeI restriction enzymes producing products with sticky ends. The restriction digestion protocol for both recombinant TOPO plasmids containing *LeUBC1* and recombinant TOPO plasmids containing *SIUBC3*, and the pET 14b vector are described below.

Digestion of recombinant TOPO plasmids containing LeUBC1. An aliquot containing 3 µg of DNA was diluted with DEPC-treated water to a final volume of 100 µL. Subsequently, 10 µL of 10X buffer H, 1 µL 100 x BSA, 1 µL XhoI, and 1 µL NdeI were added, followed by incubation in a thermocycler for 4 hours at 37 °C.

Digestion of recombinant TOPO plasmids containing SIUBC3. An aliquot containing 3 µg of DNA was diluted with DEPC-treated water to a final volume of 30 µL. Subsequently, 3 µL of 10X buffer H, 1 µL XhoI, and 1 µL NdeI were added, followed by incubation in a thermocycler for 4 hours at 37 °C.

Digestion of the pET14b vector. The digestion reaction mixture and protocol was the same as the digestion protocol for *SIUBC3*, except 3 µg of pET14b vector was added instead of *SIUBC3*. To dephosphorylate the digested pET14b vector, 0.5-1 µL of 0.2 U/µL of calf intestinal alkaline phosphatase was added, and was then incubated at 37 °C for 5 min. To inactivate the

phosphatase, EDTA was added to an equal final concentration of MgCl₂ found in 10 x buffer H followed by incubation at 65 °C for 15 min. The digestion products were separated on 0.8% agarose gels, stained with 0.5 mg/mL EtBr and visualized using the AlphaImager™ 2200 under UV light. Gel extraction to isolate digested *LeUBC1*, *SlUBC3*, and pET14b vector was performed according to the user's manual of Wizard SV Gel and PCR Clean-Up System (Promega, USA). The digested *LeUBC1* and *SlUBC3* were each cloned into the XhoI and NdeI sites of the digested pET14b vector by ligation. The ligation reaction conditions including the ligation controls are shown below in Table 2.

The ligation reactions and the ligation controls were then transformed in BL21 cells (Novagen, EMD Millipore, USA) and grown on Lysogeny Broth (LB) agar plates. The LB agar plates were prepared using LB medium (1.0 % tryptone, 0.5 % yeast extract, 1.0 % sodium chloride pH 7.0) but 1.5 % agar was added before autoclaving. After autoclaving it was cooled to 55 °C, added 100 µg/mL ampicillin, and poured into plates. They were left to harden, then inverted and stored at 4 °C in the dark. Transformation and plating techniques were done as described in the user's protocol in the pET System Manual (10th edition Novagen, EMD Millipore, USA). After the plating, a few colonies from the ligation reaction plate were each inoculated in 3 mL liquid LB medium containing 100 µg/mL ampicillin and were kept in a 37 °C shaker at 250 rpm overnight. Recombinant pET14b plasmids were harvested as described in the user's protocol of Wizard Plus SV Minipreps DNA Purification System (Promega) and stored at -20 °C freezer until used for bacterial gene expression.

Determination of the correct insert orientation in pET14b vector. RT-PCR was used to determine the orientation of *LeUBC1* and *SlUBC3* in the pET14b vector. A forward primer specific to pET14b was designed and used in combination with the forward primer of *LeUBC1*

and *SUBC3*, which acted as a reverse primer (Figure 5). The PCR primers, the optimal melting temperature (T_M), the amplified PCR product size, and the corresponding PCR protocol are shown below in Table 3.

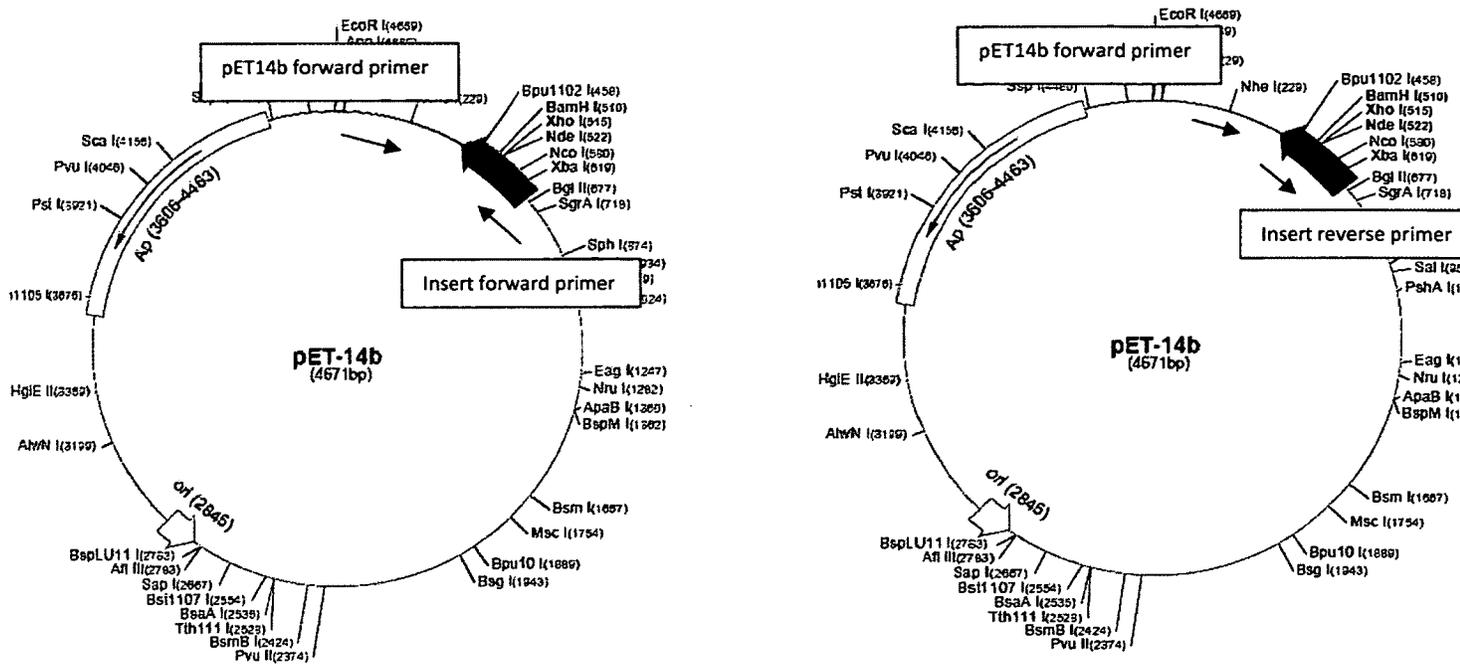


Figure 5. A schematic representation of correct insert orientation determination. If the RT-PCR illustrated on the left image produces a clear banding pattern and the one on right produces an irregular banding pattern, then the target insert is in the correct orientation.

Table 1. Optimal RT-PCR conditions for the transcript level analysis of *Le*UBC1 and *S*/UBC3

Gene	Primer Sequence ¹	T _M	Size (bp)	PCR Protocol
<i>Le</i> UBC1	Forward 5'- <u>catat</u> gatgggtggacttgcttaggt-3' Reverse 5'- <u>ctcgagt</u> tagctggacaacagctttca-3'	60.7 60.0	585	94 °C, 4 min (first cycle); 94 °C, 45 sec; 58 °C, 30 sec; 72 °C, 30 sec (30 cycles); 72 °C, 5 min (last cycle)
<i>S</i> /UBC3	Forward 5'- <u>catat</u> gatggcgtcgaagaggatatt-3' Reverse 5'- <u>ctcgagt</u> catccattgcatatttctg-3'	58.6 58.4	447	94 °C, 3 min (first cycle); 94 °C, 45 sec; 54.5 °C, 1 min; 72 °C, 1 min 30 sec (28 cycles); 72 °C, 10 min (last cycle)
Actin	Forward 5'-tg gc atcatactttctacaatg-3' Reverse 5'-ctaata tc ccacgtcacattcat-3'	50.6 50.7	615	94 °C, 3 min (first cycle); 94 °C, 45 sec; 60.2 °C, 1 min; 72 °C, 1 min 30 sec (28 cycles); 72 °C, 10 min (last cycle)

¹ The underlined sequence of the forward primers corresponds to NdeI restriction site and for the reverse primers it corresponds to XhoI restriction site.

Table 2. Reaction conditions for the ligation of *Le*UBC1 and *S/U*BC3 into pET14 vector

Gene	Ligation Reaction (digested insert and digested vector)	Ligation Control 1 (undigested vector and no insert)	Ligation Control 2 (digested vector and no insert)	Ligation Control 3 (digested insert and no vector)
<i>Le</i> UBC1	2 μ L digested pET14b vector 11 μ L digested <i>Le</i> UBC1 insert 1.5 μ L 10 x ligase buffer (Promega, USA) 0.5 μ L T4 DNA ligase Incubate at 4 °C overnight	2 μ L undigested pET14b vector 1.5 μ L 10 x ligase buffer 11.5 μ L DEPC-treated water Incubate at 4 °C overnight	2 μ L digested pET14b vector 1.5 μ L 10 x ligase buffer 0.5 μ L T4 DNA ligase 11 μ L DEPC-treated water Incubate at 4 °C overnight	11 μ L digested <i>Le</i> UBC1 insert 1.5 μ L 10 x ligase buffer 0.5 μ L T4 DNA ligase 2 μ L DEPC-treated water Incubate at 4 °C overnight
<i>S/U</i> BC3	2.5 μ L digested pET14b vector 5.5 μ L digested <i>S/U</i> BC3 insert 1.5 μ L 10 x ligase buffer 0.5 μ L T4 DNA ligase No additional water was added Incubate at 4 °C overnight	2.5 μ L undigested pET14b vector 1.5 μ L 10 x ligase buffer 6 μ L DEPC-treated water Incubate at 4 °C overnight	2.5 μ L digested pET14b vector 1.5 μ L 10 x ligase buffer 0.5 μ L T4 DNA ligase 5.5 μ L DEPC-treated water Incubate at 4 °C overnight	5.5 μ L digested <i>S/U</i> BC3 insert 1.5 μ L 10 x ligase buffer 0.5 μ L T4 DNA ligase 2.5 μ L DEPC-treated water Incubate at 4 °C overnight

Table 3. Optimal RT-PCR conditions for determining *Le*UBC1 and *S*UBC3 orientation in pET14b vector

Gene	Primer Sequence	T_M	Size (bp)	PCR Protocol
<i>Le</i> UBC1	Forward 5'-tggagccactatcgactacg-3' Reverse 5'-catatgatggtggacttggtagggt-3'	58.9 60.7	785	94 °C, 4 min (first cycle); 94 °C, 45 sec; 58 °C, 30 sec; 72 °C, 30 sec (30 cycles); 72 °C, 5 min (last cycle)
<i>S</i> UBC3	Forward 5'-tggagccactatcgactacg-3' Reverse 5'-catatgatggcgtogaagaggatatt-3'	58.9 58.6	647	94 °C, 3 min (first cycle); 94 °C, 45 sec; 54.5 °C, 1 min; 72 °C, 1 min 30 sec (28 cycles); 72 °C, 10 min (last cycle)

2.7 Gene expression in *E.coli*

The genes *LeUBC1* and *STUBC3* were subcloned into an *E.coli* expression vector containing the T7 promoter and the His-Tag coding sequence. This plasmid, pET14b, was introduced into *E.coli* BL21-CodonPlus (DE3)-RIPL (Agilent Technologies, USA) competent cells as described in the BL21-CodonPlus competent cells transformation protocol (Agilent Technologies, USA). The cells were plated on LB agar plates containing 100 µg/mL carbenicillin and 30 µg/mL chloramphenicol antibiotics and incubated at 37 °C overnight. A single colony was inoculated with 3 mL of LB media containing 100 µg/mL carbenicillin, 30 µg/mL chloramphenicol, and 0.5%-1% glucose pH 8.2, in a culture tube. The culture was incubated in a shaker at 37 °C at 250 rpm overnight. After the overnight incubation, 1 mL of the culture was added to 100 mL of LB media containing 100 µg/mL carbenicillin antibiotic only. The culture was incubated in a shaker at 37 °C for 2-3 hours until OD₆₀₀ is 0.5-1.0, and then the culture was cooled to 18 °C on ice. Before induction, the culture was divided into two 50 mL cultures. One of them served as the uninduced control. The other 50 mL was induced by adding Isopropyl β-D-1-thiogalactopyranoside (IPTG) (BioShop, Canada) to a final concentration of 0.4 mM. Both cultures were then incubated with vigorous shaking at 18 °C for 16-20 hours.

Target protein isolation. There were several protein extraction techniques used for target protein analysis and purification. Two different fractions of protein were analyzed including the total cell protein (TCP) fraction, and the soluble cytoplasm (SC) fraction.

TCP fraction isolation. An aliquot of 1 mL of the prepared culture was transferred into a 1.5 mL microcentrifuge tube, and was then centrifuged at 10, 000 x g for 1 min. The resulting supernatant was removed, and the pellet was left to dry by inversion on a paper towel. The pellet was resuspended by mixing in 100 µL of 1 x phosphate-buffered saline (PBS) (0.8% NaCl,

0.02% potassium chloride, 0.144% Na₂HPO₄, 0.024% KH₂PO₄ pH 7.4, sterilized by autoclaving). The sample was passed through a 27^{1/2}-gauge needle several times, and was then stored at -20 °C until analysis.

