This is to certify that the
dissertation entitled

IDENTIFICATION AND CHARACTERIZATION OF TOMATO (*SOLANUM LYCOPERSICUM*) PROTEINS INVOLVED IN RESISTANCE TO INSECT HERVIBORES

presented by

ELIANA GONZALES-VIGIL

has been accepted towards fulfillment
of the requirements for the

Doctoral degree in Genetics

Greg Howe

Major Professor's Signature

Aug. 17, 2009

Date

IDENTIFICATION AND CHARACTERIZATION OF TOMATO (*SOLANUM LYCOPERSICUM*) PROTEINS INVOLVED IN RESISTANCE TO INSECT HERVIBORES

By

Eliana Gonzales-Vigil

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Genetics

2009

ABSTRACT

IDENTIFICATION AND CHARACTERIZATION OF TOMATO (*SOLANUM LYCOPERSICUM*) PROTEINS INVOLVED IN RESISTANCE TO INSECT HERBIVORES

By

Eliana Gonzales-Vigil

In response to wounding or herbivore attack, plants synthesize proteins that negatively affect the growth and development of arthropod herbivores. Many of these proteins are induced in plant tissue in response to herbivory and, following ingestion by the herbivore, target processes involved in insect digestive physiology. The objective of this thesis research is to identify and characterize plant proteins that impair the ability of insect herbivores to obtain nutrients from host tissue. To address this objective, liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to identify proteins in tomato (*Solanum lycopersicum* L.) that are excreted in the insect feces (frass). This approach is based on the premise that plant anti-insect proteins are stable during passage of food through the insect digestive system, and therefore enriched in the frass. The results establish the utility of insect feces as a source of material for proteomic-based discovery of defensive proteins that target insect digestive processes. Comparative proteomic analysis of frass from three tomato-reared insect species, including lepidopteran (*Manduca sexta* and *Trichoplusia ni*) and coleopteran (*Leptinotarsa decemlineata*) herbivores, provided evidence that the lepidopteran insects digest bulk tomato leaf protein more efficiently than the coleopteran insect. This study also identified

a subset of tomato leaf proteins that are highly stable in the digestive tract of all three insect species. Including in this subset were proteins previously shown to have a role in defense against insect attack. These findings are consistent with the hypothesis that plant anti-insect proteins are inherently stable in the insect digestive track.

One of the most abundant tomato proteins excreted in the frass from all three insects was a jasmonate-inducible isoform of threonine deaminase (TD2) that converts threonine (Thr) to α-ketobutyrate and ammonium. TD2 and other plant TDs contain a C-terminal regulatory domain that, upon binding isoleucine (Ile), feedback inhibits the N-terminal catalytic domain. Following ingestion of tomato foliage by lepidopteran insects, the regulatory domain of TD2 is removed by a chymotrypsin-like protease of insect origin. This processed form of TD2 efficiently degrades Thr in the presence of Ile, thereby starving the insect of an essential nutrient. The increased growth rate of *Spodoptera exigua* larvae on transgenic tomato lines silenced for TD2 expression showed that this enzyme serves a role in anti-insect defense. Tomato contains a second TD isoform (TD1) that catalyzes the committed step in the biosynthesis of Ile. Based on the comparison of the expression pattern and biochemical properties of TD1 and TD2, it is concluded that the two TD isoforms have evolved specialized functions in plant primary metabolism and anti-insect defense, respectively.

ACKNOWLEDGEMENTS

Many people have contributed one way or another during the course and completion of this thesis:

Gregg Howe let me join his lab and treat my scoleciphobia (fear of worms). I thought I wouldn't have to deal much with insects in the Howe lab. However, I had to rear, touch, dissect, collect poop and spit from caterpillars throughout my research. Anyway, I think having such a nice project was worth the high price, and I thank Gregg for that and for his guidance and support all these years. I have learnt so much from him.

Barb Sears accepted my late application for the Genetics Program, led the writing group, served as a member on my committee when it was needed, and shared her passion for her job –something that I wish I could always have.

My guidance committee: Ke Dong and Sheng Yang He listened carefully to my updates and provided useful comments. Rob Last gave me advice on so many things from experiments to postdoc positions, and most importantly, he read this document in its entirety!

A special thanks to the MSU Proteomics Core: Curtis Wilkerson and Doug Whitten let me inject insect frass in their expensive LTQ-FTICR; and Kevin Carr handled the bioinformatics that made dealing with the data so much easier.

The people in the lab made the Howe lab a great place to work in. Hoo Sun Chung, was a friend when I needed it most; Marco Herde, my shitomics partner helped me make this thesis better through his discussions; Jin Ho Kang, the trichome king kept the Genetics discussions alive; Abe Koo, the senior lab member kept us all on track; Leron Katsir, the

not-so-neat grad student made lab meetings so entertaining; and Chris Bergum, the undergraduate student who was able to cope with me and still like his job, provided his technical skills that kept this research going while I was stuck on a computer. Also I want to thanks the new Howe lab generation, Christine Shyu, Javier Moreno, Lalita Patel and Tom Cooke; and the former lab members: Hui Chen, Tony Schilmiller, Bonnie McCaig and Guanghui Liu.

I also received assistance from Rob Larkin and his lab during the protein purification; and from Nora Bello in the statistical analysis of the performance assays.

My family, and specially my mom, was always supportive even when they had no idea what exactly I was doing. So many friends made Michigan the lively place that I never thought it could be. And last but not least, the one that was behind the scenes supporting, cheering me up, cooking, changing diapers and keeping me a sane person, my loving husband Erick. This wouldn't have been possible without you by my side, and now with our Luciana.

Thanks you all for making these years of frass collection unforgettable.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter I

Plant Defenses to Insect Herbivory

Introduction

Plants are exposed to various biotic stresses, including competing weeds, pathogens, and animal pests. It is estimated that 45% of crop yield is lost to these agents. Despite the increase in pesticide use, these losses have not decreased over the last 40 years (Oerke, 2006). The control of insect pests alone is estimated to cost $10 billion dollars annually. Moreover, global warming will likely result in greater damage by insect herbivores to crops and forests (Currano et al., 2008; DeLucia et al., 2008). Insects can reduce plant fitness directly through removal of photosynthetic tissues, or indirectly by reducing photosynthetic rates and the plant's competitive ability (Bernays, 1998; Zangerl et al., 2002). Most current approaches to controlling insect pests involve the use of chemical pesticides and synthetic pheromones. However, concerns about the effect of these chemicals on the environment as well as the development of insecticide-resistant insects have promoted the search for more environmentally friendly control measures.

One such alternative is the use of transgenic approaches to generate insect resistant crops. The first generation of insect-resistant transgenic plants was engineered to express *Cry* genes encoding so-called Bt toxins from *Bacillus thuringiensis*. This approach has proven successful in the development of insect resistance in cotton (*Gossypium hirsutum*) and maize (*Zea mays*) (Gatehouse, 2008). Different *Bt* strains

produce toxins that are effective against specific insects in the orders lepidoptera, coleoptera, and diptera. However, Cry proteins are not suitable for the control of all insect pests, and laboratory experiments have demonstrated that several insect pests can develop resistance to the toxin (Tabashnik et al., 2008).

Another approach for developing insect resistant crop plants has relied on the overproduction of plant compounds that have anti-insect activity. Because this approach relies on understanding specific plant defense mechanisms, it has been limited to a few genes that encode proteins with a role in plant defense, including protease inhibitors, polyphenol oxidase, α-amylase inhibitor, and lectins (Hilder et al., 1987; Johnson et al., 1989; Shade et al., 1994; Xu et al., 1996; Gatehouse et al., 1997; Rao et al., 1998; Foissac et al., 2000; Wang and Constabel, 2004; Thipyapong et al., 2007; Bhonwong et al., 2009). Transgenic manipulation of genes involved in the biosynthesis of toxic secondary metabolites, caffeine, hydrogen cyanide, and terpenoids (Tattersall et al., 2001; Aharoni et al., 2003; Uefuji et al., 2005), successfully enhanced plant resistance to certain insect pests. A limitation of this approach is that the strength of resistance is relatively weak, and insects can quickly adapt their physiology to cope with the toxic compounds. Insect adaptation to these chemical defenses can be retarded by engineering plants with a gene from a non-host plant of the target insect or combining multiple defense mechanisms (Jongsma and Bolter, 1997).

A promising alternative approach to controlling insect herbivores is the use of double stranded RNA (dsRNA) to suppress insect genes. In this case, the crop plant is transformed with a construct that targets an essential insect gene for suppression. This technique has been successfully utilized to suppress a cytochrome P450 involved in

gossypol tolerance in cottom bollworm (*Helicoverpa armigera*) (Mao et al., 2007), and a V-type ATPase subunit and β-tubulin from the Western corn rootworm (*Diabrotica virgifera virgifera*) (Baum et al., 2007). This approach has the potential to produce the next generation of insect-resistant crops. A current limitation of this technology is the identification of insect target genes that would confer plant protection (Price and Gatehouse, 2008).

Sounding the alarm: the jasmonate pathway

Upon insect attack, the wounded plant perceives signals derived from herbivore oral secretions and the damaged leaf (Schilmiller and Howe, 2005; Tumlinson and Lait, 2005; Schmelz et al., 2009). These signals promote calcium ion fluxes, kinase cascades, the formation of reactive oxygen species (ROS), and the activation of the octadecanoid pathway leading to synthesis of the plant defense hormone jasmonate (Howe and Jander, 2008; Mithofer and Boland, 2008). The octadecanoid pathway converts linolenic acid to jasmonic acid (JA) through various steps of oxygenation, dehydration, reduction, and β-oxidation (Vick and Zimmerman, 1984). JA and its conjugated forms (collectively referred as jasmonates) are key players in the activation of plant defense responses to insect herbivory (Howe and Jander, 2008). This has been demonstrated through genetic analysis of the wound response (Lightner et al., 1993; Howe and Ryan, 1999; Li et al., 2001; Li et al., 2004a; Schilmiller and Howe, 2005) and transcriptional profiling experiments performed with several model plant species, including tomato, tobacco, and

Arabidopsis (Reymond et al., 2000; Halitschke et al., 2003; Reymond et al., 2004; Devoto et al., 2005).

COI1 (Coronatine Insensitive1) is a key component of the jasmonates signaling cascade. Mutations in this gene, which have been described in Arabidopsis, tomato (*Solanum lycopersicum*), and *Nicotiana attenuata*, result in insensitivity to jasmonate (Feys et al., 1994; Li et al., 2004b; Paschold et al., 2007). *COI1* encodes an F-box protein that participates in ubiquitin-dependent protein degradation of Jasmonate ZIM-domain proteins (JAZ) (Thines et al., 2007; Katsir et al., 2008). JAZ proteins repress the transcription of JA-responsive genes through interaction with transcription factor such as MYC2 (Chini et al., 2007). In damaged leaves, increased levels of jasmonoyl-isoleucine (JA-Ile) promote interaction between COI1 and JAZ proteins. Degradation of JAZ proteins leads to derepression of JA-responsive genes (Katsir et al., 2008). The expression of JA-responsive genes is observed in the attacked leaf, as well as in undamaged leaves of the attacked plant. This systemic wound response heightens resistance to subsequent insect attacks, and is accompanied by large-scale changes in gene expression. Local and systemic wound responses include downregulation of photosynthetic and other growth-related processes, and increased expression of various defensive traits (Kessler and Baldwin, 2002).

Jasmonates provide protection against insect herbivores from different feeding guilds, including chewers, suckers, and cell-content feeders (Walling, 2000; Thaler et al., 2002; Browse and Howe, 2008). Jasmonate-mediated defense responses reduce the fitness of generalist insects that feed on a few plant hosts, as well as specialists that feed on one of a few closely related species (Reymond et al., 2004). The overall effect of the

jasmonate pathway on host resistance results from the combined action of direct defenses that slow insect feeding and indirect defenses (e.g., volatiles) that recruit predators or parasites of the attacking insect (Thaler, 1999; Thaler, 2002; Chen et al., 2006). The induction of indirect defense responses is thought to be coordinated with the induction of direct defenses because neither strategy alone is completely effective in targeting different developmental stages of multiple herbivores (Thaler, 2002). Direct defenses are mostly effective against hatchling larvae, whereas indirect defenses frequently kill older herbivores (Cornell et al., 1998). In the absence of a functional JA-signaling pathway, plants become a host not only to opportunistic herbivores, but also to detrivorous crustaceans that do not normally feed on living plant material (Kessler et al., 2004; Farmer and Dubugnon, 2009).

Requesting backup: plant volatiles as indirect defenses

Plant volatile organic compounds (VOCs) released in response to insect herbivory perform a dual function: they exert direct toxic or repellent effects on the attacker, and they attract natural enemies of the herbivore. Wound-induced plant VOCs allow predators and parasitoids to distinguish between infested and uninfested plants, and to locate their prey (Tumlinson et al., 1993; Takabayashi and Dicke, 1996). The ecological role of VOCs in tritrophic interactions has been extensively studied in both laboratory and natural field conditions (Kessler and Baldwin, 2001).

The blend of volatiles released after damage varies according to the attacking herbivore (Pare and Tumlinson, 1999; Thaler, 2002). VOCs are synthesized by three

different pathways. These pathways include the octadecanoid pathway for rapid wound-induced production of green leaf volatiles, the mevalonate and nonmevalonate pathways that synthesize terpenoid volatiles, and the shikimate pathway for production of methyl salicylate (Kessler and Baldwin, 2002). Nectaries also produce extrafloral nectar that attract natural enemies of the attacking insect in response to herbivore attack (Heil et al., 2001). Manipulation of volatile emission in transgenic plants has been used to increase plant resistance to herbivores (Turlings and Ton, 2006). Increased resistance is due not only to the role of volatiles as indirect defenses, but also to the important effect of volatiles in priming plant defenses for future attack (Baldwin et al., 2006).

Close combat fighting: defensive compounds that directly target insect herbivores

Direct defenses include not only metabolites and proteins that thwart insect attack (Kessler and Baldwin 2002), but also physical barriers that impede access to nutritious tissues (Hanley et al., 2007). Morphological traits such as spines, trichomes, and leaf toughness are among the direct defenses (Hanley et al., 2007). Trichomes are thought to have evolved as protection against radiation and water loss, but they are also widely regarded as the first line of defense against herbivores (Hanley et al., 2007). Trichomes act as a mechanical barrier to limit access to nutritious tissue, and also as chemical barriers that produce anti-insect compounds. Trichome-borne defensive compounds include secondary metabolites and proteins (Yu et al., 1992; Amme et al., 2005; Simmons and Gurr, 2005; Liu et al., 2006; Schilmiller et al., 2008). Trichomes provide

protection against aphids, whiteflies, chewing insects and leafminers; but are less effective against larger insects such as grasshoppers (Hanley et al., 2007).

Secondary metabolites with direct roles in plant defense

Secondary metabolites are not only responsible for the plant's "cry-for-help" as discussed above, but they can also act as feeding deterrents, repellents, and post-ingestive defenses against insects. An insect's choice of host plant is based largely on the content of secondary metabolites (Hadacek, 2002). Toxic compounds are usually characterized by their ability to repel herbivores, reduce the fitness of generalist herbivores, and force specialist herbivores to invest in detoxification mechanisms that incur fitness costs in growth and development (Kessler and Baldwin, 2002). Terpenoids, phenolics, and various nitrogen-containing compounds, including glucosinolates, alkaloids, and cyanogenic glycosides, have insecticidal properties (Howe and Jander, 2008). The synthesis of these compounds can be either constitutive or inducible in response to insect herbivory (Jansen et al., 2009).

Glucosinolates are a classical example of plant anti-insect compounds. Their production is almost exclusive to the Brassicaceae. Glucosinolates are classified according to the chemical nature of the amino acid side chain, and include indole (derived from tryptophan), aliphatic (derived from methionine), and aromatic glucosinolates (derived from phenylalanine or tyrosine). Additional chemical diversity is achieved through chain elongation, oxidation, and hydroxylation of the side chain (Fahey et al., 2001). The enzyme myrosinase catalyzes the breakdown of glucosinolates into

compounds that are toxic and pungent. This breakdown process does not occur *in planta* because myrosinase and its glucosinolate substrates are located in separate cellular compartments. Upon tissue disruption, for example by insect chewing, mixing of myrosinase with vacuole-stored glucosinolates results in the formation of compounds such as thiocyanates, isothiocyanates, and isonitriles (Hopkins et al., 2009). Glucosinolates can act as oviposition and feeding stimulants for a number of insect specialists. In the case of generalist insects, however, these compounds have potent insecticidal properties (Barth and Jander, 2006; Schlaeppi et al., 2008). Specialist herbivores use various mechanisms to cope with glucosinolates, including enzymatic detoxification, excretion, and sequestration (Mainguet, 2000), as well as with behavioral responses to avoid consumption of plant tissues that contain high glucosinolate content (Shroff et al., 2008).

Terpenoids constitute the most diverse group of anti-insect metabolites that serve roles in direct and indirect defense (Frelichowski and Juvik, 2001). Terpenoid-based defenses against insect herbivores have been extensively studied in conifers (Keeling and Bohlmann, 2006). In conifers, terpenoids accumulate in large amounts in oleoresins that are stored in specialized resin-producing resin cells. The extraordinary diversity of terpenoids is generated in large part by the action of two enzyme families, namely the terpenoid synthases and cytochrome P450-dependent monooxygenases (Bohlmann et al., 1998). Despite the importance of terpenoids in plant defense, little information is available on the mode of action of terpenoids as direct defenses.

In many plants, most notably oak, tannins have long been thought to serve as a key chemical defense against insect herbivores (Feeny, 1970). Upon oxidation in the

midgut, tannins produce semiquinone radicals that could potentially lead to oxidative stress. Recent studies, however, indicate that semiquinones do not contribute to oxidative stress in the midgut, and consequently do not reduce larval performance (Barbehenn et al., 2009).

Plant anti-insect proteins

Plant proteins are another layer of defense that has received less attention compared to secondary metabolites. Insect digestion and nutrition are important targets of plant anti-insect proteins. In the following sections, information on plant proteins that affect post-ingestive targets in the insect will be reviewed. Plants can also limit the insect's access to nutrients prior to ingestion of plant food through fortification of the cell wall and physico-chemical barriers such as epicuticular waxes (Chen, 2008; Müller, 2008). Several genes that encode proteins for cell wall modification are regulated by insect attack. This group includes pectin esterases, expansins, xyloglucan endotransglycosylases/hydrolases, and cellulose synthases (Goggin, 2007; Liu et al., 2007).

Plant proteins that serve a post-ingestive role in defense are typically synthesized in response to wounding and herbivore attack. This was established early on in tomato (*Solanum lycopersicum*) by Ryan and colleagues (Farmer et al., 1992; Constabel et al., 1995; Bergey et al., 1999; Moura et al., 2001; Diez-Diaz et al., 2004). A protein's effectiveness as a post-ingestive defense depends in part on its resistance to inactivation by insect digestive proteases. Therefore, another common feature of plant anti-insect

proteins is their stability in the gut (Chen et al., 2005). Post-ingestive defenses can be further subdivided into proteins that directly disrupt the insect digestive system (toxic proteins) and proteins that limit the access to essential nutrients (anti-nutritive proteins).

Eliminate the enemy: Toxic proteins

Lectins

Proteins are classified as lectins if they possess at least one non-catalytic domain that binds reversibly to specific carbohydrates. This relaxed definition allowed the grouping of diverse lectin-like proteins into seven families of structurally and evolutionary related proteins. Several lectins are induced by jasmonate treatment or insect attack (Zhu-Salzman et al., 1998; Williams et al., 2002). Insecticidal activity of these proteins was deduced from their ability to bind glycosylated proteins and chitin, a polymer present in the peritrophic membrane that protects the delicate midgut cells, in the insect gut (Van Damme, 2008). Lectins are highly stable under harsh conditions, including the proteolytic environment in the gut, and were reported to cross the gut epithelium to reach the hemolymph (Peumans and Van Damme, 1995; Zhu-Salzman and Salzman, 2001; Fitches et al., 2002). The anti-insect role of lectins was demonstrated with the use of transgenic plants expressing foreign lectins, and *in vitro* assays in which purified lectins are added to artificial insect diet (Carlini and Grossi-de-Sa, 2002). The exact mechanism of the toxicity of lectins remains to be determined. However, there is evidence to indicate that some lectins impair formation of the peritrophic membrane by binding to glycoproteins in midgut epithelial cells, or by binding to glycosylated insect proteins involved in food

digestion (Figure 1.1.A) (Peumans and Van Damme, 1995). The harmful effects of lectins on the peritrophic membrane was verified in the European corn borer (*Ostrinia nubilalis*) fed with the lectin wheat germ agglutinin (WGA). In contrast to a continuous peritrophic membrane protecting the midgut epithelial cells in control larvae, WGA-fed larvae showed a multilayered and disorganized peritrophic membrane, and disintegration of the midgut microvilli resulting from abrasive food particles and microorganisms that penetrated the peritrophic membrane (Harper et al., 1998) (Figure 1.1.A). The utility of transgenically expressed lectins for insect control has been limited by the high level of expression required to inhibit insect growth, as well as the fact that some lectins can be toxic to mammals (Estruch et al., 1997; Carlini and Grossi-de-Sa, 2002).

Cysteine proteases

Cysteine proteases are defined by the presence of a cysteine residue at the catalytic site (Shindo and Van der Hoorn, 2008). The insecticidal activity of cysteine proteases has been studied in several plant-insect interactions. Papain and ficin are cysteine proteases found in the latex of the Papaya tree (*Carica papaya*) and fig (*Ficus virgata*), respectively. The toxicity of latex from these plants is abolished by the cysteine-specific protease inhibitor E-64, indicating that cysteine proteases are responsible for the toxic effects on lepidopterans (Konno et al., 2004). Mir1-CP (Maize inbred resistance 1) from maize (*Zea mays*) is one of the most thoroughly characterized cysteine proteases. The anti-insect role of this protease was established by overexpression of the *mir1* gene in maize callus, which resulted in growth reduction of the fall armyworm (*Spodoptera*

frugiperda) that fed on the callus (Pechan et al., 2000). The *Mir1* gene of maize is upregulated in response to insect feeding. The mode of action of Mir-CP involves disruption of the peritrophic membrane (Figure 1.1.B) (Pechan et al., 2002). Perforation of the peritrophic membrane by Mir-CP increases the membrane's permeability to toxic proteins and microorganisms, resulting in damage to the midgut microvilli (Mohan et al., 2006). This mechanism of toxicity is comparable to that of the Bt toxin Bt-CryIIA (Mohan et al., 2008).

Cyclotides

Cyclotides are insecticidal peptides (28-37 amino acids in length) identified in species from the Violaceae, Rubiaceae, Cucurbitaceae, and Apocynaceae (Gruber et al., 2008). These disulphide-rich compounds contain a series of cyclic peptide bonds and are stabilized by six cysteine residues that form a cystine knot (Craik et al., 1999). Cyclotides are highly stable at extreme pH and temperatures, and are resistant to the activity of proteases (Colgrave and Craik, 2004). These structural features of cyclotides allow them to tolerate multiple mutations in the backbone sequence as long as the disulfide bonds are maintained, giving rise to a large diversity of variants. Discovered as the active component in *Oldenlandia affinis*, cyclotides have a range of medicinal properties, including anti-HIV, uterotonic, haemolytic, and cytotoxic activity (Craik et al., 2004). Nevertheless, their primary role in plants appears to be protection from insects (Jennings et al., 2001). The insecticidal properties of the cyclotides kalata B1 and B2 were demonstrated by incorporation of the peptides in the diet of two *Helicoverpa*

species (Jennings et al., 2005). The growth retardation effect on *Helicoverpa armigera* was caused by disruption of midgut epithelial cells (Figure 1.1.C). Ingestion of cyclotides causes thickening of the epithelial cells caused by blebbing of cell fragments into the gut lumen, and swelling and lysis of the columnar cells that form the microvilli. The peritrophic membrane in larvae reared on cyclotide-containing diet was highly degenerated. Similar to *Bt* toxins, cyclotides are hypothesized to form pores in the plasma membrane of epithelial cells (Barbeta et al., 2008).

Canatoxin

Canatoxin (CNTX) was identified in the seed of jackbean (*Canavalia ensiformis*). The widespread occurrence of CNTX-like proteins in Fabaceae seeds and their accumulation pattern during seed maturation suggested a protective role in the plant (Carlini et al., 1988; Barcellos et al., 1993). Subsequent studies demonstrated that CNTXs have fungicidal and insecticidal properties (Carlini et al., 1997; Oliveira, 1999). CNTX is a variant of the enzyme urease. Although urease also displays insecticidal activity, it is not as potent as CNTX (Follmer et al., 2004). CNTX is lethal to insects that use cathepsin B- and D-type proteases as their main digestive enzymes; insects with trypsin-based digestive systems are not affected by CNTX. The insect target and mode of CNTX action remain to be determined. Interestingly, however, CNTX-mediated toxicity requires proteolytic activation of CNTX by insect cathepsins. Differential proteolytic digestion of CNTX in different insects may account for the toxic effect of CNTX on some insects but not on others (Carlini and Grossi-de-Sa, 2002; Staniscuaski et al., 2005).

Neutralizing the enemy: Anti-nutritional proteins

Protease Inhibitors

In 1972, Green and Ryan reported for the first time that wounding of tomato and potato causes the systemic accumulation of Protease Inhibitors (PI) (Green and Ryan, 1972). Since then, PIs have been widely used as markers for the plant wound responses, and extensively studied for their role in defense against herbivores (Schilmiller and Howe, 2005). PIs accumulate constitutively in seeds, tubers, and flowers, but are massively produced in leaves after insect attack. PIs that are active against each of the four main classes (serine, cysteine, aspartic, and metalloproteases) of proteases have been found in plants.

Digestive proteases release amino acids and peptides from dietary protein. PIs work in the gut by binding to proteases and blocking their activity (Figure 1.2.A). Insects overproduce proteases in response to PIs in the diet in an attempt to compensate for reduced protease activity. Overproduction of proteases depletes the availability of amino acids needed for the synthesis of other insect proteins, which results in reduced growth rates (Jongsma and Bolter, 1997; Schilmiller and Howe, 2005). Serine and cysteine PIs frequently have deleterious effects when fed to lepidoptera and coleoptera, respectively. The effects of dietary PIs range from reduced fecundity and decreased weight to increased mortality and severe developmental defects (Gruden et al., 1998; Wilhite et al., 2000).

Reduced growth of insect larvae reared on PI-containing diets is not always observed, even in the case of larvae reared on transgenic plants expressing high PI levels (Gruden et al., 2004). This phenomenon is explained by the adaption of insects to PIs. In response to PIs in the diet, insects can induce the synthesis of proteases that break down the PIs (Giri et al., 1998; Gruden et al., 1998; Yang et al., 2009). In addition, many insect have the capacity to synthesize novel proteases that are insensitive to dietary PIs (Jongsma et al., 1995; Volpicella et al., 2000; Gruden et al., 2004; Bayes et al., 2005). A third insect adaptive strategy to dietary PIs is simply to increase food consumption (Cloutier et al., 2000; Winterer and Bergelson, 2001). As part of the evolutionary "arms race" between plants and insects, plants have expanded the diversity of PI genes to target the multitude of insect digestive proteases (Baldwin and Karban, 1997; Jongsma and Beekwilder, 2008). Transgenic expression of PIs containing domains targeting different proteases may reduce the ability of insects to adapt to the existing complement of PIs in a given host plant (Outchkourov et al., 2004). This approach has the additional advantage of protecting the PIs from degradation by insect proteases, an effect known as cross-protection (Jongsma and Bolter, 1997).

Polyphenol oxidase

PPOs are copper metalloenzymes that catalyze O_2-dependent oxidation of mono- and *o*-diphenols to *o*-diquinones. The high reactivity of the quinone products leads to secondary reactions that cause damage to proteins, lipids, and DNA, thereby reducing the nutritional quality of the tissue (Felton et al., 1992; Duffey and Stout, 1996). The wound inducibility

of PPO expression has been reported for several species including tomato, potato, and hybrid Poplar (Constabel et al., 2000; Thipyapong et al., 2007). A defensive role for PPO was demonstrated with the use of transgenic plants altered in the expression of PPO (Wang and Constabel, 2004; Mahanil et al., 2008; Bhonwong et al., 2009). Results of experiments performed with tomato suggest that PPO can act defensively against lepidoptera and coleopteran insects, which have very different gut physiologies (Thipyapong et al., 2007). The mechanisms involved in PPO-mediated plant resistance to insects are not fully understood. The quinone products of PPO may be directly toxic to insects, participate as signaling molecules that activate other plant defenses, or may promote cell wall fortification. A leading hypothesis is that highly reactive quinones alkylate dietary protein and, as a consequence, reduce the nutritional quality of the plant tissue (Felton, 1989). Recently, the role of PPO as a post-ingestive defense has been questioned on the grounds that the anaerobic environment and high ascorbate content of the lepidopteran gut severely limit the activity of PPO and other oxidases (Barbehenn et al., 2007; Barbehenn et al., 2008). This finding suggests that the effect of PPO on herbivore performance is likely caused by a pre-ingestive effect (Constabel and Barbehenn, 2008). In this context, it is worth noting that glandular trichomes of tomato and potato contain high levels of PPO (Yu et al., 1992). Upon disruption of the trichome gland, PPO can rapidly oxidize phenolic substrates that are also stored in the gland (Kennedy, 2003). Reactive quinones formed in this manner could be responsible for repelling or deterring insect herbivores prior to tissue ingestion.

Amino acid degrading enzymes

The low protein content of plant tissue represents a major challenge for herbivorous insects whose rapid growth rate depends on the assimilation of relatively high levels of amino acids from dietary protein (Bernays, 1998). This challenge is exacerbated by the presence of plant defense enzymes that degrade essential amino acids in the insect gut (Chen et al., 2005; Felton, 2005). Proteomic analysis of the fate of leaf proteins in the *M. sexta* gut showed that the tomato enzymes arginase and threonine deaminase, which previously were thought to be involved in primary metabolism, serve a role in restricting the availability of essential amino acids (Chen et al., 2005).

Arginase catalyzes the conversion of L-arginine (Arg) to urea and ornithine. An important form of storage nitrogen, free Arg and protein-bound Arg accounts for a large proportion of nitrogen reserves in storage organs (Pollaco and Holland, 1993). During seed germination, arginase activity is implicated in the breakdown of Arg to release nitrogen (Goldraij and Polacco, 1999, 2000). Arg also is a substrate for putrecsine biosynthesis. Tomato has two arginase-encoding genes designated *ARG1* and *ARG2*. *ARG2* gene expression is induced in response to wounding, JA treatment, and the *Pseudomonas syringae*-derived toxin coronatine. *ARG2* is expressed to high levels in reproductive tissues under basal conditions. *ARG1* is expressed is tissues throughout the plant and is not induced by stress. Despite these differences in expression pattern and mode of regulation, the two isoforms exhibit very similar substrate specificity, pH optimum, and kinetic parameters (Chen et al., 2004). The high pH optimum of ARG2 (Chen et al., 2008) suggested that this wound-inducible isoform might be active in the alkaline environment of the lepidopteran midgut. Support for this hypothesis came from the finding that total arginase activity in the midgut of tomato-reared *M. sexta* larvae was

inversely proportional to Arg levels (Chen et al., 2005). Moreover, transgenic tomato plants overexpressing *ARG2* were more resistant to attack by *M. sexta* larvae. This information, together with the accumulation of ARG2 in the *M. sexta* gut, are consistent with a post-ingestive role for tomato ARG2 in degrading the essential amino acid Arg from the insect midgut (Figure 1.2.B) (Chen et al., 2005).

It has been speculated for several reasons that the anti-insect function of ARG2 in the lepidopteran gut may be facilitated by leucine aminopeptidase (LAP-A), a tomato exopeptidase that releases Arg from protein and peptide substrates (Figure 1.2.B) (Chen et al., 2005; Felton, 2005). First, the expression of LAP-A in tomato leaves is co-regulated with ARG2 in response to wounding and JA treatment (Hildmann et al., 1992; Pautot et al., 1993; Chao et al., 1999). Second, LAP-A has a high pH and temperature optimum (Gu et al., 1999), and the enzyme is highly stable during passage of tomato leaf tissue through the insect gut (Chen et al., 2005). Finally, transgenic tomato plants that either over- or underexpress LAP-A exhibit decreased and increased resistance, respectively, to attack by *M. sexta* larvae (Fowler et al., 2009). Although these observations are consistent with a role for LAP-A in insect resistance, direct evidence that LAP-A provides Arg substrate for ARG2 is lacking.

Threonine deaminase (TD), which catalyzes the conversion of threonine (Thr) to α-ketobutyrate and ammonia, is another example of a host plant enzyme that accumulates in the gut of tomato-reared *M. sexta* (Chen et al., 2005). This reaction constitutes the first step in the biosynthesis of isoleucine (Ile) and is negatively regulated by Ile. In solanaceous species, TD is expressed constitutively to high levels in floral organs. In leaves, however, TD expression is induced via the JA/COI1 signaling pathway in

response to mechanical wounding and insect herbivory (Samach et al., 1991; Samach et al., 1995; Li et al., 2004b; Kang et al., 2006a). Plant and bacterial TDs consist of an N-terminal catalytic domain and a C-terminal regulatory domain. The ability of tomato TD to efficiently degrade Thr in the insect gut is associated with proteolytic removal of the regulatory domain. This post-translational modification renders TD insensitive to feedback inhibition by Ile, thereby allowing the enzyme to efficiently deplete Thr from the midgut (Figure 1.2.C) (Chen et al., 2005). This hypothesis is supported by the finding that the midgut of *M. sexta* larvae reared on TD-expressing tomato leaves contained lower Thr levels (and higher ammonia levels) than larvae reared on TD-deficient leaves (Chen et al., 2005). Additional evidence for the role of TD in plant defense against *M. sexta* attack was obtained through analysis of transgenic *Nicotiana attenuata* plants that were silenced in the expression of TD (Kang et al., 2006b).

Vegetative Storage Proteins

Vegetative storage proteins (VSPs) accumulate to high levels in storage organs of vegetative tissues and seeds. Based on this expression pattern, it was suggested that VSPs may function as an amino acid reserve that is utilized during seed germination (Staswick et al., 1994). This hypothesis was not supported, however, by transgenic studies showing that VSP-deficient soybean (*Glycine max*) lines do not exhibit phenotypes related to seed development or seedling establishment (Staswick et al., 2001). Arabidopsis VSPs share overall sequence similarity with soybean VSP. Recent insight into the physiological function of VSPs has come from studies performed in *Arabidopsis thaliana*. Similar to

the expression pattern of *VSP* genes in soybean, *VSP* expression in *A. thaliana* is induced by methyl-JA, wounding, insect feeding, osmotic and nutritional stress, and phosphate starvation (Utsugi et al., 1998; Gong et al., 2001; Berger et al., 2002; Reymond et al., 2004; Liu et al., 2005). This pattern of expression, together with high level accumulation of VSPs in the vacuole (Franceschi et al., 1983), is consistent with a role for these proteins in insect resistance. Direct support for this idea came from experiments showing that recombinant VSPs from *A. thaliana* are highly toxic to coleopteran and dipteran insect species that have an acidic midgut (Liu et al., 2005). Interestingly, the acid phosphatase activity exhibited by these VSPs is required for the insecticidal property of the proteins. It has been proposed that VSPs may interfere with phosphate metabolism in the gut of target insects (Zhu-Salzman et al., 2008).

Plants exploit insect proteases for the activation of defense

Limited proteolysis of plant proteins in the insect gut adds another level of complexity to plant-insect interactions. Defensive enzymes such as TD are presumably kept latent in the plant and subsequently activated upon exposure to insect digestive proteases. Full activity of PPO from hybrid Poplar (*Populus trichocarpa* x *Populus deltoides*) requires treatment with detergent or proteases (Constabel et al., 1995; Constabel et al., 2000). Treatment with trypsin, for example, effectively activates PPO, which appears to be latent inside the chloroplast. These findings are consistent with the observation that poplar PPO is activated upon passage through the insect gut (Wang and Constabel, 2004). Biochemical studies have shown that this phenomenon involves removal of an inhibitory peptide from

the active site of the enzyme (Gandia-Herrero et al., 2005). Cry toxins provide another remarkable example of insecticidal proteins that are activated by insect digestive proteases. The Cry pro-toxin is activated by removal of an N-terminal peptide that blocks access of the protein to its receptor on the membrane of the midgut epithelium cells (Bravo et al., 2007). Binding of the proteolytically actived Cry protein to the receptor facilitates insertion of the toxin into the membrane.