SC fraction isolation. An aliquot of 1 mL - 1.5 mL of the prepared culture in a 1.5 mL microcentrifuge tube was centrifuged at 14, 000 - 16, 000 x g for 10 min. The resulting supernatant was removed, and the pellet was left to dry by inversion on a paper towel. The wet weight of the pellet was determined using a scale. The pellet was resuspended in room temperature BugBuster Master Mix (Novagen, EMD Chemicals, USA) by pipetting, using 5 mL reagent per gram of wet cell paste. The mixture was then incubated in a shaker for 20 min at room temperature. The insoluble cell debris was removed by centrifugation at 16, 000 x g for 20 min at 4 °C, and the soluble supernatant fraction was then transferred to a fresh tube and stored at -20 °C until analysis.

2.8 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The expression of *Le*UBC1 and *Sl*UBC3 was assessed by analyzing the various protein fractions on a SDS-PAGE followed by Coomassie blue staining and western blotting.

Preparation of samples. The protein material of the TCP and the soluble cytoplasm fraction was dissolved in an equal volume of 4X SDS-PAGE sample buffer (0.25 M Tris-HCl pH 6.8, 8% SDS, 10% 2-mercaptoethanol, 30% glycerol, and 0.02% bromophenol blue). The dissolved protein was denatured at 85°C for 3 min. After denaturation, the mixture was centrifuged to retain the condensed water on the inner wall of the tube in the sample. The samples were then frozen at -20 °C until SDS-PAGE analysis.

Gel casting. The gel apparatus consisted of two glass plates (short and tall), a casting frame, and a casting stand (BioRad Laboratories Inc., USA). The short glass plate is placed against the tall

glass plate and was secured into the casting frame. The casting frame was placed onto the casting stand and ready for polyacrylamide gels to be poured in. The polyacrylamide gels are a short stacking gel consisting of 4% acrylamide followed by a 15% running (separating) gel. The 4% stacking gel contained 0.5 M Tris-HCl pH 6.8, 20% (W/V) SDS, acrylamide/bis-acrylamide (30%/0.8% W/V), 10% (W/V) ammonium persulfate (APS), tetramethylethylenediamine (TEMED), and distilled water, whereas the 15% separating gel contained 1.5 M Tris-HCl pH 8.8, 20% (W/V) SDS, acrylamide/bis-acrylamide (30%/0.8% W/V), 10% (W/V) APS, TEMED, and distilled water. The polymerization time for each gel was 1 hour.

Electrophoresis. The inner and outer chambers of the electrophoresis apparatus were filled with 1 x running buffer containing 25 mM Tris, 192 mM glycine, and 0.2% (W/V) SDS adjusted to pH 8.5. The protein material was normalized for loading based on the OD₆₀₀ at harvest so that a comparison of Coomassie-stained band intensity accurately reflects the relative amounts of target protein in various fractions. To estimate the size of the proteins, an aliquot of BenchMark™ pre-stained protein ladder (Life Technologies, USA) was loaded into one well. Electrophoresis was run at 180V for 1 hour using the BioRad Mini-PROTEAN 3 System.

Coomassie blue staining. The gels were stained in 0.1% Coomassie R or G-250 (BioRad Laboratories Inc., USA), 10% acetic acid, and 40% methanol for 1 hour in a suitable container. Destaining was in 20% methanol and 10% acetic acid in a suitable container overnight. For quicker destaining, knotted kimwipes can be put in the corners of the container. An image of the gel was visualized using the AlphaImager™ 2200 (Alpha Innotech Corporation, USA).

Western blotting. The SDS-PAGE protein was transferred to nitrocellulose membrane (BioRad Laboratories Inc., USA) by wet transfer with pre-chilled transfer buffer containing 25 mM Tris, 192 mM glycine, and 20% V/V methanol adjusted to pH 8.5, at 4 °C for 2 hours at 70V. During

transfer, sealed icepacks were placed in the buffer chambers to overcome overheating of the buffer and magnetic stir bars were used to ensure even circulation of the transfer buffer. To prevent non-specific binding, the membrane was incubated in 5% skim milk powder in 1 x TBST (20 mM Tris, 150 mM NaCl, and 0.05% Tween-20 adjusted to pH 7.5) for 1 hour at room temperature. The membrane was washed three times, 5 minutes each, with 1 x TBST. For membrane hybridization, the membrane was incubated with 1:1000 diluted His-Tag primary polyclonal antibody (Cell Signaling Technology, USA) in 5% W/V BSA, 1 x TBST at 4 °C with gentle shaking, overnight. This was followed by three 5-minute washes with 1 x TBST. The secondary antibody (anti-rabbit IgG, HRP-linked) (Cell Signaling Technology, USA) was bound by incubating the membrane in a 1:2000 (*Le*UBC1 detection) or 1:6000 (*Sl*UBC3 detection) dilution of the antibody in 5% W/V BSA, 1 x TBST at room temperature with gentle agitation, for 1 hour. The membrane was washed three times for 10 minutes with 1 x TBST. The target protein was detected using chemiluminescent methods, 20 x LumiGLO reagent and 20 x peroxide (Cell Signaling Technology, USA), following the manufacturer's directions. Chemiluminescent signals were captured using the FluoroChem Q imaging system (Alpha Innotech Corporation, USA).

2.9 Bioinformatics and statistical analysis

Bioinformatics. Various bioinformatic tools were used to understand the structure and function of *Le*UBC1 and *Sl*UBC3. Below is a list of the tools that were used and their descriptions.

Scansite: Scansite searches for motifs within the amino acid sequence of the target protein that are likely to be phosphorylated by specific protein kinases or bind to domains. It can also show predicted domains in the sequence of the target protein. The search can be at low, medium, or high stringency.

Clustal Omega and Clustal Phylogeny: Clustal Omega is a multiple sequence alignment program that uses three or more biological sequences (protein, DNA, RNA) to describe the homology and the evolutionary relationships between the sequences examined. Clustal Phylogeny is a tool used for phylogenetic tree generation using the Clustal Omega multiple sequence alignment as the input.

Genevestigator: A useful tool for investigating gene expression and gene regulation. In this project this tool was used to investigate how *LeUBC1* and *SlUBC3* expression was regulated under the influence of development, tissue type, diseases, and other external stimuli. Below is a description of the different factors that were used to examine *LeUBC1* and *SlUBC3* expression:

1. **Development:** this tool summarizes the expression of the gene of interest across different stages of development. The tool displays the results in a linear or log-scale.
2. **Anatomy:** this tool shows the expression level of the target gene across the different tissue types. For each tissue type, the expression value is the average expression value calculated from all samples of each tissue type. The results can be displayed in a linear or log-scale.
3. **Stress:** this tool shows the target gene expression in response to a variety of biotic and abiotic stresses. The responses to these various stresses only compare experimental versus control samples from the same experiment. The responses are then calculated as log ratios between the experimental and control samples. The calculated values represent a down- or up-regulation of the genes in response to a particular stress and are expressed as ratios (linear scale) or log ratios (log₂ scale).

Statistical analysis. Quantification for RT-PCR band intensity was performed using ImageJ. The band intensities for each sample were normalized to a corresponding actin band from the same

sample. RT-PCR sample sizes were 4 independent samples while the enzyme assay sample sizes were 6 independent samples. The RT-PCR control samples were normalized to a value 1. The RT-PCR results were presented as a ratio of the experimental band intensities over the control band intensities. All values were reported as mean +/- S.E.M. The means were analyzed with one-way ANOVA and two-way ANOVA for significance and a p-value ≤ 0.05 indicated a significant difference between the compared means. Student's t-test was applied to my results as well.

Chapter 3

***Le*UBC1**

3.1 Bioinformatic analysis of LeUBC1 and its homologues

The nucleotide sequence of *LeUBC1* (accession X82938) was 923 base pairs (bp). The cDNA sequence of *LeUBC1* had a 5' flanking region of 65 bp and a 3' untranslated region of 279 bp, including the 49 bp of the poly(A) tail [9]. The open reading frame encodes a protein, E2_{21K}, with 194 amino acids (aa). The molecular mass of E2_{21K} was 21.37 kDa. In region 6-145 of the amino acid sequence was a UBC catalytic (UBC_c) domain, which is part of the ubiquitin-mediated degradation pathway in which a thiol ester bond forms between a conserved cysteine residue and the C-terminus of ubiquitin. Another conserved domain was the ubiquitin associated domain (UBA) located in region 157-193 of the amino acid sequence [9]. Its specific role is so far unknown, but it has been suggested that it may be involved in conferring protein target specificity. Based on an amino acid comparison in Feussner *et al.* [9], the *LeUBC1* protein showed homology to the following UBCs: *Bos taurus* (bovine) UBC1, *Saccharomyces cerevisiae* (yeast) ScUBC1, *Lycopersicon esculentum* (tomato) UBC, *Saccharomyces cerevisiae* (yeast) ScUBC4, *Arabidopsis AtUBC11*, *Saccharomyces cerevisiae* (yeast) ScUBC5, and *Arabidopsis* EST.Z25704.

Bioinformatic tools can help understand genes and their corresponding proteins and as a result are useful in designing experiments. For example, motif analysis tools can help identify motifs in the target protein sequence which can aid in predicting the possible function of the target protein. It is also possible to predict the expression level changes of the target protein as the model organism goes through its different modes of growth, as well as when exposed to biotic and abiotic stresses.

Motif analysis of LeUBC1

Motif analysis of *LeUBC1* using the Scansite tool predicted the presence of an ERK-docking domain at site I109 that ERK type MAP kinases interact with (Figure 6). A protein kinase C motif at site T153 was also predicted to be present in the *LeUBC1* protein sequence.

Amino acid sequence homology tree for LeUBC1

Using the Clustal Omega multiple alignment tool and the Clustal Phylogeny tool, a homology tree was created from the amino acid sequences of UBCs previously mentioned in section 3.1 shows the strongest similarity was between *LeUBC1* and the hypothetical *Arabidopsis thaliana* EST.Z25704 at 74%. There were also homologies seen between *LeUBC1* and *Arabidopsis thaliana* AtUBC11, *Bos taurus* (bovine) UBC1, *Saccharomyces cerevisiae* (yeast) ScUBC5, and *Saccharomyces cerevisiae* (yeast) ScUBC4 at 51%, 48%, 48%, and 47% respectively. *LeUBC1* was related to *Saccharomyces cerevisiae* (yeast) ScUBC1, and *Lycopersicon esculentum* (tomato) *LeUBC* with a 46%, and 45% sequence similarity respectively (Figure 7).

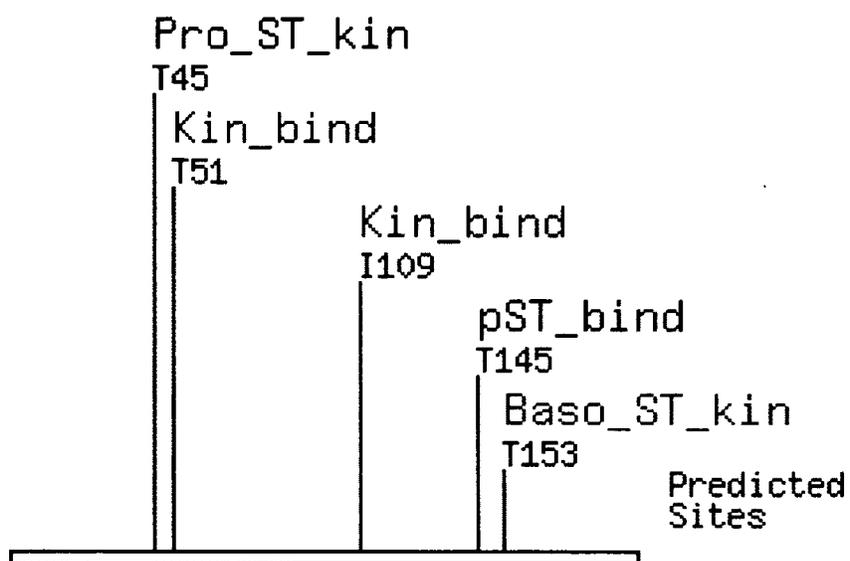


Figure 6. Medium stringency motif analysis of *LeUBC1* using the Scansite bioinformatics tool predicted the existence of T153 and I109, a protein kinase C and an ERK docking-domain respectively.

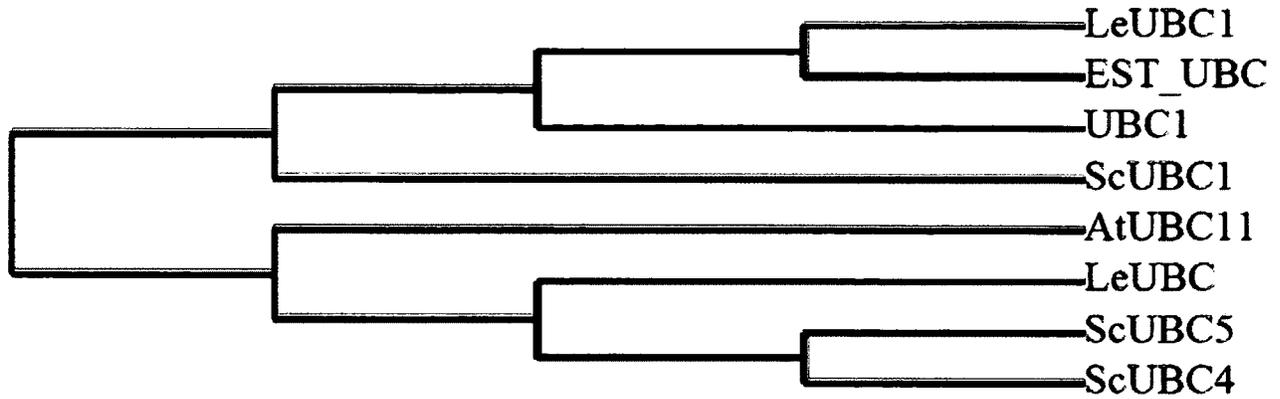


Figure 7. Homology tree from analysis of amino acid sequences of *LeUBC1* and other related UBC genes. Genbank accession numbers for *LeUBC1* and the other UBC sequences are: *Lycopersicon esculentum* (tomato) *LeUBC1* (CAA58111), *Arabidopsis thaliana* hypothetical gene (EST.Z25704), *Bos taurus* (bovine) UBC1 (AAB19536), *Saccharomyces cerevisiae* (yeast) *ScUBC1* (CAA39812), *Arabidopsis thaliana* *AtUBC11*(CAA78716), *Lycopersicon esculentum* (tomato) *LeUBC* (CAA51821), *Saccharomyces cerevisiae* (yeast) *ScUBC5* (CAA35529), *Saccharomyces cerevisiae* (yeast) *ScUBC4* (CAA35528).

LeUBC1 expression across the tomato life cycle

According to the data compiled at Genevestigator, *LeUBC1* was constitutively expressed throughout the developmental stages of tomato. No significant differences in the levels of the *LeUBC1* transcripts were observed among the different stages (Figure 8).

LeUBC1 expression across the tomato tissue types

Using data obtained from Genevestigator *LeUBC1* was found to have a stable level of expression in all tissues (Figure 9).

LeUBC1 response to abiotic and biotic stresses

Analysis of stress-response data from Genevestigator showed no significant changes from control *LeUBC1* expression levels in response to the various biotic stimuli (Figure 10B). The expression of *LeUBC1* was also not significantly changed in response to various pathogens, and abiotic stressors (Figure 10A).

3.2 *LeUBC1* transcription

The effect of FB1 and MG-115 on LeUBC1 transcription

LeUBC1 relative transcript levels did not change significantly when comparing the treated groups to the control (0 hour H₂O). Changes in relative transcript levels between the 0 hour point and 48 hours after treatment were only statistically significant with the MG-115 treatment where *LeUBC1* was significantly decreased after the 48 hour treatment ($p=0.01401$) (Figure 11).

The effect of FB1 and ERKI on LeUBC1 transcription

Because the *LeUBC1* motif analysis indicated a possibility of its interaction with ERK-type MAP kinases, we considered a possibility that the expression of *LeUBC1* in tomato infected leaves is modulated by ERK-type MAP kinases. To address this possibility, their mRNA accumulation levels were investigated under mock (0 hour and 48 hour H₂O) and treated (0 hour and 48 hour FB1, and FB1+ERKI) conditions by RT-PCR. There were no significant differences in the mRNA levels between the mock and other treatments and between the 0 hour and 48 hour samples within treatments (Figure 12).

3.3 *LeUBC1* protein detection

E. coli host strain BL21-CodonPlus (DE3)-RIPL cells transformed with the expression vector pET14b-*LeUBC1* produced a recombinant fusion protein of about 21.37 kDa after IPTG induction. Western blot using rabbit his-tag polyclonal antibody showed that the majority of the fusion protein was present in the induced TCP fraction. There was some fusion protein in the uninduced TCP fraction and the induced SC fraction, but no fusion protein was detected in the uninduced SC fraction (Figure 13).

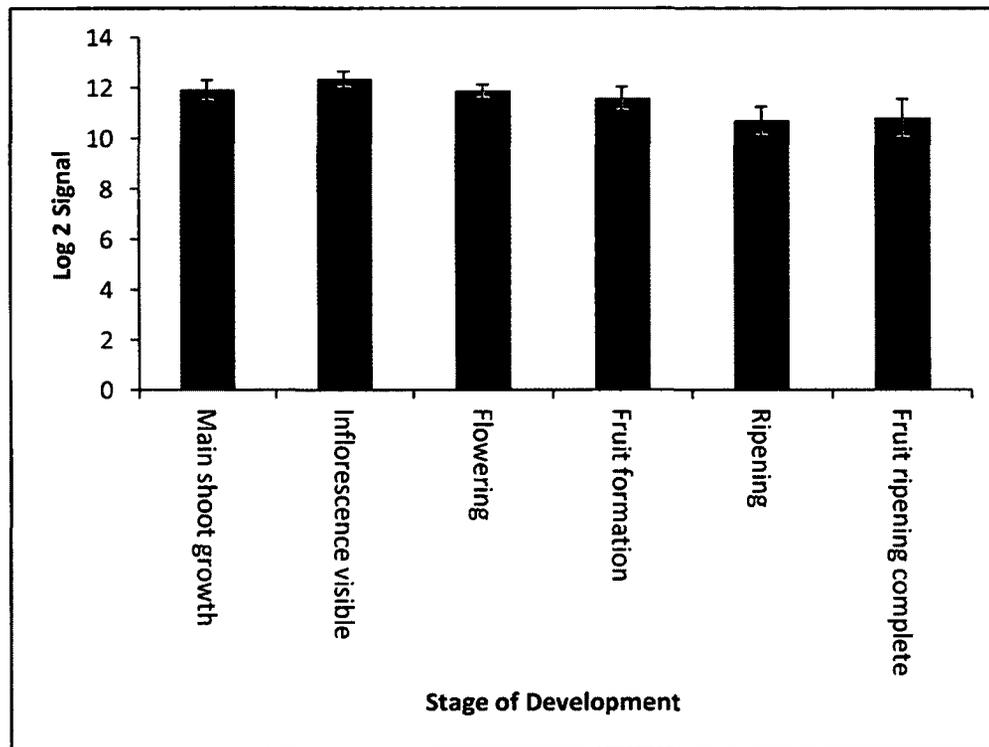


Figure 8. Expression analysis of *LeUBC1*. Relative expression level of *LeUBC1* in the indicated tomato life stages was determined using *Lycopersicon esculentum* pre-existing microarray data obtained from Genevestigator. Results are a mean of different sample sizes for each developmental stage \pm S.E.

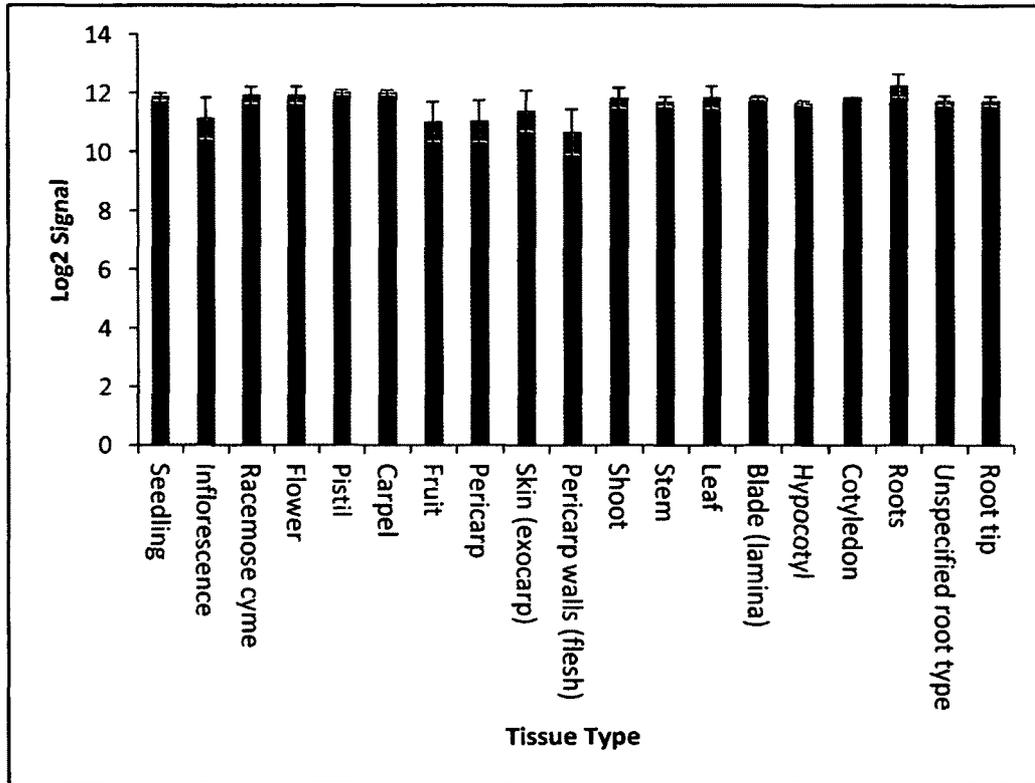


Figure 9. Expression analysis of *LeUBC1*. Relative expression level of *LeUBC1* in the indicated tomato tissues types was determined using *Lycopersicon esculentum* pre-existing microarray data obtained from Genevestigator. Results are a mean of three repetitions \pm S.E.

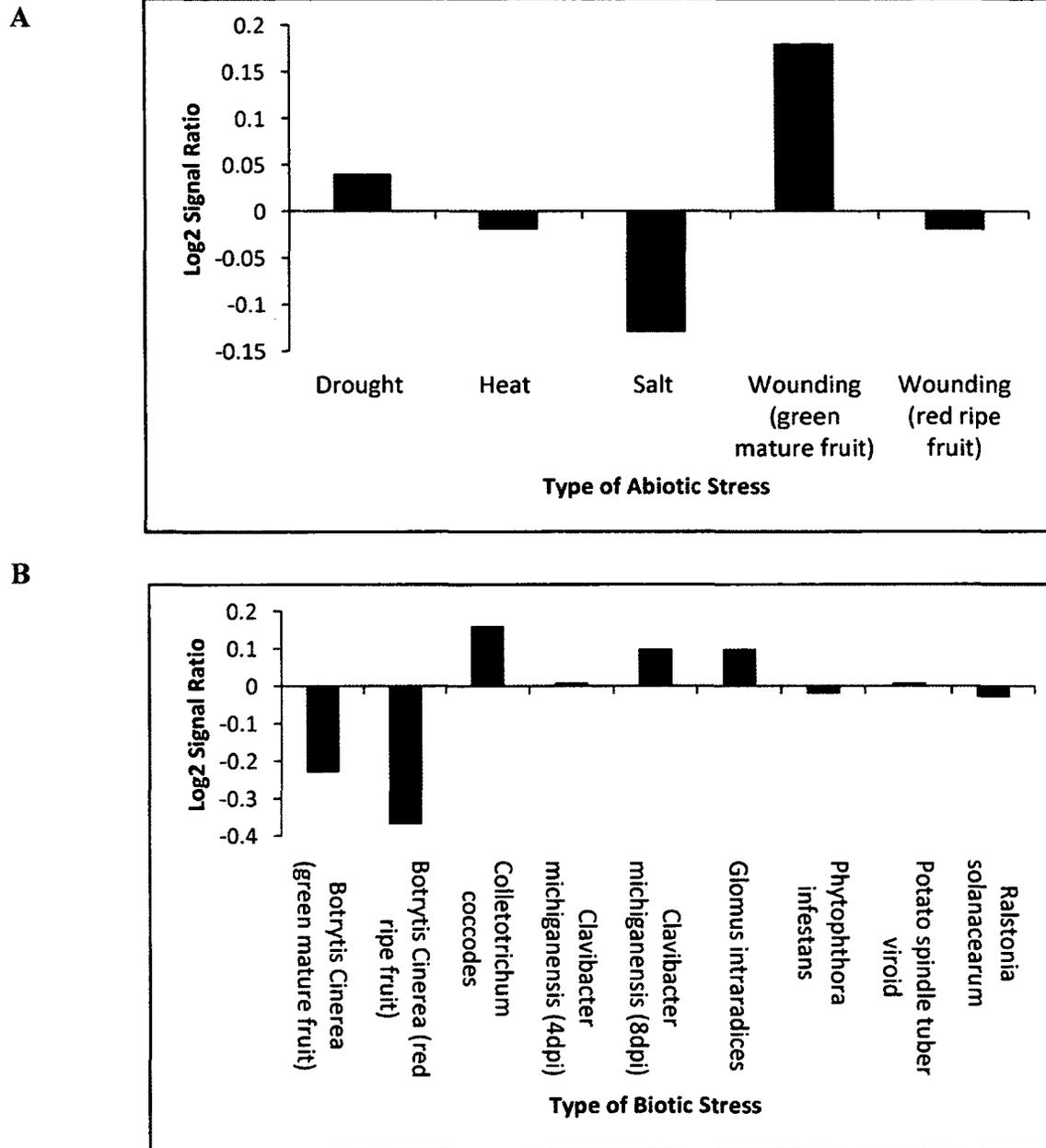
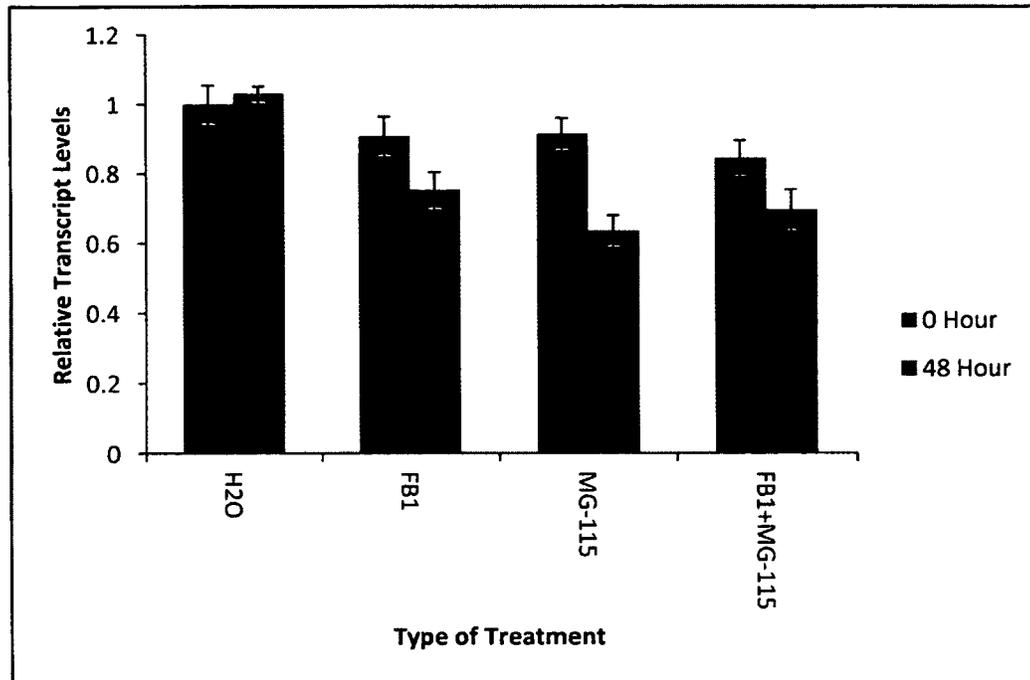