Recent studies have also shown that peptide products derived from digestion of plant proteins in the insect gut can function as signals for activation of plant defense responses. The disulfide-bridged peptide inceptin, which was isolated from oral secretions of the fall armyworm (*Spodoptera frugiperda*) fed on cowpea (*Vigna unguiculata*), elicits the production of ethylene, JA, and salicylic acid (Schmelz et al., 2006). Inceptins are derived from the regulatory domain of the chloroplastic ATP synthase γ subunit (Schmelz et al., 2006). Interestingly, digestion of intact ATP synthase by insect gut proteases is required to release the active peptide signal (Schmelz et al., 2007). Because inceptin is a plant-derived signal whose production requires the action of insect proteases, this mechanism of elicting plant defense responses is consistent with the guard hypothesis of plant immunity (Dangl and Jones, 2001; Schmelz et al., 2006).

Thesis rationale and overview

The work presented here expands our current knowledge of the mechanisms of plant defense against insect herbivores. Most previous research aimed at understanding the chemical basis of plant-insect interactions has been focused on plant secondary

metabolites. More recently, however, there is growing emphasis on understanding insecticidal proteins that exert toxic or anti-nutritional effects on insect pests. Modern proteomic technologies have greatly facilitated the identification of these proteins (Chen et al., 2005). Tomato was used for all the experiments in this research because it has been extensively used as a model system for the study of plant-insect interactions. In addition, cultivated tomato is a host for a large number of arthropod herbivores that attack roots, leaves, and fruit (Lange, 1981). The results from experiments described in this thesis may help in the design of effective pest control measures. Because the genes identified are of plant origin, these genes may be used to develop insect-transgenic plants with good public acceptance since the genes are of plant origin.

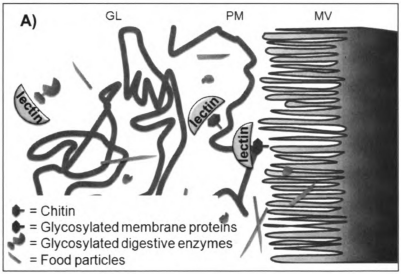
Chapter 2 describes the use of insect feces (frass) as a source of enriched plant proteins with potential roles in defense. The use of frass for proteomic analyses is based on the premise that midgut-active defense proteins are stable during passage of plant food through the insect and thus are excreted in the frass. This study identified a TD isoform (TD2) of tomato that likely serves an antinutritional role in defense. The expression pattern of TD2 and its closely related paralog TD1 is also described in this chapter. Chapter 3 examines the role of tomato TD2 as an herbivore defense in more detail. The results provide direct evidence for the contribution of TD2 to insect resistance, as well as new insight into the mechanism by which TD2 is activated in the gut by proteolysis. This chapter also compares the biochemical properties of tomato TD1 and TD2, and provides evidence for functional specialization of these two enzymes. Chapter 4 describes the results of a comparative proteomics study of frass from three different insect species

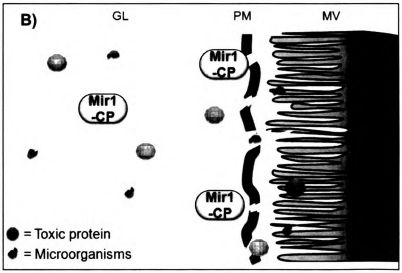
reared on tomato. The results obtained provide insight into how physicochemical conditions in the gut may affect the stability and digestibility of dietary protein.

Figure 1.1. Mode of action of toxic plant proteins.

(A) Lectins cause the formation of a multi-layered peritrophic membrane that allows abrasive food particles to access the midgut epithelium.
(B) The cysteine protease Mir1-CP impairs nutrient utilization by degrading the peritrophic membrane and allowing access of microorganisms and toxic proteins to the microvilli.
(C) Cyclotides form pores at the membrane of the midgut epithelium, which causes blebbing, swelling and, ultimately, rupture of the cells.

GL: gut lumen; PM: peritrophic membrane; MV: microvilli of the midgut epithelial cells


A)
GL
PM
MV
lectin
lectin
lectin
= Chitin
= Glycosylated membrane proteins
= Glycosylated digestive enzymes
= Food particles


B)
GL
PM
MV
Mir1 -CP
Mir1 -CP
Mir1 -CP
= Toxic protein
= Microorganisms

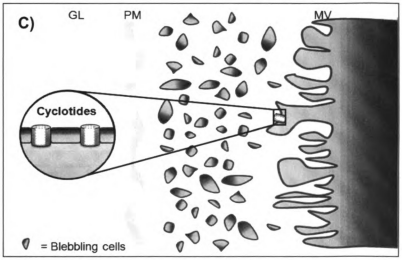
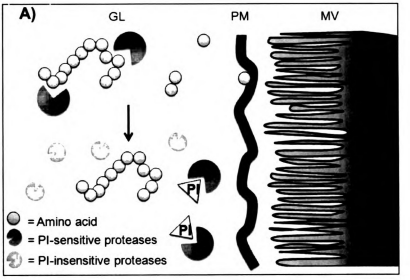
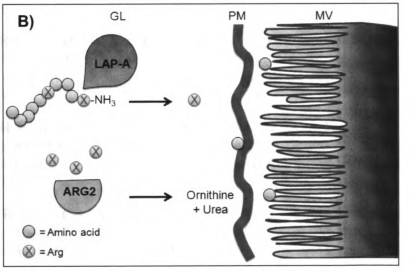

C)
GL
PM
MV
Cyclotides
= Blebbling cells

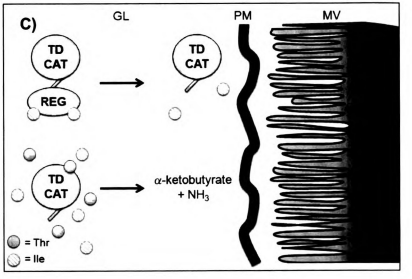
Figure 1.2. Mode of action of anti-nutritional plant proteins.

(A) Protease inhibitors (PI) induce a compensatory mechanism in insects to overproduce proteases, which depletes amino acid pools for the synthesis of other insect proteins.
(B) Leucine aminopeptidase-A (LAP-A) and Arginase2 (ARG2) may work synergistically to deplete arginine (Arg) from the midgut. LAP-A releases Arg from the N-terminus of polypeptides, whereas ARG2 catabolizes the resulting free Arg.
(C) Threonine deaminase (TD) is activated by removal of the regulatory domain that mediates inhibition of TD activity by isoleucine (Ile). Upon processing, TD degrades the essential amino acid threonine (Thr).

GL: gut lumen; PM: peritrophic membrane; MV: microvilli of the midgut epithelial cells


A)
GL
PM
MV
PI
PI
= Amino acid
= PI-sensitive proteases
= PI-insensitive proteases


B)
GL
PM
MV
LAP-A
-NH3
ARG2
Ornithine
+ Urea
= Amino acid
= Arg


C)
GL
PM
MV
TD
CAT
REG
TD
CAT
TD
CAT
α-ketobutyrate
+ NH3
= Thr
= Ile

LITERATURE CITED

Aharoni, A., Giri, A.P., Deuerlein, S., Griepink, F., de Kogel, W.J., Verstappen, F.W., Verhoeven, H.A., Jongsma, M.A., Schwab, W., and Bouwmeester, H.J. (2003). Terpenoid metabolism in wild-type and transgenic Arabidopsis plants. PLANT CELL **15,** 2866-2884.

Amme, S., Rutten, T., Melzer, M., Sonsmann, G., Vissers, J.P.C., Schlesier, B., and Mock, H.P. (2005). A proteome approach defines protective functions of tobacco leaf trichomes. PROTEOMICS **5,** 2508-2518.

Baldwin, I.T., and Karban, R. (1997). Induced Responses to Herbivory. (Chicago: The University of Chicago Press).

Baldwin, I.T., Halitschke, R., Paschold, A., von Dahl, C.C., and Preston, C.A. (2006). Volatile signaling in plant-plant interactions: "Talking trees" in the genomics era. Science **311,** 812-815.

Barbehenn, R.V., Jones, C.P., Yip, L., Tran, L., and Constabel, C.P. (2007). Limited impact of elevated levels of polyphenol oxidase on tree-feeding caterpillars: assessing individual plant defenses with transgenic poplar. OECOLOGIA **154,** 129-140.

Barbehenn, R.V., Jaros, A., Yip, L., Tran, L., Kanellis, A.K., and Constabel, C.P. (2008). Evaluating Ascorbate Oxidase as a Plant Defense Against Leaf-Chewing Insects Using Transgenic Poplar. JOURNAL OF CHEMICAL ECOLOGY **34,** 1331-1340.

Barbehenn, R.V., Jaros, A., Lee, G., Mozola, C., Weir, Q., and Salminen, J.P. (2009). Hydrolyzable tannins as "quantitative defenses": limited impact against Lymantria dispar caterpillars on hybrid poplar. J Insect Physiol **55,** 297-304.

Barbeta, B.L., Marshall, A.T., Gillon, A.D., Craik, D.J., and Anderson, M.A. (2008). Plant cyclotides disrupt epithelial cells in the midgut of lepidopteran larvae. Proc Natl Acad Sci U S A **105,** 1221-1225.

Barcellos, G.B.S., Almeida, L.M., Moreira, R.A., Cavada, B.S., Deoliveira, J.T.A., and Carlini, C.R. (1993). Canatoxin-Cross-Reactive, Concanavalin-a-Cross-Reactive and Canavalin-Cross-Reactive Materials during Maturation of Canavalia-Brasiliensis (Mart) Seeds. Planta **189,** 397-402.

Barth, C., and Jander, G. (2006). Arabidopsis myrosinases TGG1 and TGG2 have redundant function in glucosinolate breakdown and insect defense. PLANT JOURNAL **46,** 549-562.

Baum, J.A., Bogaert, T., Clinton, W., Heck, G.R., Feldmann, P., Ilagan, O., Johnson, S., Plaetinck, G., Munyikwa, T., Pleau, M., Vaughn, T., and Roberts, J. (2007). Control of coleopteran insect pests through RNA interference. Nat Biotechnol **25,** 1322-1326.

Bayes, A., Comellas-Bigler, M., Rodriguez de la Vega, M., Maskos, K., Bode, W., Aviles, F.X., Jongsma, M.A., Beekwilder, J., and Vendrell, J. (2005). Structural basis of the resistance of an insect carboxypeptidase to plant protease inhibitors. Proc Natl Acad Sci U S A **102,** 16602-16607.

Berger, S., Mitchell-Olds, T., and Stotz, H.U. (2002). Local and differential control of vegetative storage protein expression in response to herbivore damage in Arabidopsis thaliana. Physiol Plant **114,** 85-91.

Bergey, D.R., Orozco-Cardenas, M., de Moura, D.S., and Ryan, C.A. (1999). A wound- and systemin-inducible polygalacturonase in tomato leaves. Proc Natl Acad Sci U S A **96,** 1756-1760.

Bernays, E.A. (1998). Evolution of feeding behavior in insect herbivores - Success seen as different ways to eat without being eaten. Bioscience **48,** 35-44.

Bhonwong, A., Stout, M.J., Attajarusit, J., and Tantasawat, P. (2009). Defensive Role of Tomato Polyphenol Oxidases against Cotton Bollworm (Helicoverpa armigera) and Beet Armyworm (Spodoptera exigua). JOURNAL OF CHEMICAL ECOLOGY **35,** 28-38.

Bohlmann, J., Meyer-Gauen, G., and Croteau, R. (1998). Plant terpenoid synthases: molecular biology and phylogenetic analysis. Proc Natl Acad Sci U S A **95,** 4126-4133.

Bravo, A., Gill, S.S., and Soberon, M. (2007). Mode of action of Bacillus thuringiensis Cry and Cyt toxins and their potential for insect control. Toxicon **49,** 423-435.

Browse, J., and Howe, G.A. (2008). New weapons and a rapid response against insect attack. Plant Physiol **146,** 832-838.

Carlini, C.R., and Grossi-de-Sa, M.F. (2002). Plant toxic proteins with insecticidal properties. A review on their potentialities as bioinsecticides. Toxicon **40,** 1515-1539.

Carlini, C.R., Barcellos, G.B.S., Baetaneves, A.D.V., and Guimaraes, J.A. (1988). Immunoreactivity for Canatoxin and Concanavalin-a among Proteins of Leguminous Seeds. Phytochemistry **27,** 25-30.

Carlini, C.R., Oliveira, A.E., Azambuja, P., Xavier-Filho, J., and Wells, M.A. (1997). Biological effects of canatoxin in different insect models: evidence for a proteolytic activation of the toxin by insect cathepsinlike enzymes. J Econ Entomol **90,** 340-348.

Chao, W.S., Gu, Y.Q., Pautot, V.V., Bray, E.A., and Walling, L.L. (1999). Leucine aminopeptidase RNAs, proteins, and activities increase in response to water deficit, salinity, and the wound signals systemin, methyl jasmonate, and abscisic acid. Plant Physiol **120,** 979-992.

Chen, H., Jones, A.D., and Howe, G.A. (2006). Constitutive activation of the jasmonate signaling pathway enhances the production of secondary metabolites in tomato. FEBS Lett **580,** 2540-2546.

Chen, H., Gonzales-Vigil, E., and Howe, G. (2008). Action of plant defensive enzymes in the insect midgut. In Induced plant resistance to herbivory, A. Schaller, ed (Berlin: Springer.

Chen, H., McCaig, B.C., Melotto, M., He, S.Y., and Howe, G.A. (2004). Regulation of plant arginase by wounding, jasmonate, and the phytotoxin coronatine. J Biol Chem **279,** 45998-46007.

Chen, H., Wilkerson, C.G., Kuchar, J.A., Phinney, B.S., and Howe, G.A. (2005). Jasmonate-inducible plant enzymes degrade essential amino acids in the herbivore midgut. Proc Natl Acad Sci U S A **102,** 19237-19242.

Chen, M. (2008). Inducible direct plant defense against insect herbivores: A review. Insect Sci **15,** 101-114.

Chini, A., Fonseca, S., Fernandez, G., Adie, B., Chico, J.M., Lorenzo, O., Garcia-Casado, G., Lopez-Vidriero, I., Lozano, F.M., Ponce, M.R., Micol, J.L., and Solano, R. (2007). The JAZ family of repressors is the missing link in jasmonate signalling. Nature **448,** 666-671.

Cloutier, C., Jean, C., Fournier, M., Yelle, S., and Michaud, D. (2000). Adult Colorado potato beetles, Leptinotarsa decemlineata compensate for nutritional stress on oryzacystatin I-transgenic potato plants by hypertrophic behavior and over-production of insensitive proteases. Arch Insect Biochem **44,** 69-81.

Colgrave, M.L., and Craik, D.J. (2004). Thermal, chemical, and enzymatic stability of the cyclotide kalata B1: the importance of the cyclic cystine knot. Biochemistry **43,** 5965-5975.

Constabel, C.P., and Barbehenn, R. (2008). Defensive roles of polyphenol oxidase in plants In Induced plant resistance to herbivory (Berlin: Springer), pp. 462.

Constabel, C.P., Bergey, D.R., and Ryan, C.A. (1995). Systemin activates synthesis of wound-inducible tomato leaf polyphenol oxidase via the octadecanoid defense signaling pathway. Proc Natl Acad Sci U S A **92,** 407-411.

Constabel, C.P., Yip, L., Patton, J.J., and Christopher, M.E. (2000). Polyphenol oxidase from hybrid poplar. Cloning and expression in response to wounding and herbivory. Plant Physiol **124,** 285-295.

Cornell, H.V., Hawkins, B.A., and Hochberg, M.E. (1998). Towards an empirically-based theory of herbivore demography. ECOLOGICAL ENTOMOLOGY **23,** 340-349.

Craik, D.J., Daly, N.L., Bond, T., and Waine, C. (1999). Plant cyclotides: A unique family of cyclic and knotted proteins that defines the cyclic cystine knot structural motif. JOURNAL OF MOLECULAR BIOLOGY **294,** 1327-1336.

Craik, D.J., Daly, N.L., Mulvenna, J., Plan, M.R., and Trabi, M. (2004). Discovery, structure and biological activities of the cyclotides. Curr Protein Pept Sc **5,** 297-315.

Currano, E.D., Wilf, P., Wing, S.L., Labandeira, C.C., Lovelock, E.C., and Royer, D.L. (2008). Sharply increased insect herbivory during the Paleocene-Eocene thermal maximum. P Natl Acad Sci USA **105,** 1960-1964.

Dangl, J.L., and Jones, J.D. (2001). Plant pathogens and integrated defence responses to infection. Nature **411,** 826-833.

DeLucia, E.H., Casteel, C.L., Nabity, P.D., and O'Neill, B.F. (2008). Insects take a bigger bite out of plants in a warmer, higher carbon dioxide world. P Natl Acad Sci USA **105,** 1781-1782.

Devoto, A., Ellis, C., Magusin, A., Chang, H.S., Chilcott, C., Zhu, T., and Turner, J.G. (2005). Expression profiling reveals COI1 to be a key regulator of genes involved in wound- and methyl jasmonate-induced secondary metabolism, defence, and hormone interactions. Plant Mol Biol **58,** 497-513.

Diez-Diaz, M., Conejero, V., Rodrigo, I., Pearce, G., and Ryan, C.A. (2004). Isolation and characterization of wound-inducible carboxypeptidase inhibitor from tomato leaves. Phytochemistry **65,** 1919-1924.

Duffey, S.S., and Stout, M.J. (1996). Antinutritive and toxic components of plant defense against insects. Arch Insect Biochem **32,** 3-37.

Estruch, J.J., Carozzi, N.B., Desai, N., Duck, N.B., Warren, G.W., and Koziel, M.G. (1997). Transgenic plants: an emerging approach to pest control. Nat Biotechnol **15,** 137-141.

Fahey, J.W., Zalcmann, A.T., and Talalay, P. (2001). The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. Phytochemistry **56,** 5-51.

Farmer, E.E., and Dubugnon, L. (2009). Detritivorous crustaceans become herbivores on jasmonate-deficient plants. Proc Natl Acad Sci U S A **106,** 935-940.

Farmer, E.E., Johnson, R.R., and Ryan, C.A. (1992). Regulation of Expression of Proteinase Inhibitor Genes by Methyl Jasmonate and Jasmonic Acid. Plant Physiol **98,** 995-1002.

Feeny, P. (1970). Seasonal changes in oak leaf tannins and nutrients as a cause of spring feeding by winter moth caterpillars. Ecology **51,** 565-581.

Felton, G.W. (2005). Indigestion is a plant's best defense. Proc Natl Acad Sci U S A **102,** 18771-18772.

Felton, G.W., Donato, K.K., Broadway, R.M., and Duffey, S.S. (1992). Impact of Oxidized Plant Phenolics on the Nutritional Quality of Dietary-Protein to a Noctuid Herbivore, Spodoptera-Exigua. Journal of Insect Physiology **38,** 277-285.

Felton, J.W.D., K.; Del Vecchio, R. J. and Duffey S. S. (1989). Activation of plant foliar oxidases by insect feeding reduces nutritive quality of foliage for noctuid herbivores JOURNAL OF CHEMICAL ECOLOGY **15,** 2667-2694.

Feys, B., Benedetti, C., Penfold, C., and Turner, J. (1994). Arabidopsis Mutants Selected for Resistance to the Phytotoxin Coronatine Are Male Sterile, Insensitive to Methyl Jasmonate, and Resistant to a Bacterial Pathogen. Plant Cell **6,** 751-759.

Fitches, E., Audsley, N., Gatehouse, J.A., and Edwards, J.P. (2002). Fusion proteins containing neuropeptides as novel insect contol agents: snowdrop lectin delivers fused allatostatin to insect haemolymph following oral ingestion. Insect Biochem Mol Biol **32,** 1653-1661.

Foissac, X., Loc, N.T., Christou, P., Gatehouse, A.M.R., and Gatehouse, J.A. (2000). Resistance to green leafhopper (Nephotettix virescens) and brown planthopper (Nilaparvata lugens) in transgenic rice expressing snowdrop lectin (Galanthus nivalis agglutinin; GNA). Journal of Insect Physiology **46,** 573-583.

Follmer, C., Real-Guerra, R., Wasserman, G.E., Olivera-Severo, D., and Carlini, C.R. (2004). Jackbean, soybean and Bacillus pasteurii ureases: biological effects unrelated to ureolytic activity. Eur J Biochem **271,** 1357-1363.

Fowler, J.H., Narvaez-Vasquez, J., Aromdee, D.N., Pautot, V., Holzer, F.M., and Walling, L.L. (2009). Leucine Aminopeptidase Regulates Defense and Wound Signaling in Tomato Downstream of Jasmonic Acid. PLANT CELL.

Franceschi, V.R., Wittenbach, V.A., and Giaquinta, R.T. (1983). Paraveinal Mesophyll of Soybean Leaves in Relation to Assimilate Transfer and Compartmentation : III. Immunohistochemical Localization of Specific Glycopeptides in the Vacuole after Depodding. Plant Physiol **72,** 586-589.

Frelichowski, J.E., and Juvik, J.A. (2001). Sesquiterpene carboxylic acids from a wild tomato species affect larval feeding behavior and survival of Helicoverpa zea and Spodoptera exigua (Lepidoptera : Noctuidae). Journal of Economic Entomology **94,** 1249-1259.

Gandia-Herrero, F., Jimenez-Atienzar, M., Cabanes, J., Garcia-Carmona, F., and Escribano, J. (2005). Evidence for a common regulation in the activation of a polyphenol oxidase by trypsin and sodium dodecyl sulfate. Biol Chem **386,** 601-607.

Gatehouse, A.M.R., Davison, G.M., Newell, C.A., Merryweather, A., Hamilton, W.D.O., Burgess, E.P.J., Gilbert, R.J.C., and Gatehouse, J.A. (1997). Transgenic potato plants with enhanced resistance to the tomato moth, Lacanobia oleracea: Growth room trials. Mol Breeding **3,** 49-63.

Gatehouse, J.A. (2008). Biotechnological prospects for engineering insect-resistant plants. PLANT PHYSIOLOGY **146,** 881-887.

Giri, A.S., Harsulkar, A.M., Deshpande, V.V., N., S.M., S., G.V., and P.K., R. (1998). Chickpea Defensive Proteinase Inhibitors Can Be Inactivated by Podborer Gut Proteinases. Plant Physiology **116,** 393-401.

Goggin, F.L. (2007). Plant-aphid interactions: molecular and ecological perspectives. Curr Opin Plant Biol **10,** 399-408.

Goldraij, A., and Polacco, J.C. (1999). Arginase is inoperative in developing soybean embryos. Plant Physiol **119,** 297-304.

Goldraij, A., and Polacco, J.C. (2000). Arginine degradation by arginase in mitochondria of soybean seedling cotyledons. Planta **210,** 652-658.

Gong, Z., Koiwa, H., Cushman, M.A., Ray, A., Bufford, D., Kore-eda, S., Matsumoto, T.K., Zhu, J., Cushman, J.C., Bressan, R.A., and Hasegawa, P.M. (2001). Genes that are uniquely stress regulated in salt overly sensitive (sos) mutants. Plant Physiol **126,** 363-375.

Green, T.R., and Ryan, C.A. (1972). Wound-Induced Proteinase Inhibitor in Plant Leaves: A Possible Defense Mechanism against Insects. Science **175,** 776-777.

Gruber, C.W., Elliott, A.G., Ireland, D.C., Delprete, P.G., Dessein, S., Goransson, U., Trabi, M., Wang, C.K., Kinghorn, A.B., Robbrecht, E., and Craik, D.J. (2008). Distribution and Evolution of Circular Miniproteins in Flowering Plants. PLANT CELL **20,** 2471-2483.

Gruden, K., Kuipers, A.G.J., Guncar, G., Slapar, N., Strukelj, B., and Jongsma, M.A. (2004). Molecular basis of Colorado potato beetle adaptation to potato plant defence at the level of digestive cysteine proteinases. Insect Biochem Molec **34,** 365-375.

Gruden, K., Strukelj, B., Popovic, T., Lenarcic, B., Bevec, T., Brzin, J., Kregar, I., Herzog-Velikonja, J., Stiekema, W.J., Bosch, D., and Jongsma, M.A. (1998). The cysteine protease activity of Colorado potato beetle (Leptinotarsa decemlineata Say) guts, which is insensitive to potato protease inhibitors, is inhibited by thyroglobulin type-1 domain inhibitors. Insect Biochem Molec **28,** 549-560.

Gu, Y.Q., Holzer, F.M., and Walling, L.L. (1999). Overexpression, purification and biochemical characterization of the wound-induced leucine aminopeptidase of tomato. Eur J Biochem **263,** 726-735.

Hadacek, F. (2002). Secondary metabolites as plant traits: Current assessment and future perspectives. Crit Rev Plant Sci **21,** 273-322.

Halitschke, R., Gase, K., Hui, D.Q., Schmidt, D.D., and Baldwin, I.T. (2003). Molecular interactions between the specialist herbivore Manduca sexta (Lepidoptera, Sphingidae) and its natural host Nicotiana attenuata. VI. Microarray analysis reveals that most herbivore-specific transcriptional changes are mediated by fatty acid-amino acid conjugates. PLANT PHYSIOLOGY **131,** 1894-1902.

Hanley, M.E., Lamont, B.B., Fairbanks, M.M., and Rafferty, C.M. (2007). Plant structural traits and their role in anti-herbivore defence. Perspect Plant Ecol **8,** 157-178.

Harper, M.S., Hopkins, T.L., and Czapla, T.H. (1998). Effect of wheat germ agglutinin on formation and structure of the peritrophic membrane in european corn borer (Ostrinia nubilalis) larvae. Tissue Cell **30,** 166-176.

Heil, M., Koch, T., Hilpert, A., Fiala, B., Boland, W., and Linsenmair, K. (2001). Extrafloral nectar production of the ant-associated plant, Macaranga tanarius, is an induced, indirect, defensive response elicited by jasmonic acid. Proc Natl Acad Sci U S A **98,** 1083-1088.

Hilder, V.A., Gatehouse, A.M.R., Sheerman, S.E., Barker, R.F., and Boulter, D. (1987). A Novel Mechanism of Insect Resistance Engineered into Tobacco. Nature **330,** 160-163.

Hildmann, T., Ebneth, M., Peña-Cortés, H., Sánchez-Serrano, J.J., Willmitzer, L., and Prat, S. (1992). General roles of abscisic and jasmonic acids in gene activation as a result of mechanical wounding. Plant Cell **4,** 1157-1170.

Hopkins, R.J., van Dam, N.M., and van Loon, J.J. (2009). Role of glucosinolates in insect-plant relationships and multitrophic interactions. Annu Rev Entomol **54,** 57-83.

Howe, G.A., and Ryan, C.A. (1999). Suppressors of systemin signaling identify genes in the tomato wound response pathway. Genetics **153,** 1411-1421.

Howe, G.A., and Jander, G. (2008). Plant immunity to insect herbivores. Annu Rev Plant Biol **59,** 41-66.

Jansen, J.J., Allwood, J.W., Marsden-Edwards, E., van der Putten, W.H., Goodacre, R., and van Dam, N.M. (2009). Metabolomic analysis of the interaction between plants and herbivores. Metabolomics **5,** 150-161.

Jennings, C., West, J., Waine, C., Craik, D., and Anderson, M. (2001). Biosynthesis and insecticidal properties of plant cyclotides: The cyclic knotted proteins from Oldenlandia affinis. P Natl Acad Sci USA **98,** 10614-10619.

Jennings, C.V., Rosengren, K.J., Daly, N.L., Plan, M., Stevens, J., Scanlon, M.J., Waine, C., Norman, D.G., Anderson, M.A., and Craik, D.J. (2005). Isolation,

solution structure, and insecticidal activity of Kalata B2, a circular protein with a twist: Do Mobius strips exist in nature? Biochemistry **44,** 851-860.

Johnson, R., Narvaez, J., An, G.H., and Ryan, C. (1989). Expression of Proteinase Inhibitor-I and Inhibitor-Ii in Transgenic Tobacco Plants - Effects on Natural Defense against Manduca-Sexta Larvae. P Natl Acad Sci USA **86,** 9871-9875.

Jongsma, M.A., and Bolter, C. (1997). The adaptation of insects to plant protease inhibitors. Journal of Insect Physiology **43,** 885-895.

Jongsma, M.A., and Beekwilder, J. (2008). Plant Protease Inhibitors: Functional Evolution for Defense. In Induced plant resistance to herbivory, A. Schaller, ed (Berlin: Springer), pp. 462.

Jongsma, M.A., Bakker, P.L., Peters, J., Bosch, D., and Stiekema, W.J. (1995). Adaptation of Spodoptera exigua larvae to plant proteinase inhibitors by induction of gut proteinase activity insensitive to inhibition. Proc Natl Acad Sci U S A **92,** 8041-8045.

Kang, J., Wang, L., Giri, A., and Baldwin, I. (2006a). Silencing threonine deaminase and JAR4 in Nicotiana attenuata impairs jasmonic acid-isoleucine-mediated defenses against manduca sexta. Plant Cell **18,** 3303-3320.

Kang, J.H., Wang, L., Giri, A., and Baldwin, I.T. (2006b). Silencing threonine deaminase and JAR4 in Nicotiana attenuata impairs jasmonic acid-isoleucine-mediated defenses against manduca sexta. Plant Cell **18,** 3303-3320.

Katsir, L., Chung, H.S., Koo, A.J.K., and Howe, G.A. (2008). Jasmonate signaling: a conserved mechanism of hormone sensing. CURRENT OPINION IN PLANT BIOLOGY **11,** 428-435.

Keeling, C.I., and Bohlmann, J. (2006). Genes, enzymes and chemicals of terpenoid diversity in the constitutive and induced defence of conifers against insects and pathogens. New Phytol **170,** 657-675.

Kennedy, G.G. (2003). Tomato, pests, parasitoids, and predators: tritrophic interactions involving the genus Lycopersicon. Annu Rev Entomol **48,** 51-72.

Kessler, A., and Baldwin, I.T. (2001). Defensive function of herbivore-induced plant volatile emissions in nature. Science **291,** 2141-2144.

Kessler, A., and Baldwin, I.T. (2002). Plant responses to insect herbivory: The emerging molecular analysis. Annual Review of Plant Biology **53,** 299-328.

Kessler, A., Halitschke, R., and Baldwin, I.T. (2004). Silencing the jasmonate cascade: induced plant defenses and insect populations. Science **305,** 665-668.

Konno, K., Hirayama, C., Nakamura, M., Tateishi, K., Tamura, Y., Hattori, M., and Kohno, K. (2004). Papain protects papaya trees from herbivorous insects: role of cysteine proteases in latex. PLANT JOURNAL **37,** 370-378.

Lange, W.B., L. (1981). Insect pests of tomato. ANNUAL REVIEW OF ENTOMOLOGY **26,** 345-371.

Li, L., Li, C., and Howe, G.A. (2001). Genetic analysis of wound signaling in tomato. Evidence for a dual role of jasmonic acid in defense and female fertility. Plant Physiol **127,** 1414-1417.

Li, L., Zhao, Y., McCaig, B.C., Wingerd, B.A., Wang, J., Whalon, M.E., Pichersky, E., and Howe, G.A. (2004a). The tomato homolog of CORONATINE-INSENSITIVE1 is required for the maternal control of seed maturation, jasmonate-signaled defense responses, and glandular trichome development. Plant Cell **16,** 126-143.

Li, L., Zhao, Y., McCaig, B., Wingerd, B., Wang, J., Whalon, M., Pichersky, E., and Howe, G. (2004b). The tomato homolog of CORONATINE-INSENSITIVE1 is required for the maternal control of seed maturation, jasmonate-signaled defense responses, and glandular trichome development. Plant Cell **16,** 126-143.

Lightner, J., Pearce, G., Ryan, C.A., and Browse, J. (1993). Isolation of Signaling Mutants of Tomato (Lycopersicon-Esculentum). Molecular & General Genetics **241,** 595-601.

Liu, J., Xia, K.F., Zhu, J.C., Deng, Y.G., Huang, X.L., Hu, B.L., Xu, X.P., and Xu, Z.F. (2006). The nightshade proteinase inhibitor IIb gene is constitutively expressed in glandular trichomes. Plant Cell Physiol **47,** 1274-1284.

Liu, X., Bai, J., Huang, L., Zhu, L., Weng, N., Reese, J.C., Harris, M., Stuart, J.J., and Chen, M.S. (2007). Gene expression of different wheat genotypes during attack by virulent and avirulent Hessian fly (Mayetiola destructor) larvae. J Chem Ecol **33,** 2171-2194.

Liu, Y., Ahn, J.E., Datta, S., Salzman, R.A., Moon, J., Huyghues-Despointes, B., Pittendrigh, B., Murdock, L.L., Koiwa, H., and Zhu-Salzman, K. (2005). Arabidopsis vegetative storage protein is an anti-insect acid phosphatase. Plant Physiol **139,** 1545-1556.

Mahanil, S., Attajarusit, J., Stout, M.J., and Thipyapong, P. (2008). Overexpression of tomato polyphenol oxidase increases resistance to common cutworm. PLANT SCIENCE **174,** 456-466.

Mainguet, A.M.L., A.; El Sayed, G.; and Rollin P. (2000). Ability of a generalist insect, Schistocerca gregaria, to overcome thioglucoside defense in desert plants: tolerance or adaptation? . Entomologia Experimentalis et Applicata **94,** 309-317.

Mao, Y.B., Cai, W.J., Wang, J.W., Hong, G.J., Tao, X.Y., Wang, L.J., Huang, Y.P., and Chen, X.Y. (2007). Silencing a cotton bollworm P450 monooxygenase gene by plant-mediated RNAi impairs larval tolerance of gossypol. Nat Biotechnol **25,** 1307-1313.

Mithofer, A., and Boland, W. (2008). Recognition of herbivory-associated molecular patterns. Plant Physiol **146,** 825-831.

Mohan, S., Ma, P.W., Williams, W.P., and Luthe, D.S. (2008). A naturally occurring plant cysteine protease possesses remarkable toxicity against insect pests and synergizes Bacillus thuringiensis toxin. PLoS ONE **3,** e1786.

Mohan, S., Ma, P.W.K., Pechan, T., Bassford, E.R., Williams, W.P., and Luthe, D.S. (2006). Degradation of the S. frugiperda peritrophic matrix by an inducible maize cysteine protease. Journal of Insect Physiology **52,** 21-28.

Moura, D.S., Bergey, D.R., and Ryan, C.A. (2001). Characterization and localization of a wound-inducible type I serine-carboxypeptidase from leaves of tomato plants (Lycopersicon esculentum Mill.). Planta **212,** 222-230.

Müller, C. (2008). Resistance at the plant cuticle. In Induced plant resistance to herbivory, A. Schaller, ed (Berlin: Springer.

Oerke, E.C. (2006). Crop losses to pests. J Agr Sci **144,** 31-43.

Oliveira, A.E.A.S., M.P.; Machado, O.L.T.; Fernandes, K.V.S; and Xavier-Filho J. (1999). The toxicity of Jack bean (Canavalia ensiformis) cotyledon and seed coat proteins to the cowpea weevil (Callosobruchus maculatus) Entomologia Experimentalis et Applicata **92,** 249-255.

Outchkourov, N.S., de Kogel, W.J., Wiegers, G.L., Abrahamson, M., and Jongsma, M.A. (2004). Engineered multidomain cysteine protease inhibitors yield resistance against western flower thrips (Frankliniella occidentalis) in greenhouse trials. Plant Biotechnol J **2,** 449-458.

Pare, P.W., and Tumlinson, J.H. (1999). Plant volatiles as a defense against insect herbivores. PLANT PHYSIOLOGY **121,** 325-331.

Paschold, A., Halitschke, R., and Baldwin, I.T. (2007). Co(i)-ordinating defenses: NaCOI1 mediates herbivore- induced resistance in Nicotiana attenuata and reveals the role of herbivore movement in avoiding defenses. Plant J **51,** 79-91.

Pautot, V., Holzer, F.M., Reisch, B., and Walling, L.L. (1993). Leucine aminopeptidase: an inducible component of the defense response in Lycopersicon esculentum (tomato). Proc Natl Acad Sci U S A **90,** 9906-9910.

Pechan, T., Cohen, A., Williams, W.P., and Luthe, D.S. (2002). Insect feeding mobilizes a unique plant defense protease that disrupts the peritrophic matrix of caterpillars. P Natl Acad Sci USA **99,** 13319-13323.

Pechan, T., Ye, L.J., Chang, Y.M., Mitra, A., Lin, L., Davis, F.M., Williams, W.P., and Luthe, D.S. (2000). A unique 33-kD cysteine proteinase accumulates in response to larval feeding in maize genotypes resistant to fall armyworm and other lepidoptera. PLANT CELL **12,** 1031-1040.