Figure 10. *LeUBC1* expression levels in various *Lycopersicon esculentum* anatomical parts under control conditions and in response to a number of abiotic stresses (A) and pathogens (B). The log₂ signal ratio is obtained by taking the ln of the division of the expression of *LeUBC1* in each treated group by the expression of *LeUBC1* under control conditions. The difference of expression of *LeUBC1* between treated and control groups was tested for significance using false discovery rates with a threshold of $p = 0.05$.



LeUBC1



Actin



Figure 11. RT-PCR analysis showing the effect of 0 hour and 48 hour H₂O, FB1, MG-115, and FB1+MG-115 on *LeUBC1* mRNA transcript levels in tomato leaves. Representative PCR products on agarose gels are shown, from left to right: 0 hour H₂O, 0 hour FB1, 0 hour MG-115, 0 hour FB1+MG-115, 48 hour H₂O, 48 hour FB1, 48 hour MG-115, and 48 hour FB1+MG-115. Band intensities for *LeUBC1* were normalized against actin bands amplified from the same sample. Data was analyzed using the two way analysis of variance (ANOVA) ($p_{0-48h}=0.3889$, $p_{treatments}=0.7824$, $n=4$) and Student's t-test ($p_{0-48hFB1}=0.12429$, $p_{0-48hMG-115}=0.01401$, $p_{0-48hFB1+MG-115}=0.1394$). Data were obtained from four independent biological repeats.

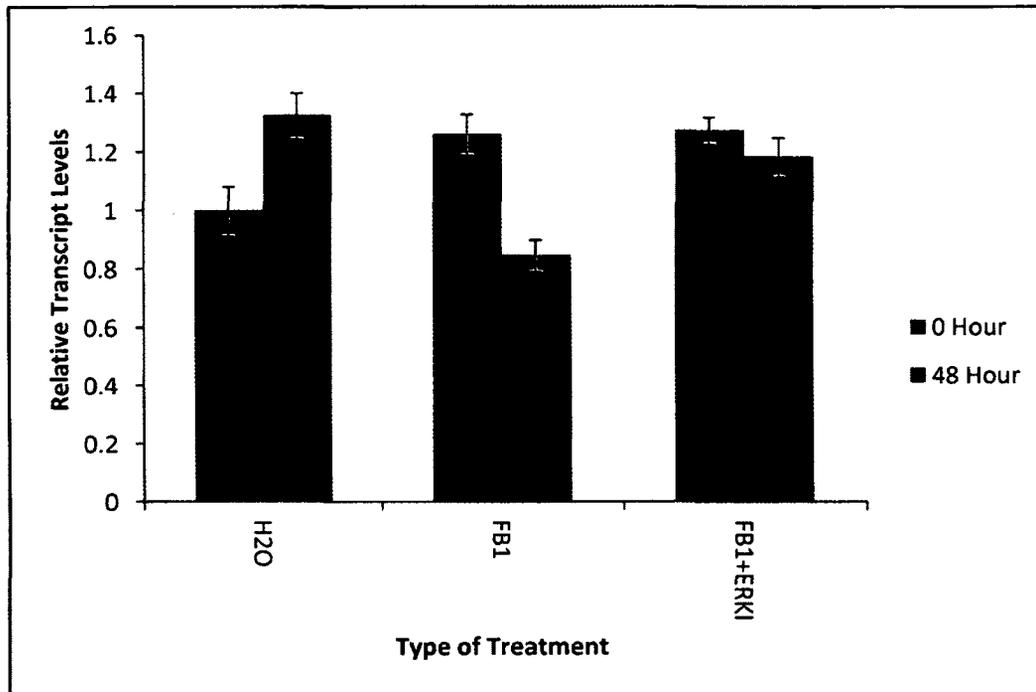


Figure 12. RT-PCR analysis showing the effect of 0 hour and 48 hour H₂O, FB1, and FB1+ERKI on *LeUBC1* mRNA transcript levels in tomato leaves. Representative PCR products on agarose gels are shown, from left to right: 0 hour H₂O, 0 hour FB1, 0 hour FB1+ERKI, 48 hour H₂O, 48 hour FB1, and 48 hour FB1+ERKI. Band intensities for *LeUBC1* were normalized against actin bands amplified from the same sample. Data was analyzed using the two way analysis of variance (ANOVA) ($p_{0-48h}=0.7676$ $p_{treatments}=0.8964$, $n=2$) and Student's t-test ($p_{0-48hFB1}=0.09452$ $p_{0-48hFB1+ERKI}=0.33301$). Data were obtained from two independent biological repeats.

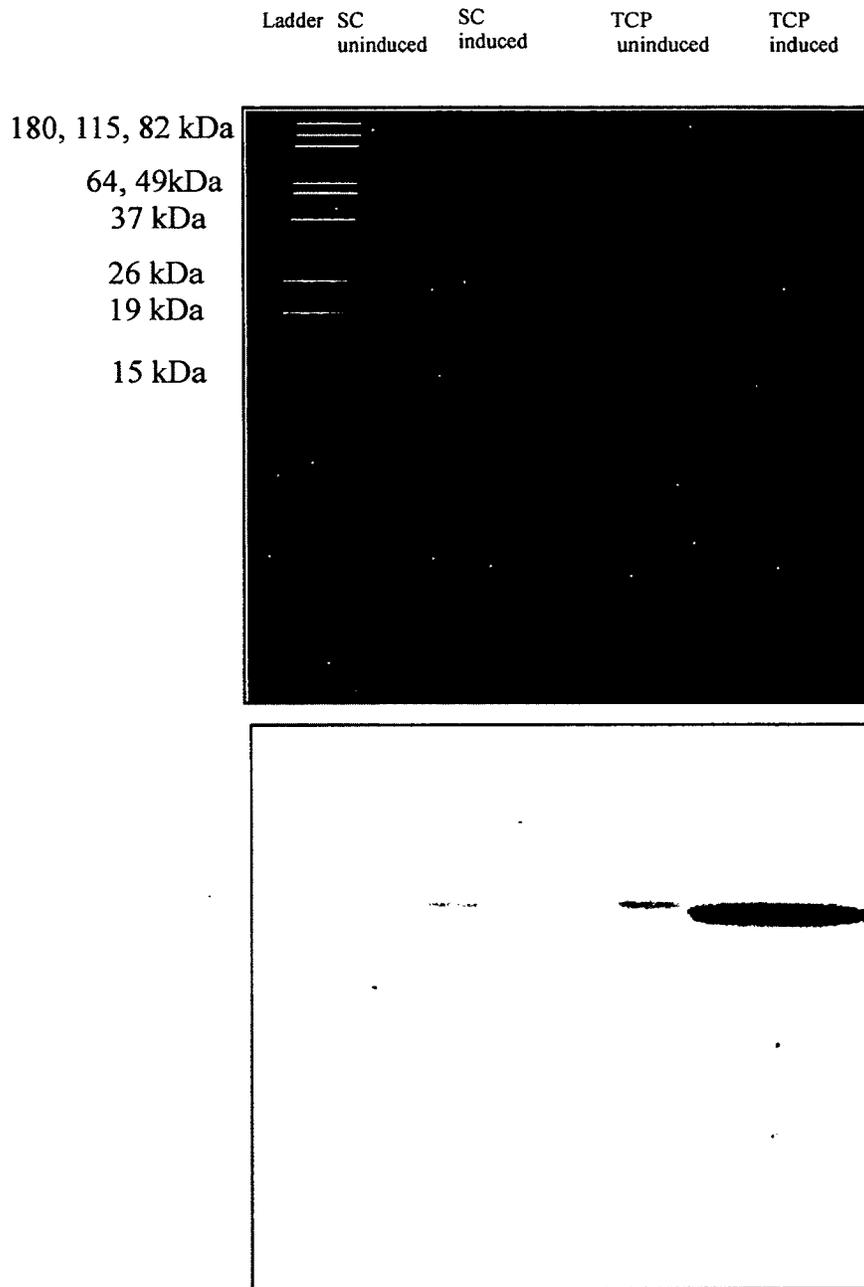


Figure 13. Analysis of the expression of the *LeUBC1*-His tag fusion protein, in the indicated protein fractions, with western blot. The *E. coli* lysate containing the expressed fusion protein was induced with IPTG. The same membrane was visualized using chemiluminescent detection (upper) and X-ray (lower).

Chapter 4

S/UBC3

4.1 Bioinformatic analysis of SIUBC3 and its homologues

The *Lycopersicon esculentum* *SIUBC3* (accession L2367) transcript was 825 bp with a 3' untranslated region from nucleotide 614 to 825 bp, and a 5' untranslated region from nucleotide 1 to 166 bp. This encodes a protein with 148 aa [8]. According to the general protein information by National Center for Biotechnology Information (NCBI), the molecular mass of the *SIUBC3* protein was approximately 17 kDa. Clustal alignment analysis of *SIUBC3* protein with *Arabidopsis thaliana* UBC members showed it was closely related to *AtUBC28*, *AtUBC10*, and *AtUBC8*. *SIUBC3* was also highly homologous to the *Gossypium hirsutum* (cotton) UBC enzyme, *GhUBC1* protein product.

Below is the description of the bioinformatic tools that were used to predict *SIUBC3* motifs, the expression across the stages of tomato development and tissue types, and the response of *SIUBC3* to abiotic and biotic stresses.

Motif analysis of SIUBC3

Using the Scansite motif analysis tool, *SIUBC3* was predicted to have an ERK-docking domain at position L97, among other motifs, at the medium stringency scan (data not shown). When a high stringency filter was applied, *SIUBC3* was predicted to have only an Src homology 3 group (SH3) at site P65 (Figure 14).

Amino acid sequence homology tree for SIUBC3

Using the Clustal Omega and Clustal Phylogeny tools, a homology tree was deduced based on the amino acid sequence of *SIUBC3* and its homologues (Figure 15). An amino acid based homology tree was also performed using the amino acid sequences of the above proteins. The amino acid sequence of *SIUBC3* was found to be 97% similar to the sequences

for *Gossypium hirsutum* (cotton) GhUBC1 and *Arabidopsis thaliana* AtUBC28. It was also 95%, 79%, and 78% similar to the proteins encoded by *Arabidopsis thaliana* AtUBC8, *Saccharomyces cerevisiae* (yeast) ScUBC4, and *Saccharomyces cerevisiae* (yeast) ScUBC5 respectively.

SIUBC3 expression across the tomato life cycle

Based on the Genvestigator compiled data, *SIUBC3* was highly expressed throughout the life cycle (Figure 16).

SIUBC3 expression across the tomato tissue types

Using data obtained from Genvestigator, *SIUBC3* was found to be highly expressed in all the tomato organs (Figure 17).

SIUBC3 response to abiotic and biotic stresses

Analysis of abiotic stress-response data from Genevestigator revealed a significant *SIUBC3* up regulation in response to heat in leaf samples, salt, and wounding in red ripe fruit (Figure 18A). In terms of biotic stresses *SIUBC3* was down regulated in *Colletotrichum coccodes* treated fruit pericarp flesh samples, and up regulated in potato spindle tuber viroid treated leaf samples (Figure 18B).

4.2 *SIUBC3* transcription

The effect of FB1 and MG-115 on SIUBC3 transcription

SIUBC3 relative transcript levels did not change significantly when comparing the treated groups to the control (0 hour H₂O). Changes in relative transcript levels between the 0 hour point and 48 hours after treatment were only statistically significant with the MG-115 treatment where *SIUBC3* was significantly decreased after the 48 hour treatment (p=0.00678) (Figure 19).