Peumans, W.J., and Van Damme, E.J. (1995). Lectins as plant defense proteins. Plant Physiol **109,** 347-352.

Pollaco, J.C., and Holland, M.A. (1993). Role of urease in plant cells. International Review of Cell & Molecular Biology **145,** 65-103.

Price, D.R., and Gatehouse, J.A. (2008). RNAi-mediated crop protection against insects. Trends Biotechnol **26,** 393-400.

Rao, K.V., Rathore, K.S., Hodges, T.K., Fu, X., Stoger, E., Sudhakar, D., Williams, S., Christou, P., Bharathi, M., Bown, D.P., Powell, K.S., Spence, J., Gatehouse, A.M.R., and Gatehouse, J.A. (1998). Expression of snowdrop lectin (GNA) in transgenic rice plants confers resistance to rice brown planthopper. PLANT JOURNAL **15,** 469-477.

Reymond, P., Weber, H., Damond, M., and Farmer, E.E. (2000). Differential gene expression in response to mechanical wounding and insect feeding in Arabidopsis. Plant Cell **12,** 707-720.

Reymond, P., Bodenhausen, N., Van Poecke, R.M., Krishnamurthy, V., Dicke, M., and Farmer, E.E. (2004). A conserved transcript pattern in response to a specialist and a generalist herbivore. Plant Cell **16,** 3132-3147.

Samach, A., Broday, L., Hareven, D., and Lifschitz, E. (1995). Expression of an amino acid biosynthesis gene in tomato flowers: developmental upregulation and MeJa response are parenchyma-specific and mutually compatible. Plant J **8,** 391-406.

Samach, A., Hareven, D., Gutfinger, T., Ken-Dror, S., and Lifschitz, E. (1991). Biosynthetic threonine deaminase gene of tomato: isolation, structure, and upregulation in floral organs. Proc Natl Acad Sci U S A **88,** 2678-2682.

Schilmiller, A., and Howe, G. (2005). Systemic signaling in the wound response. Curr Opin Plant Biol **8,** 369-377.

Schilmiller, A.L., Last, R.L., and Pichersky, E. (2008). Harnessing plant trichome biochemistry for the production of useful compounds. PLANT JOURNAL **54,** 702-711.

Schlaeppi, K., Bodenhausen, N., Buchala, A., Mauch, F., and Reymond, P. (2008). The glutathione-deficient mutant pad2-1 accumulates lower amounts of glucosinolates and is more susceptible to the insect herbivore Spodoptera littoralis. PLANT JOURNAL **55,** 774-786.

Schmelz, E.A., LeClere, S., Carroll, M.J., Alborn, H.T., and Teal, P.E. (2007). Cowpea chloroplastic ATP synthase is the source of multiple plant defense elicitors during insect herbivory. Plant Physiol **144,** 793-805.

Schmelz, E.A., Engelberth, J., Alborn, H.T., Tumlinson, J.H., 3rd, and Teal, P.E. (2009). Phytohormone-based activity mapping of insect herbivore-produced elicitors. Proc Natl Acad Sci U S A **106,** 653-657.

Schmelz, E.A., Carroll, M.J., LeClere, S., Phipps, S.M., Meredith, J., Chourey, P.S., Alborn, H.T., and Teal, P.E. (2006). Fragments of ATP synthase mediate plant perception of insect attack. Proc Natl Acad Sci U S A **103,** 8894-8899.

Shade, R.E., Schroeder, H.E., Pueyo, J.J., Tabe, L.M., Murdock, L.L., Higgins, T.J.V., and Chrispeels, M.J. (1994). Transgenic Pea-Seeds Expressing the Alpha-Amylase Inhibitor of the Common Bean Are Resistant to Bruchid Beetles. Bio-Technol **12,** 793-796.

Shindo, T., and Van der Hoorn, R.A. (2008). Papain-like cysteine proteases: key players at molecular battlefields employed by both plants and their invaders. Mol Plant Pathol **9,** 119-125.

Shroff, R., Vergara, F., Muck, A., Svatos, A., and Gershenzon, J. (2008). Nonuniform distribution of glucosinolates in Arabidopsis thaliana leaves has important consequences for plant defense. Proc Natl Acad Sci U S A **105,** 6196-6201.

Simmons, A.T., and Gurr, G.M. (2005). Trichomes of Lycopersicon species and their hybrids: effects on pests and natural enemies. Agr Forest Entomol **7,** 265-276.

Staniscuaski, F., Ferreira-Dasilva, C.T., Mulinari, F., Pires-Alves, M., and Carlini, C.R. (2005). Insecticidal effects of canatoxin on the cotton stainer bug Dysdercus peruvianus (Hemiptera: Pyrrhocoridae). Toxicon **45,** 753-760.

Staswick, P.E., Papa, C., Huang, J.F., and Rhee, Y. (1994). Purification of the Major Soybean Leaf Acid Phosphatase That Is Increased by Seed-Pod Removal. Plant Physiol **104,** 49-57.

Staswick, P.E., Zhang, Z., Clemente, T.E., and Specht, J.E. (2001). Efficient down-regulation of the major vegetative storage protein genes in transgenic soybean does not compromise plant productivity. Plant Physiol **127,** 1819-1826.

Tabashnik, B.E., Gassmann, A.J., Crowder, D.W., and Carriere, Y. (2008). Insect resistance to Bt crops: evidence versus theory. Nat Biotechnol **26,** 199-202.

Takabayashi, J., and Dicke, M. (1996). Plant-carnivore mutualism through herbivore-induced carnivore attractants. TRENDS IN PLANT SCIENCE **1,** 109-113.

Tattersall, D.B., Bak, S., Jones, P.R., Olsen, C.E., Nielsen, J.K., Hansen, M.L., Hoj, P.B., and Moller, B.L. (2001). Resistance to an herbivore through engineered cyanogenic glucoside synthesis. Science **293,** 1826-1828.

Thaler, J.S. (1999). Jasmonate-inducible plant defences cause increased parasitism of herbivores. Nature **399,** 686-688.

Thaler, J.S., Karban, R., Ullman, D.E., Boege, K., and Bostock, R.M. (2002). Cross-talk between jasmonate and salicylate plant defense pathways: effects on several plant parasites. OECOLOGIA **131,** 227-235.

Thaler, J.S.F., M.A.; Pare, P.W. and Dicke, M. (2002). Jasmonate-deficient plants have reduced direct and indirect defences against herbivores. ECOLOGY LETTERS **5,** 764–774.

Thines, B., Katsir, L., Melotto, M., Niu, Y., Mandaokar, A., Liu, G., Nomura, K., He, S.Y., Howe, G.A., and Browse, J. (2007). JAZ repressor proteins are targets of the SCF(COI1) complex during jasmonate signalling. Nature **448,** 661-665.

Thipyapong, P., Stout, M.J., and Attajarusit, J. (2007). Functional analysis of polyphenol oxidases by antisense/sense technology. Molecules **12,** 1569-1595.

Tumlinson, J., and Lait, C. (2005). Biosynthesis of fatty acid amide elicitors of plant volatiles by insect herbivores. Arch Insect Biochem Physiol **58,** 54-68.

Tumlinson, J.H., Lewis, W.J., and Vet, L.E.M. (1993). How Parasitic Wasps Find Their Hosts. Sci Am **268,** 100-106.

Turlings, T.C.J., and Ton, J. (2006). Exploiting scents of distress: the prospect of manipulating herbivore-induced plant odours to enhance the control of agricultural pests. CURRENT OPINION IN PLANT BIOLOGY **9,** 421-427.

Uefuji, H., Tatsumi, Y., Morimoto, M., Kaothien-Nakayama, P., Ogita, S., and Sano, H. (2005). Caffeine production in tobacco plants by simultaneous expression of three coffee N-methyltrasferases and its potential as a pest repellant. Plant Mol Biol **59,** 221-227.

Utsugi, S., Sakamoto, W., Murata, M., and Motoyoshi, F. (1998). Arabidopsis thaliana vegetative storage protein (VSP) genes: gene organization and tissue-specific expression. Plant Mol Biol **38,** 565-576.

Van Damme, E.J.M. (2008). Plant lectins as part of the plant defense system against insects. In Induced plant resistance to herbivory, A. Schaller, ed (Berlin: Springer), pp. 462.

Vick, B., and Zimmerman, D. (1984). Biosynthesis of Jasmonic Acid by Several Plant Species. Plant Physiol **75,** 458-461.

Volpicella, M., Schipper, A., Jongsma, M.A., Spoto, N., Gallerani, R., and Ceci, L.R. (2000). Characterization of recombinant mustard trypsin inhibitor 2 (MTI2) expressed in Pichia pastoris. FEBS Lett **468,** 137-141.

Walling, L.L. (2000). The Myriad Plant Responses to Herbivores. JOURNAL OF PLANT GROWTH REGULATION **19,** 195-216.

Wang, J., and Constabel, C.P. (2004). Polyphenol oxidase overexpression in transgenic Populus enhances resistance to herbivory by forest tent caterpillar (Malacosoma disstria). Planta **220,** 87-96.

Wilhite, S.E., Elden, T.C., Brzin, J., and Smigocki, A.C. (2000). Inhibition of cysteine and aspartyl proteinases in the alfalfa weevil midgut with biochemical and plant-derived proteinase inhibitors. Insect Biochem Mol Biol **30,** 1181-1188.

Williams, C.E., Collier, C.C., Nemacheck, J.A., Liang, C., and Cambron, S.E. (2002). A lectin-like wheat gene responds systemically to attempted feeding by avirulent first-instar Hessian fly larvae. J Chem Ecol **28,** 1411-1428.

Winterer, J., and Bergelson, J. (2001). Diamondback moth compensatory consumption of protease inhibitor-transformed plants. Mol Ecol **10,** 1069-1074.

Xu, D.P., Xue, Q.Z., McElroy, D., Mawal, Y., Hilder, V.A., and Wu, R. (1996). Constitutive expression of a cowpea trypsin inhibitor gene, CpTi, in transgenic rice plants confers resistance to two major rice insect pests. Mol Breeding **2,** 167-173.

Yang, L., Fang, Z., Dicke, M., van Loon, J.J., and Jongsma, M.A. (2009). The diamondback moth, Plutella xylostella, specifically inactivates Mustard Trypsin Inhibitor 2 (MTI2) to overcome host plant defence. Insect Biochem Mol Biol **39,** 55-61.

Yu, H.F., Kowalski, S.P., and Steffens, J.C. (1992). Comparison of Polyphenol Oxidase Expression in Glandular Trichomes of Solanum and Lycopersicon Species. PLANT PHYSIOLOGY **100,** 1885-1890.

Zangerl, A.R., Hamilton, J.G., Miller, T.J., Crofts, A.R., Oxborough, K., Berenbaum, M.R., and de Lucia, E.H. (2002). Impact of folivory on photosynthesis is greater than the sum of its holes. P Natl Acad Sci USA **99,** 1088-1091.

Zhu-Salzman, K., and Salzman, R.A. (2001). Functional mechanics of the plant defensive Griffonia simplicifolia lectin II: Resistance to proteolysis is independent of glycoconjugate binding in the insect gut. Journal of Economic Entomology **94,** 1280-1284.

Zhu-Salzman, K., Luthe, D.S., and Felton, G.W. (2008). Arthropod-inducible proteins: Broad spectrum defenses against multiple herbivores. PLANT PHYSIOLOGY **146,** 852-858.

Zhu-Salzman, K., Shade, R.E., Koiwa, H., Salzman, R.A., Narasimhan, M., Bressan, R.A., Hasegawa, P.M., and Murdock, L.L. (1998). Carbohydrate binding and resistance to proteolysis control insecticidal activity of Griffonia simplicifolia lectin II. Proc Natl Acad Sci U S A **95,** 15123-15128.

Chapter II

Chen H*, Gonzales-Vigil E*, Wilkerson CG, Howe GA (2007) Stability of plant defense proteins in the gut of insect herbivores. Plant Physiol 143: 1954-1967

* These authors contributed equally to this work and are listed in alphabetical order.

Abstract

Plant defense against insect herbivores is mediated in part by enzymes that impair digestive processes in the insect gut. Little is known about the evolutionary origins of these enzymes, their distribution in the plant kingdom, or the mechanisms by which they act in the protease-rich environment of the animal digestive tract. One example of such an enzyme is threonine deaminase (TD), which in tomato (*Solanum lycopersicum*) serves a dual role in Ile biosynthesis *in planta* and Thr degradation in the insect midgut. Here, we report that tomato uses different TD isozymes to perform these functions. Whereas the constitutively expressed TD1 has a housekeeping role in Ile biosynthesis, expression of TD2 in leaves is activated by the jasmonate signaling pathway in response to herbivore attack. Ingestion of tomato foliage by specialist (*Manduca sexta*) and generalist (*Trichoplusia ni*) insect herbivores triggered proteolytic removal of TD2's C-terminal regulatory domain, resulting in an enzyme that degrades Thr without being inhibited through feedback by Ile. This processed form (pTD2) of TD2 accumulated to high levels in the insect midgut and feces (frass). Purified pTD2 exhibited biochemical properties that are consistent with a post-ingestive role in defense. Shotgun proteomic analysis of frass from tomato-reared *M. sexta* identified pTD2 as one of the most abundant proteins in the excrement. Among the other tomato proteins identified were several jasmonate-inducible proteins that have a known or proposed role in anti-insect defense. Subtilisin-like proteases and other pathogenesis-related proteins, as well as proteins of unknown function, were also catalogued. We conclude that proteomic analysis of frass from insect herbivores provides a robust experimental approach to identify hyperstable plant proteins that serve important roles in defense.

Introduction

The optimal growth of leaf-eating insects depends on their ability to acquire essential amino acids from dietary protein. The low protein content of plant tissue, however, poses a major nutritional challenge to phytophagous insects; protein is both the major macronutrient and the most commonly limiting nutrient for insect growth (Mattson, 1980; Bernays and Chapman, 1994). In addition to factors affecting protein quantity, evidence indicates that dietary protein *quality* also has a significant impact on plant-insect relations (Broadway and Duffey, 1988; Felton, 1996). Insect diets containing nutritionally unbalanced proteins pose a serious impediment to herbivory and may also influence patterns of host plant utilization among insect herbivores (Moran and Hamilton, 1980; Karowe and Martin, 1989; Haukioja et al., 1991; Berenbaum, 1995). The idea that variation in protein quality has evolved as a plant defense is supported by studies showing that certain classes of allelochemicals, such as tannins and phenolic resins, impair herbivore performance by interfering with the digestibility of dietary protein (Feeny, 1976; Rhoades, 1976).

Plants also produce defensive proteins that disrupt nutrient acquisition and other aspects of insect digestive physiology. Proteinase inhibitors (PIs) that impair the activity of digestive proteases are perhaps the best example of this type of post-ingestive defense (Green and Ryan, 1972; Ryan, 1990). Because PIs are not catalytic, their capacity to slow herbivore growth is dependent on accumulation to relatively high concentrations inside the gut lumen. Enzymes have the potential to exert defensive effects at much lower concentrations, but this hypothesis has received relatively little attention until recently (Duffey and Stout, 1996; Felton, 1996; Chen et al., 2005; Felton, 2005). Research on

midgut-active plant enzymes has focused mainly on polyphenol oxidase (PPO) and other oxidative enzymes that covalently modify dietary protein, thus reducing the digestibility of plant food (Constabel et al., 1995; Duffey and Stout, 1996; Felton, 1996; Wang and Constabel, 2004). Other defensive proteins directly target structural components of the insect digestive apparatus. Members of the cysteine protease family of enzymes, for example, are thought to disrupt the integrity of the peritrophic membrane that protects the gut epithelium (Pechan et al., 2002; Konno et al., 2004; Mohan et al., 2006). These collective studies indicate that enzymes play a pivotal role in host plant defense, and thus broaden the traditional view that secondary metabolites are the major determinants of host plant utilization and specialization (Fraenkel, 1959; Berenbaum, 1995).

Many plant anti-insect proteins are synthesized in response to wounding and herbivore attack. Induced expression of the vast majority of these proteins is regulated by the jasmonate signaling pathway (Walling, 2000; Gatehouse, 2002; Kessler and Baldwin, 2002; Howe, 2004; Schilmiller and Howe, 2005). Examples of jasmonate-inducible proteins (JIPs) that have a confirmed or proposed role in post-ingestive defense include PPO, arginase, leucine amino peptidase A (LAP-A), lipoxygenase, and a battery of PIs (Duffey and Felton, 1991; Felton et al., 1994; Constabel et al., 1995; Felton, 1996; Chen et al., 2005; Walling, 2006; Lison et al., 2006). A JA-inducible acid phosphatase (VSP2) in Arabidopsis was recently shown to exert insecticidal activity against coleopteran and dipteran insects (Liu et al., 2005). These observations indicate that a primary function of the jasmonate signaling pathway is to promote the expression of proteins that act post-ingestively to impair the growth and development of insect herbivores (Chen et al., 2005).

Biosynthetic threonine deaminase (TD) is a pyridoxal phosphate-dependent enzyme that converts L-threonine (Thr) to α-ketobutyrate and ammonia. Plant TDs function in the chloroplast to catalyze the committed step in the biosynthesis of Ile. The enzyme contains an N-terminal catalytic domain and a C-terminal regulatory domain and is subject to negative feedback regulation by Ile (Gallagher et al., 1998). The physiological importance of TD in plant growth and development was demonstrated by studies of TD-deficient mutants of *Nicotiana plumbaginifolia* and, more recently, *N. attenuata* (Sidorov et al., 1981; Colau et al., 1987; Kang et al., 2006). TD expression in leaves of several solanaceous plants is massively induced by the jasmonate signaling pathway in response to wounding and herbivory (Hildmann et al., 1992; Samach et al., 1995; Hermsmeier et al., 2001; Li et al., 2004). In contrast to this expression pattern, TD is constitutively expressed to high levels in reproductive organs (Hildmann et al., 1992; Kang and Baldwin, 2006). TD is reported to be the most abundant protein in tomato flowers (Samach et al., 1991). The high level of TD expression in reproductive tissues is similar to the expression pattern of PIs and other JIPs that impair insect growth.

Direct evidence for the hypothesis that TD has a role in anti-insect defense came initially from studies showing that the enzyme accumulates in the midgut of tomato-reared *Manduca sexta* larvae (Chen et al., 2005). TD activity in the midgut was correlated with reduced levels of free Thr, which is a dietary requirement for phytophagous insects. A jasmonate-insensitive mutant (*jai1*) of tomato that fails to express TD is more susceptible to attack by *M. sexta* larvae. Because this mutant is defective in all jasmonate-signaled processes, however, decreased resistance of *jai1* plants could not be linked directly to loss of TD function (Li et al., 2004; Chen et al., 2005). A recent study by Kang

et al. (2006) showed that mutants of *N. attenuata* engineered specifically for TD deficiency are compromised in resistance to *M. sexta* larvae. Supplementation of *N. attenuata* leaves with Thr led to increased larval performance, indicating that Thr availability in the leaf diet is limiting for larval growth. The Ile deficiency in TD-silenced *N. attenuata* plants also resulted in decreased production of jasmonoyl-Ile (JA-Ile), which is an important signal for induced defense responses to pathogens (Staswick et al., 1998) and insects (Kang et al., 2006). Thus, TD's defensive function in *N. attenuata* was attributed both to its involvement in JA-Ile synthesis and its role in post-ingestive defense (Kang et al., 2006).

TD's dual function in primary metabolism and defense makes it an attractive subject for research aimed at understanding the evolutionary origins of plant enzymes that exert toxic or antinutritional effects on insect herbivores. Here, we show that tomato has two *TD* genes (designated *TD1* and *TD2*) whose differential expression pattern is consistent with functional divergence of the two isoforms. Second, we show that ingestion of tomato foliage by specialist and generalist herbivores triggers proteolytic removal of the TD2 regulatory domain, resulting in an enzyme (pTD2) that effectively degrades Thr in the lepidopteran gut. Third, we show that the biochemical properties of pTD2 are consistent with a post-ingestive role in defense. Finally, we employed a "shotgun" proteomic approach to demonstrate that pTD2 is one of the most abundant proteins in frass from tomato-reared *M. sexta* larvae. Nineteen additional tomato proteins were cataloged in *M. sexta* frass. Among these were JIPs that have a known role in defense against insect herbivores, pathogenesis-related proteins, and proteins of unknown function. These findings provide new insight into the evolution of plant anti-insect

proteins and establish a robust experimental approach to identify hyperstable proteins that serve important roles in plant protection against biotic stress.

Results

Tomato has two *TD* genes that are differentially expressed

Previous studies of TD-encoding genes in tomato and potato have focused on a single orthologous gene whose expression in leaves is induced by various stress conditions including wounding and jasmonate treatment (Samach et al., 1991; Hildmann et al., 1992; Samach et al., 1995; Schaller et al., 1995; Strassner et al., 2002; Li et al., 2004). We previously reported that the *jai1* mutant of tomato, which is defective in all known jasmonate responses as a consequence of a null mutation in *Coi1*, fails to express this *TD* gene but nevertheless does not exhibit symptoms (e.g., stunted growth) of Ile deficiency (Li et al., 2004). This observation led us to test the hypothesis that tomato uses a different TD isozyme for Ile biosynthesis. Indeed, searches of the tomato EST database provided evidence for a second expressed *TD* gene. The corresponding full-length cDNA is predicted to encode a 606-amino-acid protein with a molecular mass of 66,182 Da (Figure 2.1A). The recombinant protein expressed in *E. coli* converted Thr to α-ketobutyrate in a manner that was inhibited by exogenous Ile (data not shown), indicating that the enzyme is an authentic TD. For reasons explained below, we henceforth refer to this previously uncharacterized gene as *SlTD1*, and refer to the JA-inducible gene initially described by Samach et al. (1991) as *SlTD2*.

SlTD1 and SlTD2 share 48% amino acid sequence identity (Figure 2.1A). Both proteins contain a predicted chloroplast-targeting sequence, as well as canonical catalytic

and regulatory domains found in other plant and bacterial TDs. Phylogenetic analysis showed that plant TD sequences cluster into two major groups (Groups 1 and 2; Figure 2.1B). SlTD1 was more similar to TDs from Arabidopsis (66% identity), poplar (68% identity), and rice (69% identity) than it was to SlTD2. Because Arabidopsis, poplar, and rice each contain a single *TD* gene, this finding supports the idea that SlTD1 performs a housekeeping role in Ile biosynthesis. JA-inducible isozymes from tomato (SlTD2) and potato (StTD2) comprised a distinct subgroup of proteins that, interestingly, were closely related to a TD sequence from chickpea. The JA-inducible TD from *N. attenuata*, which has a dual role in Ile biosynthesis and post-ingestive defense (Kang et al., 2006), occupied an intermediate position in the phylogeny and thus was not assigned to either group.

We used RNA blot analysis to compare the developmental and stress-induced expression patterns of *SlTD1* and *SlTD2*. *SlTD1* was constitutively expressed in all tissues examined (Figure 2.2A). In contrast, *SlTD2* transcripts accumulated to very high levels in immature buds and unopened flowers, but were not detected in unstressed leaves and other vegetative tissues. Expression of *SlTD2* in leaves was massively induced in response to methyl-JA (MeJA) application, as previously reported (Hildmann et al., 1992; Samach et al., 1995; Li et al., 2004) (Figure 2.2B). MeJA had little or no effect on *SlTD1* transcript levels. *SlTD2* expression was induced locally and systemically in response to mechanical wounding, whereas *SlTD1* mRNA levels were not affected by wounding (Figure 2. 2C). Taken together, these findings provide strong support for the hypothesis that *SlTD1* and *SlTD2* serve distinct physiological roles.

Digestion of bulk tomato leaf protein in the gut lumen of *M. sexta* larvae

As a prelude to studying the fate of TD2 in the *M. sexta* digestive system, we used SDS-polyacrylamide gel electrophoresis (PAGE) to qualitatively assess changes in bulk tomato leaf protein during passage through the insect. These studies were facilitated by analysis of caterpillars raised either on wild-type (WT) plants or on mutants that are affected in the JA signaling pathway. These mutants included a transgenic line (*35S::PS*) that constitutively expresses high levels of JA-inducible proteins (JIPs) (McGurl et al., 1994; Bergey et al., 1996), and the *jai1* mutant that fails to express TD2 and other JIPs (Li et al., 2004). A phenol-based protein extraction procedure was used to isolate total protein from three sources: 1) insect-damaged leaves; 2) midgut content from actively feeding larvae; and 3) larval feces (i.e., frass). The polypeptide profile of total leaf protein was much different from that of protein isolated from midgut content or frass (Figure 2.3A). One conspicuous difference was the large subunit (RbcL) of ribulose-1,5 bisphosphate carboxylase-oxygenase (Rubisco). As the most abundant soluble protein in tomato leaves, RbcL is a major source of amino acids for phytophagous insects and a convenient marker for bulk leaf protein (Sheen, 1991; Bernays and Chapman, 1994; RF, 1994; Felton, 1996). In contrast to the high level of RbcL in herbivore-damaged leaves, midgut and frass contained very little intact RbcL. Efficient digestion of chloroplast proteins within the midgut was confirmed by western blot analysis with antibodies against the chloroplast outer envelope protein Toc75 (Figure 2.3B)(Reumann et al., 2005). We also found that a peroxisomal matrix protein, acyl-CoA oxidase1A (Acx1A), accumulated in leaves but not in the midgut content or frass (Figure 2.3C). These results

demonstrate that bulk tomato leaf protein is efficiently degraded during passage through the *M. sexta* digestive system.

Numerous discretely sized polypeptides exhibiting a wide range of molecular weights were present in frass extracts (Figure 2.3A). The polypeptide profile of frass from larvae reared on the various genotypes exhibited several reproducible differences. For example, frass extracts from larvae grown on *jai1* plants contained more discretely sized, higher-molecular-weight polypeptides in comparison to the WT and *35S::PS* frass samples (Figure 2.3A). A second host genotype-specific difference was a protein of ~40-kDa that accumulated in WT and *35S::PS* frass but not in frass from *jai1*-reared larvae (Figure 2.3A, arrow). Differential accumulation of this protein was also observed in the midgut content, but not in herbivore-damaged leaves. This pattern of accumulation suggests that the 40-kDa polypeptide is a JIP that is stable in the *M. sexta* gut.

Proteolytic processing of TD2 in the lepidopteran gut

We previously reported mass spectrometry evidence indicating that a form of TD2 lacking the enzyme's regulatory domain accumulates in *M. sexta* midgut content and frass (Chen et al., 2005). The predicted size of this TD2 variant was consistent with its identity as the above-mentioned 40-kDa protein (Figure 2.3A). To test this hypothesis, a gel slice containing the 40-kDa protein was digested with trypsin and the resulting peptides were sequenced by LC-MS/MS. Among 18 unique peptides that were confidently identified ($P<0.05$), all showed an exact match to the catalytic domain of TD2 (Figure 2.4A). The predicted molecular weight of the protein defined by LC-MS/MS

(Met52 to K418) was 38,890 Da, which was in good agreement with the size observed by SDS-PAGE.

Mature TD2 isolated from tomato tissues has an apparent molecular mass of 55 kDa (Samach et al., 1991; Samach et al., 1995). We used western blot analysis to determine whether there is a product-precursor relationship between the truncated TD2 variant, designated pTD2, and the 55-kDa protein. Anti-TD2 antibodies cross-reacted with a ~55-kDa protein in herbivore-damaged WT and *35S::PS* leaves (Figure 2.4B). The absence of this polypeptide in *jai1* leaves, in which TD2 is not expressed, confirmed the specificity of the antibody for TD2. In contrast to leaf tissue, pTD2 was the predominant form of the protein in midgut and frass extracts. These results indicate that TD2 is proteolytically processed to pTD2 following ingestion of foliage by *M. sexta*. A small amount of unprocessed TD2 in midgut extracts was observed upon prolonged development of western blots (Figure 2.4B, asterisks). Shorter exposure times showed that pTD2 migrates as a doublet, suggesting heterogeneity in the size of the processed protein.

M. sexta is highly specialized for feeding on tomato and other solanaceous plants. To determine whether proteolytic processing of TD2 occurs in the gut lumen of a generalist herbivore, we analyzed the TD2 content in frass from *Trichoplusia ni* (cabbage looper) caterpillars that were raised on tomato foliage. Western blot analysis showed that *T. ni* frass contained a form of TD2 that co-migrated with pTD2 from *M. sexta* frass (Figure 2.4C). The absence of this polypeptide in frass from *jai1*-reared *T. ni* larvae confirmed that the cross-reacting protein is derived from TD2. TD activity was detected in frass from *T. ni* larvae grown on WT plants (Figure 2.4D). Consistent with a

processing event that removes the regulatory domain, this activity was insensitive to feedback inhibition by 10 mM Ile. We conclude that ingestion of tomato foliage by both specialist (*M. sexta*) and generalist (*T. ni*) insect herbivores results in proteolytic removal of the regulatory domain of TD2.

Biochemical properties of pTD2

To investigate the biochemical properties of pTD2 in more detail, we purified the enzyme from *M. sexta* frass. An aqueous buffer system effectively extracted active pTD2 from frass (Figure 2.5A). The 40-kDa polypeptide co-purified with Ile-insensitive TD activity during subsequent purification steps (data not shown). Following the final stage of purification by gel filtration chromatography, we estimated that pTD2 was at least 90% pure as determined by SDS-PAGE (Figure 2.5B). The purified enzyme was active against L-Thr and L-Ser. Kinetic analysis showed that the apparent *K*m of L-Thr and L-Ser was 2.3 and 3.0 mM, respectively. The Vmax for L-Thr was ~5000 μmol/mg protein/hr, which was about 1.5 times higher than the Vmax for L-Ser. The enzyme was highly active in an alkaline pH range that matches that of the lepidopteran midgut; little or no activity was observed at pH values below 6.0 (Figure 2.6A). pTD2 was also active over a wide range of temperatures. Optimal enzyme activity against L-Thr was observed at 58°C (Figure 2.6B).

Identification of plant defensive proteins by shotgun proteomic analysis of insect frass

Excretion of pTD2 as an active enzyme from *M. sexta* and *T. ni* led us to hypothesize that insect frass may be a useful source of material in which to identify other defense-related proteins. To test this idea, we used a shotgun proteomic approach to catalogue and quantify tomato proteins in frass from *M. sexta* caterpillars reared on tomato foliage. The total protein content of frass was digested with trypsin and the resulting peptide mixture was subjected to LC-MS/MS. Protein identifications were considered positive if at least two peptides derived from the same protein were confidently detected in searches of the MS/MS data against the tomato EST database. These stringent criteria resulted in identification of 20 distinct tomato proteins with probability scores of $P<10^{-4}$ (Table 2.1).

Wound- and jasmonate-inducible proteins comprised the largest group of tomato proteins in *M. sexta* frass (Table 2.1). Among the proteins previously implicated in defense against lepidopteran insects were TD2, LAP-A (Gu et al., 1999; Chen et al., 2005), cathepsin D inhibitor (CDI) (Lison et al., 2006), and a germin-like protein (GLP) similar to a GLP isozyme from *N. attenuata* (Lou and Baldwin, 2006). Two stress-inducible proteins of unknown function were also identified. One of these is a member of a plant-specific group of stress-related proteins that contain a lipoxygenase homology (LH) domain (Coker et al., 2005). The second uncharacterized protein is a chloroplast-targeted member of the highly conserved YjgF family of proteins (Leitner-Dagan et al., 2006). We previously showed that the gene encoding this protein, which is annotated in the tomato EST database as a protein translation inhibitor, is regulated by the jasmonate signaling pathway (Li et al., 2004).

Proteins implicated in plant defense against pathogens were also identified in *M. sexta* frass (Table 2.1). Among the pathogenesis-related (PR) proteins identified were

the P69A and B members of the subtilisin-like family of endoproteases (PR-7) (Tornero et al., 1996, 1997), β-1,3-glucanase (PR-2) (Domingo et al., 1994), lignin-forming peroxidase (PR-9) (Vera et al., 1993), and a hevein-like protein P2 (PR-4) (Linthorst et al., 1991). A xyloglucan-specific fungal endoglucanase inhibitor protein previously reported from tomato, potato, and tobacco (Naqvi et al., 2005) was also identified. All of these proteins contain an N-terminal signal peptide for targeting to the secretory pathway (Table 2.1), and most have been shown to be expressed in response to pathogen infection or wounding.

All other tomato proteins identified in *M. sexta* frass, with the exception of mitochondrial malate dehydrogenase, were chloroplastic metalloproteins (Table 2.1). These included plastocyanin, ferredoxin, superoxide dismutase, and carbonic anhydrase. Given that TD2, LAP-A, and the YjgF-related protein (see above) are also plastid-localized, it would appear that chloroplast proteins are highly represented in the frass. The failure to identify peptides corresponding to Rubisco in this experiment argues against the possibilty that this phenomenon results from passage of the intact plastids through the insect digestive tract.

The number of mass spectral counts obtained for a given protein by LC-MS/MS provides a quantitative measure of the protein's abundance in the extract (Old et al., 2005; Gilchrist et al., 2006). Based on this information, pTD2 and LAP-A were among the most abundant tomato proteins in the frass (Table 2.1). LAP activity assays were performed to determine whether LAP-A, like pTD2, is excreted as an active enzyme. Both LAP and TD activity was detected in frass from larvae grown on WT plants (Figure 2.7). The lack of activity in frass from *jai1*-reared larvae indicated that the activity was

specific for the JA-inducible isozymes LAP-A and pTD2. LAP activity in frass from insects reared on *35S::PS* plants was significantly greater than that in the WT frass, which is consistent with the fact that LAP-A expression in tomato foliage is upregulated by systemin (Chao et al., 1999). These findings indicate that LAP-A, like TD2, is excreted from *M. sexta* as an active enzyme.

Discussion

Functional diversification of two TD isoforms in tomato

The role of TD in producing Ile for protein synthesis is essential for all aspects of plant growth and development. Ile is also required for the synthesis of JA-Ile, which is an important signal for activation of jasmonate-based defenses (Staswick et al., 1998; Kang et al., 2006). The broad distribution of jasmonates in the plant kingdom indicates that TD's participation in JA-Ile synthesis is likely conserved in all plants. In contrast, TD's function as a post-ingestive defense against insect herbivores appears to be restricted to certain plant lineages. Here, we provide evidence that tomato employs different TD isozymes to fulfill distinct roles in Ile biosynthesis *in planta* and post-ingestive defense. This contrasts the situation in native tobacco, which uses a single TD isoform to perform both functions (Kang et al., 2006).

Several observations lead us to conclude that SlTD1 performs a role in Ile biosynthesis. First, the deduced amino acid sequence of SlTD1 is more similar to TDs in plants such as Arabidopsis, rice, and poplar, which all harbor a single "housekeeping" form of the enzyme, than it is to SlTD2. Second, constitutive expression of *TD1* in all tissues is consistent with a general role in amino acid biosynthesis. Third, recombinant

TD1 expressed in *E. coli* exhibits Ile-sensitive TD activity. Finally, the *jai1-1* mutant, which lacks detectable TD2 expression in leaves (Figure 2.4B), does not exhibit chlorosis or other signs of Ile deficiency (Li et al., 2004). This finding provides functional evidence that TD1 can produce Ile pools that are utilized for normal growth and development in the absence of TD2.

A specialized role for TD2 in post-ingestive defense is supported by the fact that this isozyme accumulates in the midgut and frass of tomato-reared caterpillars (Figures 2.3 and 2.4). The gut-accumulating form of the enzyme (i.e., pTD2) has biochemical features that presumably facilitate its action in the midgut environment. These features include protease-resistance, an alkaline pH optimum, and the capacity to degrade Thr in the presence of high concentrations of Ile. The high temperature optimum of pTD2 indicates that the enzyme would be active at elevated body temperatures, which for *M. sexta* caterpillars in natural field conditions can easily exceed 35°C (Casey, 1976). The expression pattern of TD2 also supports a role in anti-insect defense. *TD2* is coordinately induced with other defensive genes in response to wounding and JA treatment (Hildmann et al., 1992; Samach et al., 1995; Li et al., 2004). In reproductive tissues, TD2 is expressed constitutively at extraordinarily high levels (Samach et al., 1991; Samach et al., 1995). Many other JA-regulated defensive proteins including PIs, arginase, LAP-A, and AtVSP2 exhibit a similar expression pattern (Hildmann et al., 1992; Utsugi et al., 1998; Chao et al., 1999; Chen et al., 2004). These observations support the idea that accumulation of TD2 and other JIPs in floral tissues protects reproductive structures from insect herbivores. Induction of TD2 expression by diverse types of biotic and abiotic stress (Hildmann et al., 1992; Zhao et al., 2003) raises the possibility that the enzyme

performs other physiological roles *in planta*. For example, it is possible that a proteolytically processed form of TD2 accounts for the biodegrative TD activity observed in senescing tomato leaves (Szamosi et al., 1993).