The effect of FB1 and ERKI on SIUBC3 transcription

Because the *SIUBC3* motif analysis indicated a possibility of its interaction with ERK-type MAP kinases, we considered a possibility that the expression of *SIUBC3* in tomato infected leaves is modulated by ERK-type MAP kinases. To address this possibility, their mRNA accumulation levels were investigated under mock (0 hour and 48 hour H₂O) and treated (0 hour and 48 hour FB1, and FB1+ERKI) conditions by RT-PCR. There were no significant differences in the mRNA levels between the mock and other treatments and between the 0 hour and 48 hour samples within treatments (Figure 20).

4.3 *SIUBC3* protein detection

The *E. coli* host strain BL21-CodonPlus (DE3)-RIPL cells transformed with the expression vector pET14b-*SIUBC3* produced a recombinant fusion protein of about 17 kDa after IPTG induction. Western blot using rabbit his-Tag polyclonal antibody showed that the majority of the fusion protein was present in the induced TCP fraction (Figure 21). There was some fusion

protein in the uninduced TCP fraction and the induced SC fraction, but no fusion protein was detected in the uninduced SC fraction (Figure 21).



Figure 14. High stringency motif analysis of *STUBC3* using the Scansite bioinformatics tool predicted the existence of an SH3 group at position P65.

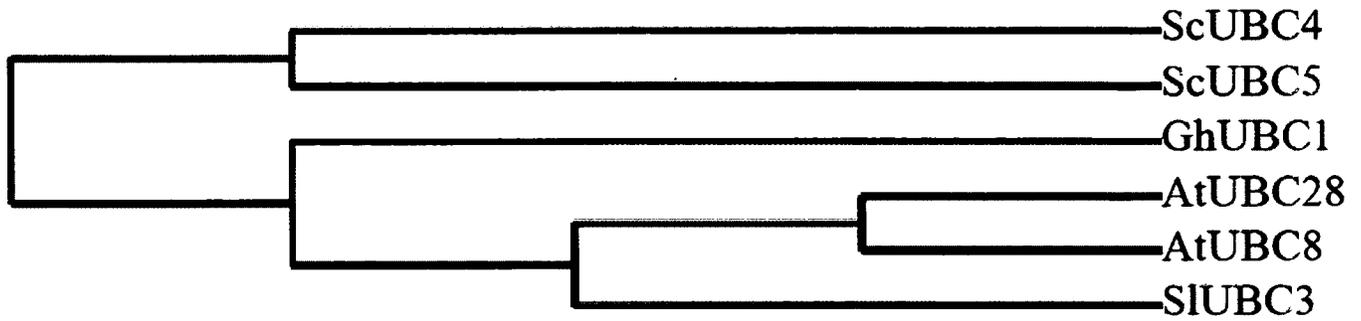


Figure 15. Homology tree from analysis of amino acid sequences of *SIUBC3* and other related UBC genes. Genbank accession numbers for *SIUBC3* and the other UBC sequences are: *Saccharomyces cerevisiae* (yeast) *ScUBC4* (NP_009638), *Saccharomyces cerevisiae* (yeast) *ScUBC5* (NP_010344), *Gossypium hirsutum* (cotton) *GhUBC1* (AAL99219), *Arabidopsis thaliana* *AtUBC28* (NP_564828), *Arabidopsis thaliana* *AtUBC8* (NP_001190447), and *Lycopersicon esculentum* (tomato) *SIUBC3* (AAA34125).

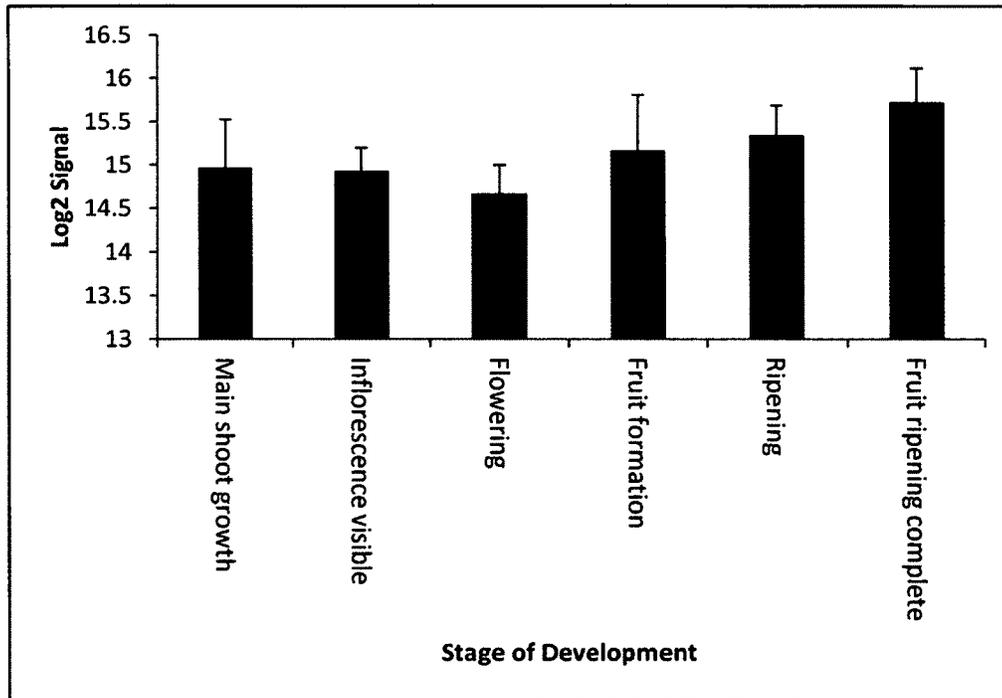


Figure 16. Expression analysis of *SlUBC3*. Relative expression level of *SlUBC3* in the indicated tomato life stages was determined using *Lycopersicon esculentum* pre-existing microarray data obtained from Genevestigator. Results are a mean of different sample sizes for each developmental stage \pm S.E.

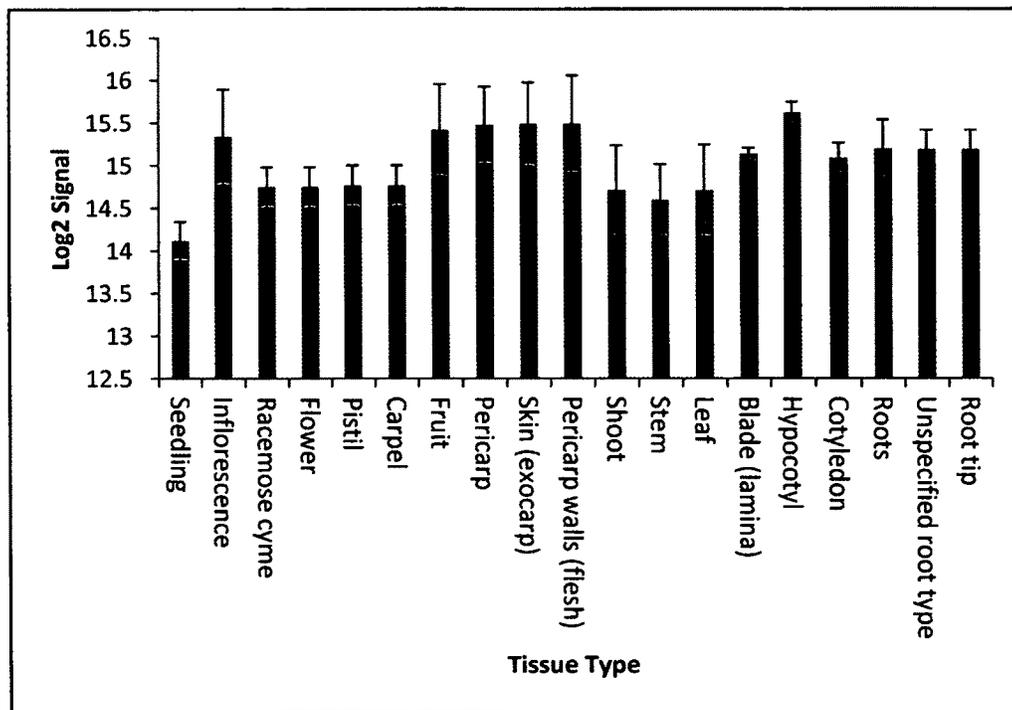


Figure 17. Expression analysis of *SlUBC3*. Relative expression level of *SlUBC3* in the indicated tomato tissues types was determined using *Lycopersicon esculentum* pre-existing microarray data obtained from Genevestigator. Results are a mean of three repetitions \pm S.E.

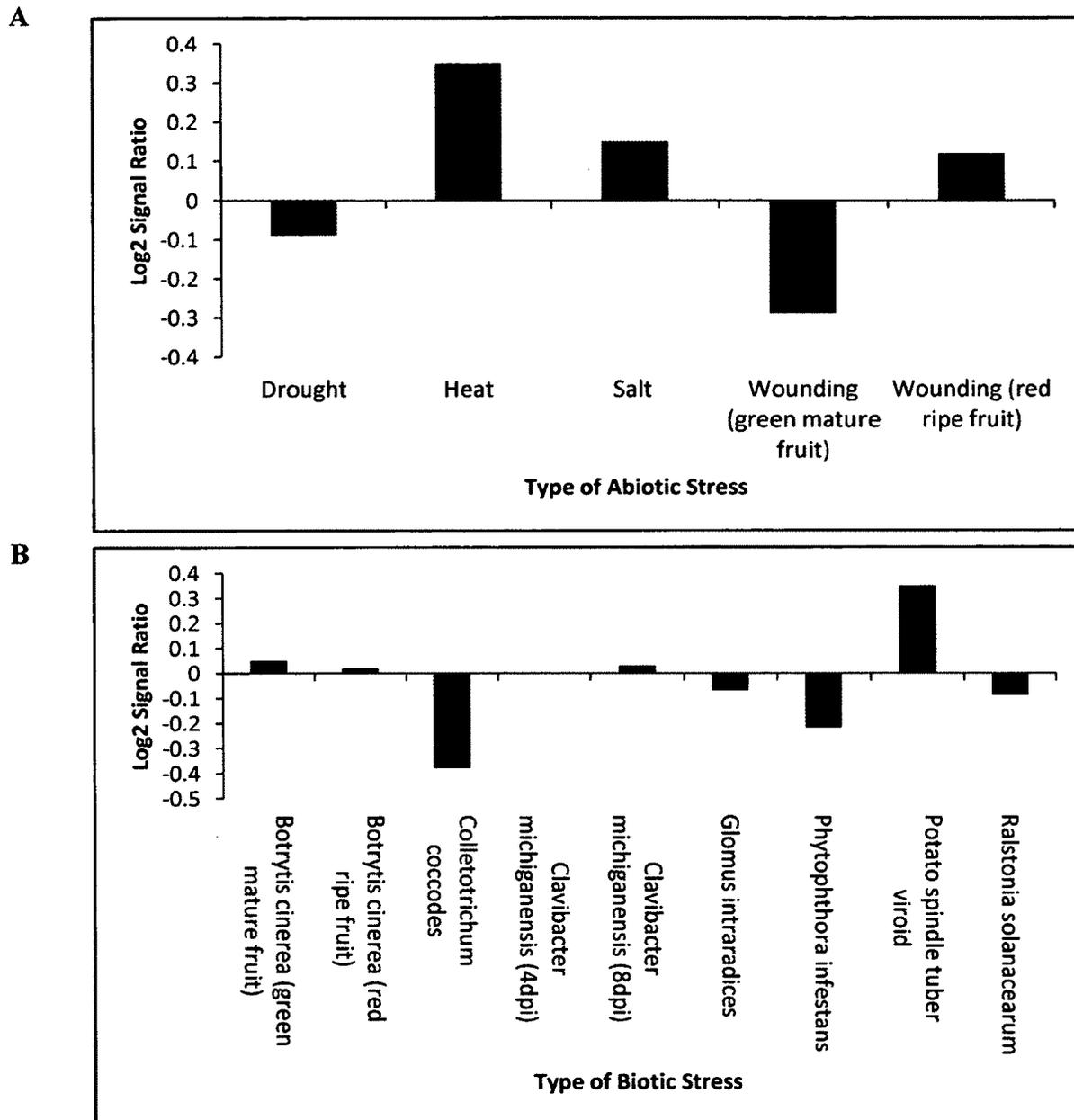
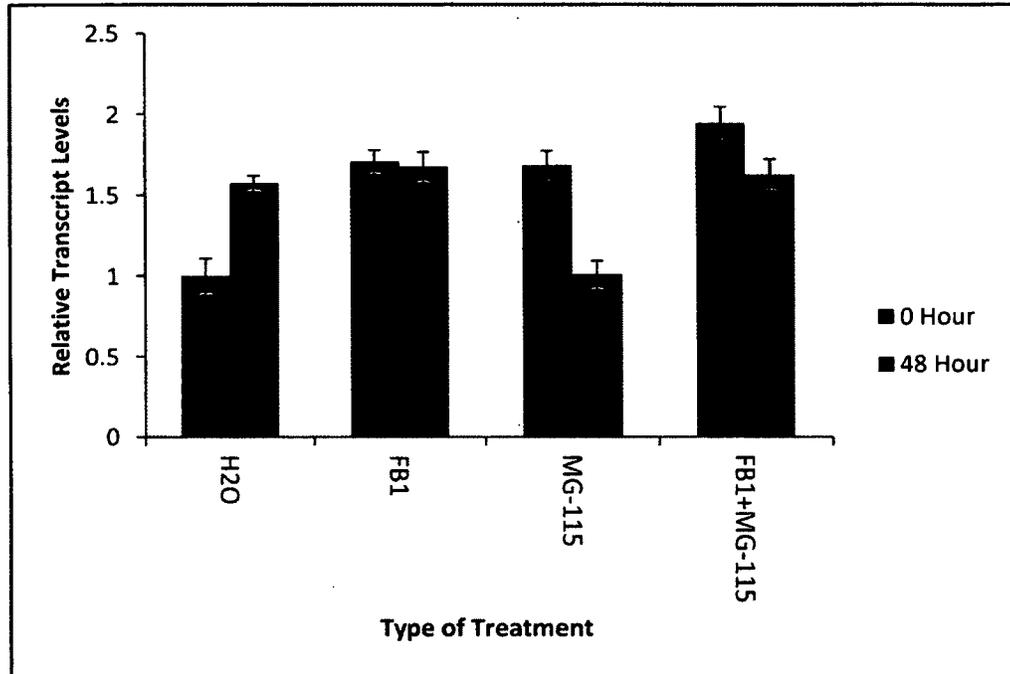


Figure 18. *S/UBC3* expression levels in various *Lycopersicon esculentum* anatomical parts under control conditions and in response to a number of abiotic stresses (A) and pathogens (B). The log₂ signal ratio is obtained by taking the ln of the division of the expression of *S/UBC3* in each treated group by the expression of *S/UBC3* under control conditions. The difference of expression of *S/UBC3* between treated and control groups was tested for significance using false discovery rates with a threshold of $p=0.05$.