Functional divergence of two TD isozymes in tomato raises interesting questions about the evolutionary origins of plant TD's that participate in post-ingestive defense. It is reasonable to assume that SlTD2 arose from a gene duplication event, and that selective pressure imposed by insect herbivores led to the evolution of this isoform as a defensive enzyme. A key feature acquired by both SlTD2 and *N. attenuata* TD during evolution was regulation via the jasmonate signaling pathway. Whether or not these enzymes evolved novel biochemical or structural features that enhance their ability to impair insect digestive physiology is unclear. Future studies aimed at comparing the structure, stability, and activity of SlTD1 and SlTD2 promise to provide insight into this question. The dual role of *N. attenuata* TD in Ile synthesis and post-ingestive defense (Kang et al., 2006) is consistent with the intermediate position of this protein in the TD phylogenetic tree (Figure 2.1A). The evolution of *N. attenuata* TD as a midgut-active enzyme may be constrained, however, by its essential role in Ile biosynthesis. Tomato TD2 is presumably not subjected to such constraint, and thus may be better adapted to function in the lepidopteran gut.

Proteolytic processing of TD2

Our results confirm and extend previous evidence (Chen et al., 2005) indicating that TD2 is proteolytically processed following ingestion of foliage by the herbivore. LC-MS/MS and other biochemical data demonstrated that the midgut-active form of the enzyme

(pTD2) contains the entire catalytic domain, but lacks the C-terminal regulatory domain. That very little unprocessed TD2 was observed in midgut content suggests that the processing reaction occurs rapidly upon maceration of leaf tissue by the caterpillar. Based on the structural organization of TD2 into distinct catalytic and regulatory domains, we propose that processing involves an endoprotease that cleaves the neck region between the two domains. Additional work is needed to test this hypothesis and to determine whether the processing enzyme is of plant or insect origin. The finding that TD2 is processed to an Ile-insensitive enzyme in the digestive tract of the generalist caterpillar *T. ni* indicates that the processing phenomenon likely occurs in a broad range of tomato-insect interactions.

An important consequence of proteolytic removal of the regulatory domain is loss of feedback inhibition by Ile. The midgut content of *M. sexta* larvae reared on tomato plants contains levels of Ile (~2.5 mM) that are sufficient to inhibit TD2 activity (HC and GAH, unpublished data). Thus, proteolytic cleavage of TD2 is required to activate the enzyme's ability to degrade Thr in the amino acid-rich environment of the midgut. This interpretation is consistent with the fact that the Thr content in the midgut of *M. sexta* larvae reared on TD2-containing tomato foliage is much less than that in the gut of insects grown on TD2-deficient foliage (Chen et al., 2005). Given that dietary Thr is limiting for *M. sexta* growth on native tobacco (Kang et al., 2006), these results strongly support the notion that post-ingestive processing of TD2 has evolved as a plant defense to deplete Thr levels in the midgut. It is also possible that the defensive function of pTD2 is related to its ability to produce ammonia, which at alkaline pH is highly toxic to biological systems (Visek et al., 1984).

Herbivore-induced processing of TD2 provides support for the more general concept that proteolysis of dietary protein is part of the plant's overall defense response against insect attack. Other examples of plant defensive proteins that are activated by digestive proteases include polyphenol oxidase (Wang and Constabel, 2004) and urease (Ferreira-DaSilva et al., 2000). Schmelz and co-workers (Schmelz et al., 2006) identified a peptide elicitor from the oral secretion of insect herbivores that promotes the expression of plant defense responses. Interestingly, this elicitor is a proteolytic fragment of the γ-subunit of chloroplastic ATP synthase. It was thus proposed that proteolysis of dietary proteins by insect digestive proteases can generate peptide signals that are introduced to the host plant via insect oral secretions. The accumulation of tomato LAP-A and P69 proteins in *M. sexta* frass leads us to suggest that plant proteases, in addition to insect proteases, play a role in the digestion of dietary protein in the insect gut.

Shotgun proteomic analysis of insect frass

Anal droppings of insect herbivores are a rich repository of biological information (Gangwere, 1993; Weiss, 2006). It is well established, for example, that frass is an important source of compounds involved in host selection by insect parasitoids (Vinson, 1976). To our knowledge, the composition of plant proteins excreted by insect herbivores has not been previously described. The proteomic analysis reported herein shows that insect frass is enriched in pTD2 and other hyperstable plant proteins that serve defense-related functions. Many of these proteins were previously shown to accumulate in the *M. sexta* midgut (Chen et al., 2005) and, significantly, have an established role in anti-insect defense. CDI is a Ser PI (rather than an Asp PI) that exerts potent growth-inhibiting

effects on lepidopteran caterpillars (Lison et al., 2006). The frass-accumulating GLP is closely related to a MeJA-inducible GLP from *N. attenuata* that has a role in resistance to *M. sexta* attack (Lou and Baldwin, 2006). Detection of this protein in frass raises the possibility that GLPs exert defensive effects (e.g., H_2O_2 production) in the herbivore gut. A role for LAP-A in post-ingestive defense is supported by the stability of the protein in the lepidopteran digestive tract (Chen et al., 2005; this study), the enzyme's high pH optimum (Gu et al., 1999), co-expression with other midgut-active defensive proteins (Li et al., 2004; Chen et al., 2005), and the increased susceptibility of *LapA*-silenced plants to herbivory (Walling, 2006). There is also evidence indicating that LAP-A performs a signaling role in jasmonate-induced expression of defensive proteins (Walling, 2006).

Shotgun proteomic analysis also identified proteins that had not previously been implicated in plant defense. These included stress-inducible isoforms of an LH2 domain protein that may participate in lipid metabolism (Coker et al., 2005), and a member of the YjgF family of proteins that is conserved in bacteria, yeast, animals, and plants. In the context of TD function, it is noteworthy that YjgF and related proteins have been implicated in the regulation of Ile biosynthesis and Thr degradation (Datta et al., 1987; Kim et al., 2001; Parsons et al., 2003). A recent study (Leitner-Dagan et al., 2006) showed that the YjgF-related tomato protein accumulates in chloroplasts of stressed leaves, and is required for optimal photosynthetic function. Additional work is needed to test the hypothesis that this protein has a role in defense against insects.

The expression of many tomato proteins identified in *M. sexta* frass is promoted by the jasmonate signaling pathway. Genes encoding these JIPs tend to be among the most highly induced following wounding or jasmonate treatment. For example, a DNA

microarray study identified *TD2* and *LapA* as the most highly expressed JA-responsive genes among all elements on the array (Li et al., 2004), whereas proteomic analysis showed that TD2 and LAP-A are two of the most abundant tomato proteins in the midgut and frass of tomato-reared *M. sexta* larvae (Chen et al., 2005; this study). Thus, there is a strong correlation between the level of induced mRNA accumulation in leaves and protein accumulation in the insect gut. Similar correlations hold for the YjgF-related protein, CDI, and other PIs. We suggest that jasmonate-induced accumulation of defensive proteins in leaves, together with the stability of these proteins in the gut lumen, provide complementary mechanisms to maximize the effectiveness of post-ingestive plant defense. The correlation between gene and protein expression suggests that microarray data can be used as a starting point to identify novel anti-insect proteins.

Several tomato PR proteins were excreted in *M. sexta* frass. Nearly all of these proteins have been shown to be highly expressed in response to pathogen infection or wounding, and secreted into the extracellular space where they presumably interact directly with invading pathogens (van Loon et al., 2006). The biological significance of PR protein accumulation in frass is unclear. It is possible that these proteins accumulate in frass simply because they are highly resistant to proteolysis. This idea is supported by studies showing that PR proteins are extremely stable (Ferreira et al., 2001; Flamini and De Rosso, 2006; van Loon et al., 2006). It is also possible that PR proteins perform a physiological role in the digestive system of insect herbivores. The alkaline pH optimum of subtilisin-like P69 proteases (Vera and Conejero, 1988), which appear to be the most abundant PR proteins in *M. sexta* frass, indicates that these extracellular proteases may be activated upon entry of macerated leaf tissue into the lepidopteran gut. Studies of cysteine

proteases establish a precedent for the role of plant proteases in post-ingestive defense against insect herbivores (Pechan et al., 2002; Konno et al., 2004).

An important conclusion from this and previous (Chen et al., 2005) work is that foliar proteins have a wide range of stability in the gut lumen of phytophagous insects. Whereas bulk dietary protein (e.g., Rubisco) is efficiently degraded in the *M. sexta* midgut, other plastidic proteins such as TD2 and LAP-A remain active following passage through the gut. The most straightforward interpretation of these results is that midgut-active defensive proteins are highly resistant to digestive proteases and, as a consequence, are selectively enriched during passage of the food bolus through the animal. The biophysical properties that allow pTD2 and LAP-A to accumulate and function in the extreme environment of the lepidopteran gut remain to be determined. In this context, it is worth noting that hyperstable (as well as alkaliphilic) enzymes are of significant commercial interest for their use as industrial biocatalysts (Hough and Danson, 1999). Although research in this area has focused mainly on extremophilic bacteria and Archaea, our results suggest that frass-accumulating plant proteins can be exploited as a new source of hyperstable enzymes.

Seminal work by Green and Ryan (1972) introduced the idea that wound-inducible plant proteins act directly in the insect gut as a defense. The recent discovery of TD, arginase, VSP, and proteases as anti-insect proteins extends this concept to include plant enzymes that impair digestive physiology (Pechan et al., 2002; Chen et al., 2005; Liu et al., 2005; Kang et al., 2006). Proteomic-based technologies provide a powerful tool to address the question of how variation in the quantity and quality of dietary protein influences plant-insect relations. Only recently have these approaches been used to assess

the effects of herbivory on large-scale changes in plant protein content (Francis et al., 2006; Giri et al., 2006; Lippert et al., 2007). Our results demonstrate that proteomic analysis of midgut content (Chen et al., 2005) and frass (this study) can be used to track the fate of the plant proteome during passage through the insect digestive tract, thus providing insight into how the plant proteome interacts with components of the insect gut. Additional work is needed to determine the limitations of this approach for identifying midgut-active plant defense proteins. For example, it conceivable that some defensive proteins are degraded by microbial flora in the insect gut or frass, or that covalent modification of dietary polypeptides in the insect gut (Felton, 1996) prevents protein identification by MS. These limitations notwithstanding, we conclude that proteomic analysis of frass has general utility for large-scale identification of plant defensive proteins in virtually any plant-insect interaction for which appropriate sequence databases are available.

Materials and Methods

Biological material and growth conditions

Solanum lycopersicum cv Castlemart was used as the wild type (WT) for all experiments except where otherwise noted. *35S::PS* and *jai1* mutant lines and conditions for plant growth were previously described (Chen et al., 2005). *M. sexta* eggs were obtained from the Department of Entomology, North Carolina State University (Raleigh, NC). Newly hatched larvae were transferred directly to three-week-old tomato plants. Larval midguts were dissected from cold-anesthetized larvae (4^{th} - 5^{th} instar) that were actively feeding

at the time of collection. Total midgut content was isolated by removing the food bolus from the dissected midgut. Care was taken to avoid mixing the midgut content with insect tissue. Midgut content from three to five larvae was pooled and frozen at -20°C until further use for protein extraction. For collection of *M. sexta* frass, 3rd to 4th instar larvae were transferred to a Tupperware box containing cut leaves from ~6-week-old tomato plants that were heavily damaged by *M. sexta* feeding. The petiole of the cut leaf was inserted through the closed cap (in which a hole was punctured) of a 1.5 ml plastic microcentrifuge tube containing water. Cut leaves were replaced on a daily basis. Frass was collected at least once daily, and stored at -20°C until needed for protein extraction. The use of host genotypes that are non-inducible (*jai1*) or constitutively induced (*35S::PS*) helped to control for possible effects of wounds that were generated by leaf cutting. Care was taken to avoid contamination of frass with intact leaf tissue.

T. ni eggs were obtained from Benzon Research (Carlisle, PA) and hatched at 30°C. Within 8 h of hatching, larvae were transferred to three-week-old tomato plants. Frass pellets were collected daily from 4th to 5th instar larvae grown on cut tomato leaves. Leaves were replaced daily. Pellets were stored at -20°C until further use.

Protein extraction and enzyme assays

A modified version of a phenol-based protein extraction method (Constabel et al., 1995) was used to isolate total protein for SDS-PAGE (Figure 2. 3A) and immunoblot analysis. Frozen leaf tissue, midgut content, and frass were ground in liquid nitrogen to fine powder. One volume equivalent of the ground tissue was mixed with two volumes of protein extraction buffer [0.7 M sucrose, 100 mM Tris-HCl (pH 6.8), 20 mM EDTA, 100

mM KCl, 2% (v/v) 2-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride]. Following the final protein precipitation step (Constabel et al., 1995), protein pellets were resuspended in solution containing 9.5 M urea, 2% (v/v) Nonidet detergent, and 5% 2-mercaptoethanol. Samples were heated to 65°C for 5 min prior to SDS-PAGE.

An aqueous buffer system (Chen et al., 2005) was used to prepare frass extracts for TD and LAP enzyme assays, purification of pTD2, and shotgun proteomic analysis. TD activity measurements in the presence or absence of Ile were as previously described (Chen et al., 2005). LAP activity was measured as previously described (Gu et al., 1999; Nampoothiri et al., 2005) with some modifications. Briefly, the reaction mixture contained 1 ml of a 2.5-mM solution of L-leucine-*p*-nitroanilide substrate in 100 mM NaOH-glycine buffer (pH 8.5), 1 ml 0.5 mM $MnCl_2$ in 100 mM NaOH-glycine buffer (pH 8.5), and 0.5 ml H_2O. Reactions were initiated by the addition of 5 µl of protein extract prepared from frass. Following 30 min incubation at 37°C, the reaction was stopped by the addition of 1 ml glacial acetic acid. The absorbance was measured at 405 nm against a mock reaction devoid of enzyme. A standard curve was prepared with *p*-nitroaniline. L-leucine-*p*-nitroanilide and *p*-nitroaniline were purchased from Sigma (St. Louis, MO).

Purification of pTD2 from *M. sexta* frass

Frass obtained from tomato-reared *M. sexta* larvae was ground in liquid nitrogen to a fine powder. Ten g of powder was extracted with approximately 2 volumes of 100 mM Tris buffer (pH 7.5) containing 1 mM EDTA, 1% (vol/vol) 2-mercaptoethanol, and 0.1 mM PMSF. The mixture was centrifuged at 20,000*g* for 10 min and the resulting supernatant

was filtered through a 0.45-μm filter (Millipore). Protein in the supernatant was brought to 30% (w/v) saturation with ammonium sulfate and stirred for 2 h at 4°C. Precipitated proteins were discarded following centrifugation at 20,000*g* for 15 min. The supernatant was adjusted to 65% saturation with ammonium sulfate and stirred for 4 h at 4°C. Following centrifugation at 20,000*g* for 15 min, the supernatant was discarded. The protein precipitate was dissolved in 15 mM Tris buffer (pH 7.5) and then desalted on a Sephadex G-25 column (Amersham Pharmacia Biosciences) that was equilibrated with the same buffer. The desalted extract was applied to a DEAE-cellulose (DE52; Maidstone, England) column (30 x 1.5 cm i.d.) that was equilibrated with the same buffer. Proteins were eluted from the column with a linear gradient of 0 to 0.5 M NaCl in 15 mM Tris·HCl (pH 7.5). Fractions (1.5 ml) were collected with a Gilson fraction collector (model FC-203B; Middletown, WI). Fractions containing the bulk of TD activity (~2/3 of the activity peak height) were pooled and concentrated with a 10-kDa molecular weight cut-off Amicon centrifugal filter (Millipore, Bedford, MA). Concentrated enzyme preparation (0.2 ml) was loaded on a Superose-12 gel filtration column (Pharmacia, Piscataway, NJ) that was pre-equilibrated with 50 mM Tris-HCl (pH 7.5) containing 100 mM NaCl. Proteins were eluted with the same buffer at a flow rate of 0.6 ml/min on a Waters HPLC system equipped with a model 600 pump, a 996 photodiode array detector, and a 717-plus autosampler. Fractions (1.0 ml) were collected manually and assayed for TD activity. The specific activity of TD increased ~30-fold during the purification procedure. Protein concentrations were determined by the Bradford method, using bovine serum albumin as a standard. The relative purity of protein samples was assessed by SDS-polyacrylamide gel electrophoresis and staining of

gels with Coomassie Brilliant Blue R-250. A Superose-12 gel filtration column equilibrated with a 50 mM Tris-HCl (pH 7.5) solution containing 100 mM NaCl was used to estimate the native molecular weight of purified pTD2.

Cloning and expression analysis of *SlTD1* and *SlTD2*

A search of the tomato EST database (version 11.0, released on June 21, 2006) at The Gene Index Project (http://compbio.dfci.harvard.edu/tgi/plant.html) identified a tentative consensus sequence (TC176654) annotated as a *TD*. This sequence, which we designated as *SlTD1*, was distinct from the published tomato TD sequence (Samach et al., 1991). Three cDNA clones (cTOF22A12, cTOC4022, and cTOD22M4) corresponding to *SlTD1* were obtained from the Boyce Thompson Institute and sequenced in their entirety. Overlapping regions between the clones showed that the three cDNAs corresponded to the same transcript. The assembled full-length cDNA sequence of *SlTD1*, deposited in GenBank as accession number EF026088, has an 1821-bp open reading frame, a 91-bp 5' UTR upstream of the ATG initiation codon, and a 235-bp 3' UTR excluding poly(A) residues.

A full-length *SlTD2* cDNA was obtained by RT-PCR (DuraScript™, Sigma) of total RNA isolated from leaves of tomato plants (cv Castlemart) that were treated with MeJA for 24 h. The PCR primers for the cDNA amplification step were TD5 (forward) 5'-ATGGAATTCCTTTGTTTAGCCCCA-3' and TD3-2 (reverse) 5'-GCCATTACATTACATTGGATACAT-3'. The resulting PCR product was cloned into the pGEM-T Easy vector (Promega) to yield pGEM-*SlTD2*. The sequence of the cDNA insert perfectly matched that of the *TD* sequence reported by Samach et al. (1991).