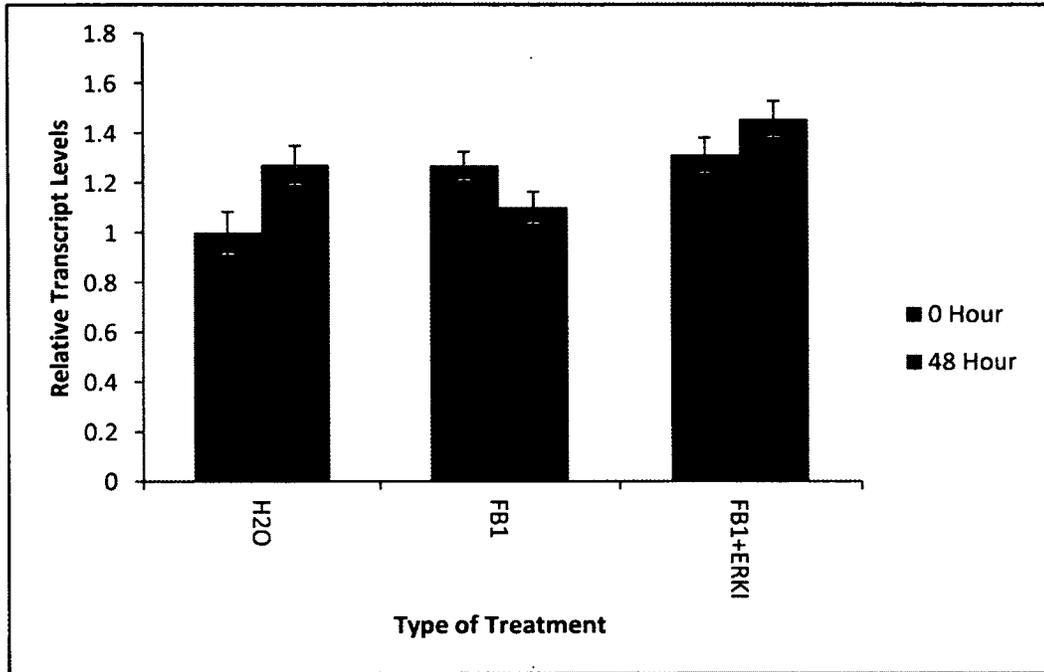
SIUBC3



Actin



Figure 19. RT-PCR analysis showing the effect of 0 hour and 48 hour H₂O, FB1, MG-115, and FB1+MG-115 on *S/UBC3* mRNA transcript levels in tomato leaves. Representative PCR products on agarose gels are shown, from left to right: 0 hour H₂O, 0 hour FB1, 0 hour MG-115, 0 hour FB1+MG-115, 48 hour H₂O, 48 hour FB1, 48 hour MG-115, and 48 hour FB1+MG-115. Band intensities for *S/UBC3* were normalized against actin bands amplified from the same sample. Data was analyzed using the two way analysis of variance (ANOVA) ($p_{0-48h}=0.7668$ $p_{treatments}=0.7265$, $n=4$) and Student's t-test ($p_{0-48hFB1}=0.80536$ $p_{0-48hMG-115}=0.00678$ $p_{0-48hFB1+MG-115}=0.09095$). Data were obtained from four independent biological repeats.



SIUBC3



Actin



Figure 20. RT-PCR analysis showing the effect of 0 hour and 48 hour H₂O, FB1, and FB1+ERKI on *S/UBC3* mRNA transcript levels in tomato leaves. Representative PCR products on agarose gels are shown, from left to right: 0 hour H₂O, 0 hour FB1, 0 hour FB1+ERKI, 48 hour H₂O, 48 hour FB1, and 48 hour FB1+ERKI. Band intensities for *S/UBC3* were normalized against actin bands amplified from the same sample. Data was analyzed using the two way analysis of variance (ANOVA) ($p_{0-48h}=0.8644$ $p_{treatments}=0.7968$, $n=2$) and Student's t-test ($p_{0-48hFB1}=0.38697$ $p_{0-48hFB1+ERKI}=0.23567$). Data were obtained from four independent biological repeats.

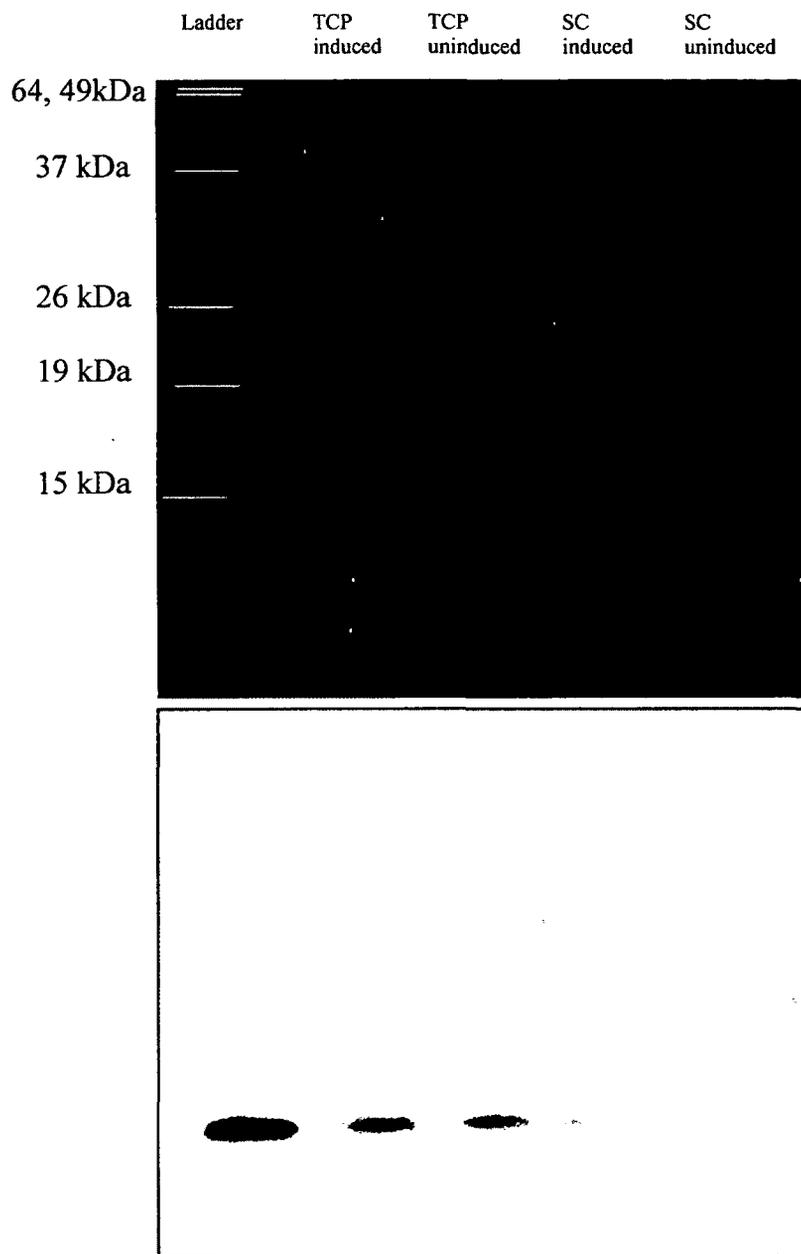


Figure 21. Analysis of the expression of the *STUBC3*-his tag fusion protein, in the indicated protein fractions, with western blot. The *E. coli* lysate containing the expressed fusion protein was induced with IPTG. The same membrane was visualized using chemiluminescent detection (upper) and X-ray (lower).

Chapter 5

β -1,3-glucanase and Glutamine Synthetase

5.1 β -1,3-glucanase total activity assay

The main objective of this study was to determine the role of FB1 as an effector of plant defense responses, particularly those involved in defense against fungal pathogens. To test this, we measured the activity of all β -1,3-glucanase isoforms in soluble protein extracts from mock (0 hour and 48 hour H₂O) and treated tomato leaves (0 hour and 48 hour FB1, MG-115, and FB1+MG-115). The activity of the β -1,3-glucanases was significantly increased, compared to their activity at the 0 hour mark, after 48 hours of treatment with H₂O, FB1, MG-115, and FB1+MG-115 (Figure 22). However, no significant difference was observed when the activities of the glucanases under the different treatments were observed (Figure 22).

5.2 Glutamine synthetase total activity assay

A glutamine synthetase assay was performed on the protein extracts from the previous section. To test for significance between the 0 hour and 48 hour samples, a Two Way ANOVA was used, which showed that there was a significant difference between the 0 hour and 48 hour samples ($p=0.043$), but no significant difference in the effects of the different treatments ($p=0.103$) (Figure 23). A One Way ANOVA was used to test for significant differences between the treatments after 48 hours of treatment. It showed that after 48 hours, the activity of glutamine synthetase was significantly different between the water and the other treatments ($p=0.038$) (Figure 23).

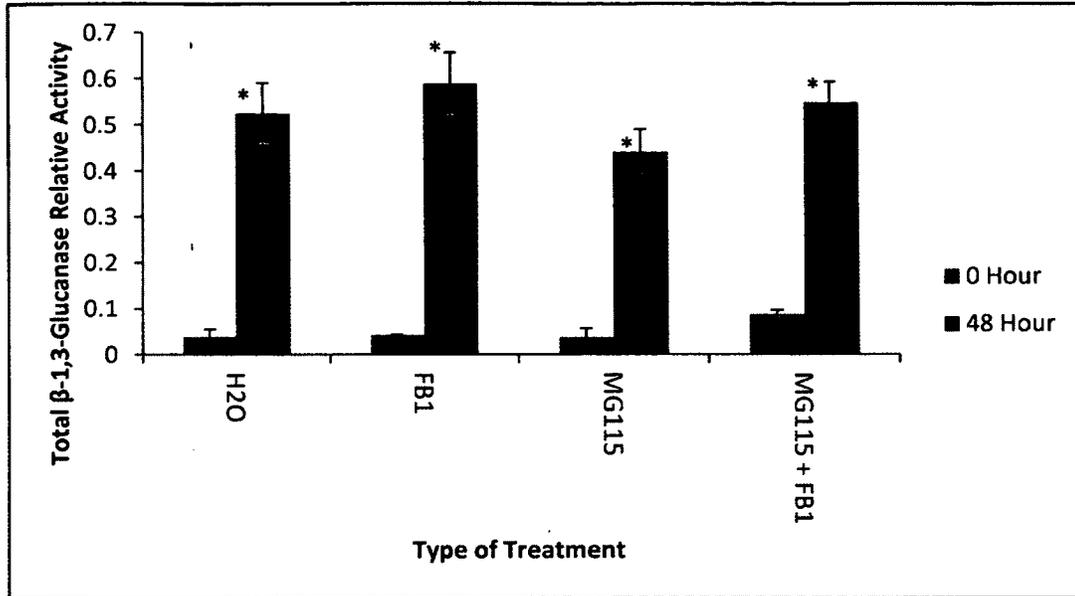


Figure 22. Total β -1,3-glucanase activity in mock and tomato leaves treated with either FB1, MG-115, or FB1+MG-115 was determined using laminarin as substrate. Values are expressed as means \pm standard error. Significance was assessed using the two way analysis of variance (ANOVA) ($p_{0-48h} < 0.0001$ $p_{treatments} = 0.1605$, $n=6$). An asterisk indicates a significant difference ($p \leq 0.05$). Data were obtained from six independent biological repeats.

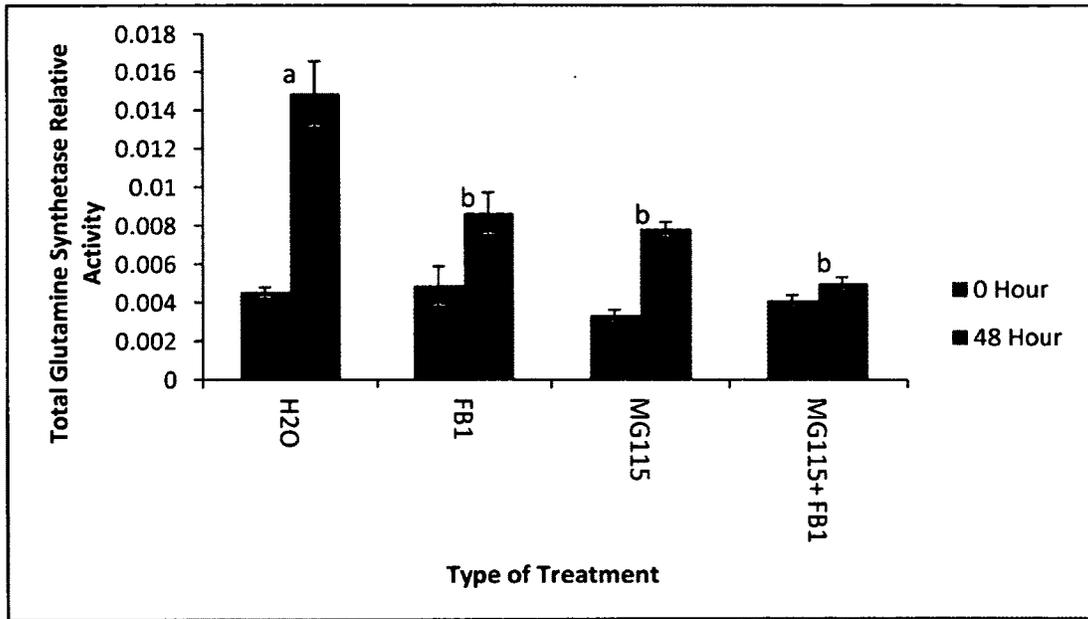


Figure 23. Total glutamine synthetase activity in mock and tomato leaves treated with either FB1, MG-115, or FB1+MG-115 was determined using glutamate as substrate. Values are expressed as means +/- standard error. Significance was assessed using the two way analysis of variance (ANOVA) and one way analysis of variance (ANOVA) ($p_{0-48h}=0.043$ $p_{treatments}=0.103$, $p_{48h\ treatments}=0.038$, $n=6$). An “a” or “b” indicates a significant difference ($p \leq 0.05$). Data were obtained from six independent biological repeats.

Chapter 6

Discussion

6.1 Bioinformatic analysis of *LeUBC1* and *SlUBC3*

LeUBC1 and *SlUBC3* expression across the tomato life cycle and tissue types

Genevestigator data analysis indicated that *LeUBC1* and *SlUBC3* are constitutively expressed throughout the developmental stages of tomato. This can be an indication that *LeUBC1* and *SlUBC3* may perform essential functions in many cell types during plant development.

LeUBC1 showed an even expression pattern in different tomato tissue types based on data available through Genevestigator. *LeUBC1* was homologous to *ScUBC4* and *ScUBC5*, which are both essential for protein turnover and stress response, so it makes sense that it is evenly expressed in all tissues since these functions are important to all tissues. In contrast, there was more variation in *SlUBC3* expression in the tomato anatomical parts. *SlUBC3* expression in inflorescence, fruit, pericarp, skin (exocarp), pericarp walls (flesh), and hypocotyl was at least 3-fold higher than in most of the other tomato anatomical parts. Since *SlUBC3* was homologous to the cotton *GhUBC1*, which is involved in plant senescence and is highly expressed in senescent leaves and flowers, it is possible that *SlUBC3* is highly expressed in these tissues with a possible function in senescence [35]. Senescence is often induced in plant tissues such as the flower and fruit through hormones like ethylene, so it would be interesting to see if *SlUBC3* is regulated by ethylene. The relatively lower *SlUBC3* expression in the other anatomical parts including seedling, flower, pistil, carpel, shoot, stem, leaf, cotyledon, roots, and root tip might be due to various reasons. One possible reason is a tighter regulation of *SlUBC3* in those tissue types. Also *SlUBC3* may have a broad specificity, thus not requiring high expression in those tissues.

Another possible reason is that the need for *SIUBC3* enzymes may be reduced as a result of a decrease in the total level of target protein ubiquitination in those anatomical parts.

Homology trees for LeUBC1 and SIUBC3

As an attempt to clarify what the role of *LeUBC1* and *SIUBC3* might be in stressed tomato, a homology tree using the amino acid sequence of *LeUBC1* and *SIUBC3* and their homologous plant UBC proteins was performed. For *LeUBC1*, the strongest similarity was between its sequence and the hypothetical *Arabidopsis thaliana* EST.Z25704. However, this does not provide any information about any possible roles of *LeUBC1* in stressed tomato. There were also homologies seen between *LeUBC1* and *Arabidopsis thaliana AtUBC11*, bovine UBC1, yeast *ScUBC5*, and yeast *ScUBC4* at 51%, 48%, 48%, and 47% respectively. *AtUBC11*, *ScUBC4* and *ScUBC5* are all heat shock inducible and essential to protein turnover [11]. *LeUBC1* transcription was also induced by heat shock as shown in Feussner *et al.* [9]. *LeUBC1* was also significantly homologous to yeast *ScUBC1*, and both are induced by metal stress. Based on this structural homology, it seems likely that these UBCs serve a similar essential function in protein turnover and the response to stresses such as heat shock and heavy metal stress. *ScUBC4* and *ScUBC5* and their UBC homologs require supplementary E3s for target substrate recognition, unlike some UBCs that can ubiquitinate target proteins directly. It can be predicted that *LeUBC1* would also require an E3 to ubiquitinate their target substrate.

When compared to other plant UBC proteins, *SIUBC3* was most homologous to cotton *GhUBC1* and *Arabidopsis thaliana AtUBC28*. It has been shown that cotton *GhUBC1* plays an important role in plant senescence and is highly expressed in senescent leaves and flowers. Also, it substitutes the function of yeast *ScUBC4* and *ScUBC5*. As previously stated, yeast *ScUBC4*

and *ScUBC5* transcription was induced by heat shock and their role in targeting their substrate requires a supplementary E3. Therefore, it is likely that cotton *GhUBC1* and *SlUBC3* both perform their functions in a similar way. *AtUBC28* has been shown to function in vitro with a large number of E3s to perform a broad ubiquitination function. *SlUBC3* was also significantly similar to the proteins encoded by yeast *ScUBC4* and *ScUBC5*. As a result of these homologies, it is possible that *SlUBC3* plays a general ubiquitination function in plants as well as a more specific role in senescence.

6.2 Transcriptional regulation of LeUBC1 and SlUBC3

The effect of FB1 and MG-115 on LeUBC1 and SlUBC3 transcription

RT-PCR analysis showed that *LeUBC1* and *SlUBC3* transcript levels did not change significantly when comparing the groups treated with FB1 and/or MG-115 to the control (0 hour H₂O) groups. However *LeUBC1* and *SlUBC3* transcript levels decreased significantly compared to their 0 hour levels after the 48 hour treatment with MG-115. This agrees with our hypothesis and shows that these UBCs are down regulated by proteasome inhibition due to the accumulation of ubiquitinated substrates. For *LeUBC1*, the result differs from the Northern blotting data presented by Feussner *et al.* [9], showing an increase in its transcription levels in response to stresses such as heat shock and heavy metal stress, which supports our prediction, based on homology trees for *LeUBC1*, that *LeUBC1* has an essential function in responding to stresses such as heat shock and heavy metal stress [9]. However, our findings were supported by data from Genevestigator, which showed no significant changes from control *LeUBC1* expression levels in response to various pathogens. Therefore, it is possible that *LeUBC1* plays a role in responding to abiotic stresses but not biotic stresses.

The RT-PCR data for *S/UBC3* did not support our hypothesis. We had predicted that *S/UBC3* would play a role in the response to biological stresses such as those caused by fungal toxins. This was based on previous data which had linked *S/UBC3* to the response to both biotic and abiotic stresses. For example, transgenic expression of β Cl in tobacco plants causes the production of an unstable β Cl that should be targeted for degradation by the 26S proteasome [8]. However, the transgenic plants showed a decreased level of polyubiquitinated protein compared to wild type plants. This was not because of a decrease in *S/UBC3* expression in these plants, but possibly due to the possibility that the interaction between β Cl and *S/UBC3* may impair the activity of this UBC, resulting in a perturbation in the ubiquitin proteasome pathway. Normally, unstable proteins are targeted for degradation by the ubiquitin proteasome pathway in plant cells. However, if their expression leads to inhibition of the ubiquitin proteasome pathway, it would result in further accumulation of the proteins in infected plants. Thus, the targeting of *S/UBC3* by β Cl for inhibition may be an indication that *S/UBC3* is involved in the defense response to pathogens [8, 34]. In addition, *S/UBC3* expression levels were up regulated in potato spindle tuber viroid treated leaf samples. Also, homology trees for *S/UBC3* predicted that the protein may be involved in the response to stresses such as heat shock. This prediction was supported by Genevestigator based stress-response data that indicated a significant up regulation in response to heat as well as salt.

One possible explanation for the *LeUBC1* and *S/UBC3* RT-PCR results is that the availability of *LeUBC1* and *S/UBC3* transcript may already be high enough under normal conditions to cope with the increase in demand for ubiquitin-dependent degradation under stress conditions. Another possible explanation is that tomatoes may have UBCs other than *LeUBC1* and *S/UBC3* that respond to fungal toxin induced stress through up regulation. Different UBC

enzymes have specialized functions, mainly because they are localized to different cellular compartments and more than one UBC enzyme can interact with the same E3 in different parts of the cell. It was shown that two different UBCs, *OsUBC5a* and *OsUBC5b*, interact with the E3 EL5 in rice [27]. Upon elicitor induced stress however, only *OsUBC5b* mRNA was shown to be up regulated along with the EL5 mRNA levels. Although *OsUBC5a* does interact with EL5, its mRNA levels are not up regulated in response to stress. This shows that it is possible that certain UBC enzymes, although involved in ubiquitination and interaction with E3s, do not respond to certain types of stress, possibly explaining why we did not see a change in mRNA levels of *LeUBC1* and *SlUBC3* in response to fungal toxin induced stress.

The effect of FB1 and ERKI on LeUBC1 and SlUBC3 transcription

We hypothesized that MAP kinases may be involved in phosphorylating *LeUBC1* and *SlUBC3* during infection scenarios i.e. fungal toxin induced stress. As previously stated, previous work in our lab has indicated that constitutive activation of tMEK2 (a tomato MAPKK) enhanced phosphorylation of a UBC enzyme under heat induced stress (Xing lab, unpublished). Motif analysis at medium stringency scan predicated the existence of ERK-docking domains for *LeUBC1* and *SlUBC3* indicating that these proteins may have sites that physically interact with mitogen activated protein kinases belonging to the ERK family. RT-PCR analysis was performed after treatment with FB1 and ERKI to test whether or not MAP kinases phosphorylate the UBCs of interest. Although transcript levels are not the best indicator of protein activation through phosphorylation, it is possible that cells will not continue to produce a protein that is continuously in an inactivated state. The RT-PCR revealed that there were no significant differences in *LeUBC1* and *SlUBC3* mRNA levels between the mock (0 hour and 48 hour H₂O) and other treatments and between the 0 hour and the 48 hour samples within treatments. It seems

that ERK-type MAP kinases do not affect *LeUBC1* and *SUBC3* when examined at transcript levels. It is possible that ERK-type MAP kinases do not actually interact with *LeUBC1* and *SUBC3*. However, this result does not rule out the possibility that MAPK kinases regulate *LeUBC1* and *SUBC3* activities due to the fact that transcript levels are not the best indicator of how phosphorylation affects protein activity. Ideally, this would be done through western blot analysis of phosphorylated levels of *LeUBC1* and *SUBC3*, but antibodies are not yet available for these proteins.

6.3 Enzyme assays

β -1,3-glucanase activity

Plants have evolved to recognize and target the cell wall of pathogens through synthesis and secretion of hydrolytic enzymes such as β -1,3-glucanases. It has been shown that β -1,3-glucanase is induced upon pathogen attack, has antifungal activity in vitro, and increases resistance to fungal pathogens when overexpressed in transgenic plants. As a result this enzyme has been classified as a pathogenesis-related protein. We examined its activity in tomato seedlings in the presence of FB1 and MG-115. We predicted its activity to increase in response to those treatments at the 48 hour interval.

The findings showed that β -1,3-glucanase activity was significantly higher after a 48 hour treatment with H₂O, FB1, MG-115, and FB1+MG-115. A similar finding was reported where β -1,3-glucanase activity increased in infected tissues after 48 hour inoculation with a fungus, *Phytophthora meadii* [32]. The significant increase after 48 hour H₂O was not expected since it is expected to have little to no stress effect on the tomato seedlings. One possible explanation is that submerging the tomato leaves in water-containing petri dishes over 48 hours is a possible

environmental stress that can be classified as flooding. Dong *et al.* revealed that β -1,3-glucanase transcript accumulation occurred 24 hours after plantlets were exposed to flooding [7]. The flooding stress was applied by submerging the plantlets into sterile distilled water in petri dishes over 24 hours. While transcript accumulation does not conclusively show an increase in enzyme activity levels, it is an indicator that more enzyme is possibly being made to cope with certain stresses.

β -1,3-glucanase activity also increased in response to the 48 hour MG-115 treatment. Proteasomes degrade or process proteins by ATP/ubiquitin-mediated proteolysis. Studies in plants have shown that inhibition of the proteasome activities leads to programmed cell death accompanied by accumulation of polyubiquitinated proteins [17]. One of the indications that PCD was occurring in the affected cells is the higher production of reactive oxygen species. Oxidative stress is involved in pathogen-related processes as well as other stresses. This cell death induced the transcriptional activation of defense related proteins such as β -1,3-glucanase and PR5. MG-115 being a proteasome inhibitor may have led to the above morphological changes by mimicking PCD.

Glutamine synthetase activity

It has been reported that the expression and activity of metabolism-related proteins, like GS, decreases in response to stresses such as pathogen attacks. GS has two isoforms, a cytosolic GS1 involved in nitrogen remobilization and a chloroplastic GS2 involved in nitrogen assimilation. Due to the importance of nitrogen in plant defense, the effects of FB1 and MG-115 induced stress on GS in tomato plants was investigated in this report. It was hypothesized that total GS activity would decrease after the 48 hour incubation, and GS activity after the 48 hour

treatment with FB1, MG-115, or FB1+MG115 would be significantly lower than the 48 hour H₂O control. The latter part of the hypothesis was supported by the data, showing that after 48 hours of treatment, the FB1 and MG-115 treated plants had significantly lower GS activity than the H₂O treated plants. However, when comparing the 48 hour treated groups to the 0 hour groups, there was an increase in GS activity, which is opposite to our prediction. Further study is needed. With respect to the other treatments, an explanation is possible when the different isoforms of GS are taken into account. Previous studies have shown that in response to pathogen infection, there is a change in the GS isoform pattern; GS2 is downregulated and GS1 becomes the predominant isoform in tomato leaves [25]. A study by Perez-Garcia *et al.* reported that the GS1 isoform accumulated in infected tomato leaves during infection of tomato by *P. Syringae* pv. *tomato* [24]. It is believed that this isoform might be involved in remobilization of nitrogen released during protein degradation in infected tissues and its transport to healthy parts of the plant. The induction of these mechanisms during pathogenesis might be to save nitrogen by preventing its use in already severely damaged tissues or by the pathogen [22, 30].

6.4 Conclusion and future directions

In conclusion, we find that *LeUBC1* and *SlUBC3* expression patterns across the tomato developmental stages are an indication that both genes are required for important cellular functions in the organism. These functions may be related to the functions of UBC genes from other organisms, including *ScUBC4*, *ScUBC5*, and *GhUBC1*, all of which showed homology to *LeUBC1* and *SlUBC3*. However, these functions do not include the response to FB1 toxin, as both UBC genes did not show a change in transcriptional levels when the plants were infiltrated with the toxin. Glucanase activity increased when the plants were infiltrated with FB1 recapitulating the role of glucanase as a defense protein. Glucanase was also up regulated in

response to MG-115 treatment showing that inhibition of the proteasome causes a stress response from the plants. Finally it was shown that GS decreased in response to induced stress, but the decrease was likely offset by the up regulation of certain isoforms of the enzyme.

Unanswered questions regarding the role of *LeUBC1* and *SlUBC3* in the response to different types of stresses remain. The RT-PCR work indicated no change in *LeUBC1* and *SlUBC3* transcript level in response to FB1 and/or MG-115. Whether this result reflects a lesser role for the UBC enzymes in general in the biotic stress response of tomato is unknown. As this result was unexpected, our next step is to find answers to the following ideas that arose as a result of the RT-PCR findings: 1) the availability of other UBCs that may respond to FB1, or 2) the presence of sufficient baseline levels of *LeUBC1* and *SlUBC3* to cope with the stress. After we successfully completed protein expression and immunoblotting experiments for *LeUBC1* and *SlUBC3*, the next step would be to study proteins that interact with them using in vitro pull-down assays. If, based on these experiments, *LeUBC1* and *SlUBC3* interact with a downstream E3 enzyme, then the E3 enzyme identity can be found through mass spectrometry analysis. E3s are known to interact with more than one UBC. If the downstream E3 enzymes interact with UBCs other than *LeUBC1* and *SlUBC3*, then it is worthwhile to test whether or not those UBCs respond to stress. Other interacting proteins could also be identified and their functional analysis can be included as well.

Another area that needs further exploration is the possible ability of MAPK to phosphorylate *LeUBC1* and *SlUBC3*. To determine this possibility, we can compare the protein phosphorylation levels of *LeUBC1* and *SlUBC3* under the effects of ERKI to their phosphorylation levels under normal conditions. This would require the development of western blot antibodies for *LeUBC1* and *SlUBC3*. The ratio of phosphorylated to total protein would be

taken from western blot analysis of control samples and those treated with ERKI. We can also further explore the effects of FB1 and MG-115 on GS activity in tomato. Enzyme assays of GS1 and GS2 can be differentially separated and their activities measured using a standard GS enzyme activity assay.

References

1. Asai, T., Stone, J. M., Heard, J. E., Kovtun, Y., Yorgey, P., Sheen, J., et al. (2000). Fumonisin B1-induced cell death in *Arabidopsis* protoplasts requires jasmonate-, ethylene-, and salicylate-dependent signaling pathways . *Plant Cell*, *12*, 1823-1836.
2. Asano, T., Kimura, M., & Nishiuchi, T. (2012). The defense response in *Arabidopsis thaliana* against *Fusarium sporotrichioides*. *Proteome Science*, *10*, 61.
3. Bethard, J., Zheng, H., Roberts, L., & Eblen, S. (2011). Identification of phosphorylation sites on the E3 ubiquitin ligase UBR5/EDD. *Journal of Proteomics*, *75*, 603-609.
4. Bernard, S.M., Møller, A.L.B., Dionisio, G., Kichey, T., Jahn, T.P., Dubois, F. et al. (2008). Gene expression, cellular localisation and function of glutamine synthetase isozymes in wheat (*Triticum aestivum* L.). *Plant Molecular Biology*, *67*, 89-105.
5. Cheng, Y. T., & Li, X. (2012). Ubiquitination in NB-LRR-mediated immunity. *Current Opinion in Plant Biology*, *15*, 392-399.
6. Desjardins, A. E., & Hohn, T. M. (1997). Mycotoxins in plant pathogenesis. *Molecular Plant-Microbe Interactions*, *10*, 147-152.
7. Dong, J. Z., & Dunstan, D. I. (1997). Endochitinase and β -1, 3-glucanase genes are developmentally regulated during somatic embryogenesis in *Picea glauca*. *Planta*, *201*, 189-194.
8. Eini, O., Dogra, S., Selth, L. A., Dry, I. B., Randles, J. W., & Razaian, M. A. (2009). Interaction with a host ubiquitin-conjugating enzyme is required for the pathogenicity of a geminiviral DNA B satellite . *Molecular Plant-Microbe Interactions*, *22*(6), 737-746.
9. Feussner, K., Feussner, I., Leopold, I., & Wasternack, C. (1997). Isolation of a cDNA coding for an ubiquitin-conjugating enzyme UBC1 of tomato-the first stress-induced UBC of higher plants . *FEBS Letters*, *409*, 211-215.

10. Gautam, S., Liangying, D., Yajun, H., Xuejun, W., Lirong, Z., & Guo-Liang, W. (2010). Role of ubiquitination in plant innate immunity and pathogen virulence. *Journal of Plant Biology*, 53, 10-18.
11. Girod, P. A., Carpenter, T. B., Nocker, S., Sullivan, M. L., & Vierstra, R. D. (1993). Homologs of the essential ubiquitin conjugating enzymes UBC1, 4, and 5 in yeast are encoded by a multigene family in *Arabidopsis thaliana*. *Plant Journal*, 3, 545-552.
12. Gribble, G. W. (2013). Food chemistry and chemophobia. *Food Security*, 1-11.
13. Hammond-Kosack, K. E., & Jones, J. D. (1996). Resistance gene-dependent plant defense responses. *Plant Cell*, 8, 1773.
14. Hondo *et al.* (2007). The LeATL6-associated ubiquitin/proteasome system may contribute to fungal elicitor-activated defense response via the jasmonic acid-dependent signaling pathway in tomato. *Molecular Plant-Microbe Interactions*, 20, 72-81.
15. Joosten, M. H., & De Wit, P. J. (1989). Identification of several pathogenesis-related proteins in tomato leaves inoculated with *Cladosporium fulvum* (syn. *Fulvia fulva*) as 1,3- β -glucanases and chitinases. *Plant Physiology*, 89, 945-951.
16. Jongedijk *et al.* (1995). Synergistic activity of chitinases and B-1,3-glucanases enhances fungal resistance in transgenic tomato plants. *Euphytica*, 85, 173-180.
17. Kim, M., Ahn, J. W., Jin, U. H., Choi, D., Paek, K. H., & Pai, H. S. (2003). Activation of the programmed cell death pathway by inhibition of proteasome function in plants. *Journal of Biological Chemistry*, 278, 19406-19415.
18. Laine, A., & Ronai, Z. (2005). Ubiquitin chains in the ladder of MAPK signaling. *Sci. STKE*, 281, 1-10.

19. Mishra, N. S., Tuteja, R., & Tuteja, N. (2006). Signaling through MAP kinase networks in plants. *Archives of Biochemistry and Biophysics*, 45, 55-68.
20. Moretti, A. N. (2009). Taxonomy of Fusarium genus, a continuous fight between lumpers and splitters. *Proceedings of National Science Matica Srpska Novi Sad*, 117, 7-13.
21. Niderman, T., Genetet, I., Bruyere, T., Gees, R., Stintzi, A., Legrand, M., et al. (1995). Pathogenesis-related PR-1 proteins are antifungal. *Plant Physiology*, 108, 17-27.
22. Pageau, K., Reisdorf-Cren, M., Morot-Gaudry, J. F., & Masclaux-Daubresse, C. (2006). The two senescence-related markers, GS1 (cytosolic glutamine synthetase) and GDH (glutamate dehydrogenase), involved in nitrogen mobilization, are differentially regulated during pathogen attack and by stress hormones and reactive oxygen species in *Nicotiana tabacum* L. leaves. *Journal of Experimental Botany*, 57, 547-557.
23. Pedley, K. F., & Martin, G. B. (2005). Role of mitogen-activated protein kinases in plant immunity. *Current Opinion in Plant Biology*, 8, 541-547.
24. Perez-Garcia, A., Canovas, F. M., Gallardo, F., Hirel, B., & Vicente, A. d. (1995). Differential expression of glutamine synthetase isoforms in tomato detached leaflets infected with *Pseudomonas syringae* pv. *tomato*. *Molecular Plant-Microbe Interactions*, 8, 96-103.
25. Perez-Garcia, A., Pereira, S., Gutierrez, A. G., Cazorla, F., Salema, R., Vicente, A. d., et al. (1998). Cytosolic localization in tomato mesophyll cells of a novel glutamine synthetase induced in response to bacterial infection or phosphinothricin treatment. *Planta*, 206, 426-434.
26. Plett, J. M., Cvetkovska, M., Makenson, P., Xing, T., & Regan, S. (2009). *Arabidopsis* ethylene receptors have different roles in Fumonisin B1-induced cell death. *Physiological and Molecular Plant Pathology*, 20, 1-9.

27. Ryota, T., Noriyuki, M., Akihiko, N., Koji, H., Chiharu, A., Naoto, S., et al. (2002). EL5, a rice *N*-acetylchitooligosaccharide elicitor-responsive RING-H2 finger protein, is a ubiquitin ligase which functions *in vitro* in co-operation with an elicitor-responsive ubiquitin-conjugating enzyme, OsUBC5b. *Plant Journal*, 30, 447-455.
28. Sánchez-Rangel, D., Sánchez-Nieto, S., & Plasencia, J. (2012). Fumonisin B1, a toxin produced by *Fusarium verticillioides*, modulates maize β -1, 3-glucanase activities involved in defense response. *Planta*, 235, 965-978.
29. Shen, Q., Zhou, F., Bieri, S., Haizel, T., Shirasu, K., & Schulze-Lefert, P. (2003). Recognition specificity and RAR1/SGT1 dependence in barley Mla disease resistance genes to the powdery mildew fungus. *Plant Cell*, 15, 732-744.
30. Snoeijers, S. S., Perez-Garcia, A., Joosten, M. H., & De Wit, P. J. (2000). The effect of nitrogen on disease development and gene expression in bacterial and fungal plant pathogens. *European Journal of Plant Pathology*, 106, 493-506.
31. Stone, J. M., Heard, J. E., Asai, T., & Ausubel, F. M. (2000). Simulation of fungal-mediated cell death by fumonisin B1 and selection of fumonisin B1-resistant (*fbr*) *Arabidopsis* mutants. *Plant Cell*, 12, 1811-1822.
32. Thanseem, A. Joseph, & A. Thulaseedharan. (2005). Induction and differential expression of β -1,3-glucanase mRNAs in tolerant and susceptible *Hevea* clones in response to infection by *Phytophthora meadii*. *Tree Physiology*, 25, 1361-1368.
33. Thurston, G., Regan, S., Rampitsch, C., & Xing, T. (2005). Proteomic and phosphoproteomic approaches to understand plant-pathogen interactions. *Physiological and Molecular Plant Pathology*, 66, 3-11.

34. Trujillo, M., & Shirasu, K. (2010). Ubiquitination in plant immunity . *Current Opinion in Plant Biology*, 13, 402-408.
35. Zhang, X. D., Jenkins, J. N., Callahan, F. E., Creech, R. G., Si, Y., McCarty, J. C., & Ma, D. P. (2003). Molecular cloning, differential expression, and functional characterization of a family of class I ubiquitin-conjugating enzyme (E2) genes in cotton. *Biochimica et Biophysica Acta - Gene Structure and Expression*, 1625, 269-279.