RNA blot experiments were performed with total RNA isolated from WT tomato plants (cv Micro-Tom), as previously described (Howe et al., 2000). Roots, stems, petioles, and leaves were collected from 3-week-old plants. Floral tissues were harvested from six-week-old plants. Wound and MeJA treatments were performed according to published methods (Howe et al., 2000; Li and Howe, 2001). RNA blots were probed with ^{32}P-labeled *SlTD1* and *SlTD2* cDNAs, or with a cDNA for *eIF4A* as a loading control.

Antibody production and western-blot analysis

The pET30TD plasmid for expression of AtTD was kindly provided by Dr. Renaud Dumas (Wessel et al., 2000). This vector, which is derived from pET30a+, was digested with *Nde*I and *Sal*I to release the *AtTD* cDNA. The resulting linearized vector was ligated to a PCR product containing a modified *SlTD2* cDNA. This cDNA was prepared by PCR amplification of pGEM-*SlTD2* with the following primer sets. The forward primer (5'-TGATTAATATGATGTCACCAATTGTTTCTGTG-3') was designed with an *Ase*I restriction site that is compatible with the *Nde*I site on the vector. The underlined ATG sequence in the forward primer represents the initiation codon in the resulting recombinant protein. This ATG codon replaces the first amino acid (Lys52) of the mature protein, thus eliminating the 51-amino-acid chloroplast-targeting sequence (Wessel et al., 2000). The reverse primer was designed with a TGA stop codon upstream of the *Sal*I site (5'-ATGTCGACTCACTCACTTACTACAAGGAA-3'). The amplified PCR product was cloned into the pET30 vector (described above) to yield a plasmid called pET30-TD2. This expression vector produces a truncated form of SlTD2 that lacks 51 amino acids corresponding to the N-terminal chloroplast-targeting sequence.

TD2 expression and purification were performed as described previously (Wessel et al., 2000) with minor modifications. A 750-mL log-phase culture of *E.coli* BL21 (DE3) cells containing the pET30-TD2 plasmid grown at 37°C was induced by the addition of IPTG to a final concentration of 0.5 mM. The induced cells were incubated with agitation at 28°C for 15 h, and then harvested by centrifugation at 20,000*g* for 15 min. The cell pellet was resuspended in buffer A [50 mM Hepes (pH 7.5) and 1 mM EDTA] containing 0.1 mM PMSF and 1 mM dithiothreitol (DTT). The cell suspension was treated with lysozyme (1mg/mL) for 30 min at 30°C, followed by sonication. Cell debris was removed by centrifugation at 20,000*g* for 15 min and the supernatant (crude lysate) was saved. The crude lysate was brought to 30% (w/v) saturation with ammonium sulfate and stirred for 1 h at 4°C. The precipitated proteins were discarded, and the solution was adjusted to 65% saturation with ammonium sulfate. The precipitate was collected by centrifugation at 20,000*g* for 15 min, resuspended in buffer A, and applied to a Sephadex G-25 column, and eluted with buffer A. The eluate was collected and loaded onto a Whatman DEAE-cellulose DE52 column (30 x 1.5 cm) equilibrated with buffer A. The column was eluted with 600 mL of a 0-400 mM KCl gradient. Elution was monitored by absorbance at 280 nm and enzyme activity. Fractions containing TD activity were pooled, concentrated with an Amicon Ultra-15 30-kDa filter (Millipore), and subjected to further purification on a Pharmacia Biotech ÄKTA FPLC system. Specifically, the concentrate was applied to a HiLoad26/60 Superdex 200 column (Amersham Pharmacia Biotech) previously equilibrated with buffer A. The column was eluted with 1.5 column volumes of buffer A containing 150 mM KCl. Fractions containing TD activity were pooled and loaded directly into a HiPrep 16/10 column

(Pharmacia Biotech) equilibrated in buffer A. The column was eluted with 20 column volumes of a 0-400 mM KCl gradient. The enzyme was concentrated with an Amicon Ultra-15 30-kDa filter to a concentration of 4.5 mg/mL. The purified enzyme was determined to be >95% pure as determined by SDS-PAGE. Rabbit polyclonal antibodies against purified TD2 antigen were produced by a commercial vendor (Cocalico Biologicals, Reamstown, PA) according to their standard protocol, using 0.5 mg of the purified protein as antigen.

Western blot analysis was performed as previously described (Schilmiller et al., 2007), using anti-SlTD2 antibodies that were diluted 1:2000 in TTBS (Tris-buffered saline with 0.1% Tween 20) containing 1% nonfat milk. Blots were washed 3 times with TTBS and then incubated with a peroxidase-conjugated anti-rabbit secondary antibody (1:10,000 dilution; Sigma, St. Louis, MO). TD2 protein-antibody complexes were visualized with the SuperSignal West Pico Chemiluminescent substrate (Pierce, Rockford, IL) according to the manufacturer's instructions. Anti-Acx1A antibodies (Schilmiller et al., 2007) were used at a 1:1,000 dilution. Toc75 antibodies (Tranel et al., 1995) were used at a 1:2,000 dilution.

LC-MS/MS-based identification of tomato proteins in *M. sexta* frass

Frass was collected from *M. sexta* larvae (3^{rd} to 4^{th} instar) that were grown on WT (cv Castlemart) tomato plants as described above. Frass was frozen in liquid nitrogen, ground to a fine powder, and extracted with 100 mM Tris buffer (pH 7.5) containing 1 mM EDTA, 1% (v/v) β-mercaptoethanol, and 0.1 mM PMSF. Extracts were centrifuged at 20,000*g* for 10 min at 4°C. Approximately 60 μg protein was electrophoresed through a

4% SDS-polyacrylamide stacking gel (1.5 cm) and ~1 cm into a 12% resolving gel. Gels were stained with Coomassie Blue and the protein-stained region of the gel was excised. Proteins within the gel piece were reduced and alkylated, followed by digestion with trypsin as previously described (Chen et al., 2005).

The extracted peptides were automatically injected by a Michrom Paradigm Endurance Bio-Cool Autosampler onto a Paradigm Platinum Peptide Nanotrap (C18, 0.15 x 50 mm) and washed for 5 min. The bound peptides were eluted onto a 10 cm x 75 μm New Objectives Picofrit column packed with Microm Magic C18 AQ packing material. Peptides were eluted from this column over 90 min with a gradient of 5% B to 90% B, with constant 10% C in 76 min using a Michrom Paradigm MDLC (Buffer A, 100% water; Buffer B, 100% acetonitrile; Buffer C, 1% formic acid), at a flow rate of 300 nL/min. Eluted peptides were analyzed with a ThermoElectron LTQ Linear Ion trap mass spectrometer (Thermo Electron Crop, San Jose CA). The top five ions in each survey scan were subjected to data-dependent zoom scans followed by low-energy collision-induced dissociation (CID). The resulting MS/MS spectra were converted to peak lists using BioWorks Browser v 3.2.

The X!-tandem algorithm (Craig and Beavis, 2003, 2004) was used to search MS/MS spectra against the tomato EST database from the TIGR Gene Indices (http://compbio.dfci.harvard.edu/tgi/). Protein identifications were considered positive if two or more peptides from the same protein were identified, each with a probability score of $P \leq 0.01$ (Eriksson and Fenyo, 2004). Of the 20 tomato proteins cataloged by this procedure, 18 proteins were identified on the basis of two or more unique peptides.

Accession Numbers

The GenBank accession number for the *SlTD1* cDNA sequence is EF026088.

Acknowledgements

We thank Doug Whitten of the Michigan Proteome Consortium for assistance with proteomic experiments and bioinformatic analysis of LC-MS/MS data. Guanghui Liu and Rob Larkin (MSU) are acknowledged for assistance with the expression and purification of recombinant TD. John Froehlich (MSU) and Leron Katsir are acknowledged for the anti-Toc75 antibody and assistance with constructing SlTD homology models, respectively. Bonnie St. John provided helpful assistance with immunoblot analysis. We also thank R. Dumas for the pET30-TD expression vector for *A. thaliana* TD. Tomato EST clones were obtained from the Boyce Thompson Institute at Cornell University.

Table 2.1. Host plant proteins identified in frass of tomato-reared *M. sexta* larvae.

Protein identity or best BLAST hit[1]	Access. No.	Unigene[2]	No. Peptides	Local.[3]
Jasmonate- and wound-inducible proteins				
Threonine deaminase (pTD2)	AAA34171	U312574	25	CP
Leucine aminopeptidase (LAP-A)	AAC49456	U312377	23	CP
Trypsin inhibitor-like protein	AAA80497	U313384	4	SP
Cathepsin D inhibitor (CDI)	CAC00536	U312623	4	SP
YjgF family protein[4]	BT013249	U313029	5	CP
Stress-induced LH2 domain protein	BI209796	U315202	3	SP
Aspartic protease inhibitor	BI929912	U312622	2	SP
Germin-like protein	CN384576	U318102	2	SP
Pathogenesis-related proteins				
P69B (PR-7)	CAA71234	U313775	20	SP
P69A (PR-7)	CAA64566	U313772	7	SP
Lignin-forming peroxidase (PR-9)	CAA50597	U321126	10	SP
β-1,3-Glucanase (PR-2)	CAA52872	U314382	7	SP
Endoglucanase inhibitor protein	AAN87262	U314071	3	SP
PR protein P2 (PR-4)	CAA41439	U316008	2	SP
Other proteins				
Plastocyanin	CAA32121	U312690	16	CP
Malate dehydrogenase	AAU29198	U313128	7	MT
Ferredoxin	BI931178	U312380	3	CP
Superoxide dismutase	AAQ09007	U315384	2	CP
Carbonic anhydrase	AW093720	U319550	2	CP
Chlorophyll a/b binding protein	CAA84525	U312438	2	CP

[1] In cases where the BLAST hit did not perfectly match a known tomato protein, the best BLAST hit is listed.

[2] Unigene indicates the tomato gene nomenclature provided by the SOL Genomics Network at http://www.sgn.cornell.edu/index.pl.

[3] Local. denotes the predicted protein location. The presence of an N-terminal signal peptide (SP) for protein secretion was analyzed with the SignalP software (http://www.cbs.dtu.dk/services/SignalP). Proteins having a signal peptide probability score > 0.75 are indicated. CP, chloroplast-targeted protein; MT, mitochondrial-targeted protein.

[4] Annotated in the tomato EST database (http://compbio.dfci.harvard.edu/tgi/plant.html) as a protein translation inhibitor (Li et al., 2004).

Figure 2.1. Tomato has two distinct TD isoforms.

(A) Comparison of the deduced amino acid sequence of SlTD1 (TD1) and SlTD2 (TD2). Amino acids that are either identical (black) or similar (gray) between the two sequences are indicated. The inverted triangle denotes the site of cleavage (between Leu51 and Lys52) of the plastid-targeting peptide on TD2 (Samach et al., 1991). The dotted line denotes the "neck" region that connects the N-terminal catalytic and C-terminal regulatory domains, based on homology modeling with *E. coli* TD (Gallagher et al., 1998).

(B) Phylogenetic relationship of SlTD1 and SlTD2 to TDs from other plants. Shown is an unrooted neighbor-joining tree constructed with MEGA 3.1 (Kumar et al., 2004) from the following sequences: maize (ZmTD; CO446428); sugarcane (SoTD; CA208490); rice (OsTD; NP_001051069); poplar (PtTD; estExt_fgenesh4_pg.C_280257); Arabidopsis (AtTD; NP_187616); *Aquilegia formosa* x *pubescens* (AfTD; DT735861); tomato (SlTD1; ABK20067) (SlTD2; P25306); potato (StTD1; BI436101) (StTD2; X67846); *N. attenuata* (NaTD; AAX22214); chickpea (CaTD; Q39469). GenBank accession numbers are given in parentheses. The indicated bootstrap values (% of 1000 repeated tree reconstructions) show the reliability of each branch of the inferred tree. The two major subgroups of the tree are designated "Group 1" and "Group 2".

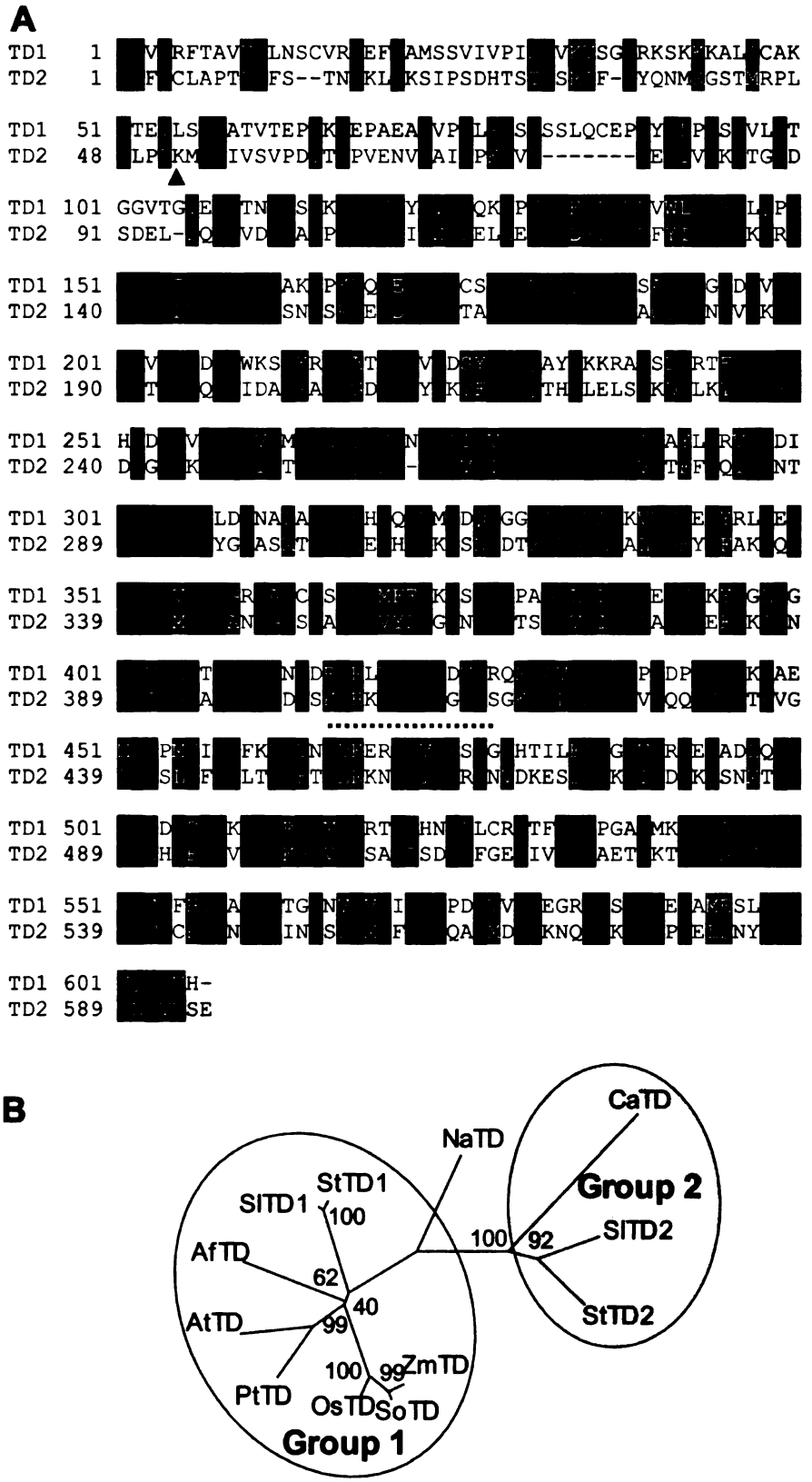
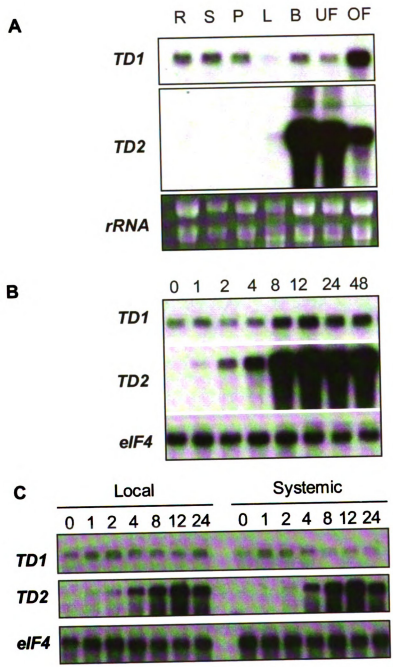

A
B
CaTD
NaTD
StTD1
SlTD1
Group 2
SlTD2
StTD2
AfTD
AtTD
PtTD
OsTD
SoTD
ZmTD
Group 1
100
92
62
40
99
100
99

Figure 2.2. Differential expression of *SlTD1* and *SlTD2* in tomato.

(A) Five μg total RNA from root (R), stem (S), petiole (P), leaf (L), immature flower bud (B), unopened flower (UF), and opened flower (OF) was immobilized to a membrane and hybridized to full-length cDNA probes for *SlTD1* and *SlTD2*. A duplicate blot (lower panel) was stained with ethidium bromide to visualize rRNA, as a means to determine the quality and quantity of the loaded RNA.

(B) Expression of *SlTD1* and *SlTD2* in response to treatment with MeJA. Four-week-old plants were exposed to MeJA vapor for the indicated length of time (h) in an enclosed box, after which leaves were harvested for RNA extraction. RNA isolated from untreated plants (0 hr time point) was analyzed as a control. RNA gel blots were hybridized to cDNA probes for *SlTD1* and *SlTD2*. Blots were also hybridized to an *eIF4A* probe as a loading control.

(C) Expression of *SlTD1* and *SlTD2* in response to mechanical wounding. Leaflets on the 2nd and 3rd fully expanded leaves (counted from the oldest leaf) of four-week-old plants were wounded three times with a hemostat, perpendicular to the main vein. Total RNA was isolated separately from the lower wounded (local) and the upper unwounded (systemic) leaves at various times (h) after wounding. RNA was also isolated from unwounded plants (0 h time point) as a control. RNA gel blots were hybridized to cDNA probes for *SlTD1* and *SlTD2*. Blots were also hybridized to an *eIF4A* probe as a loading control.


A
R S P L B UF OF
TD1
TD2
rRNA
B
0 1 2 4 8 12 24 48
TD1
TD2
eIF4
C
Local
Systemic
0 1 2 4 8 12 24
0 1 2 4 8 12 24
TD1
TD2
eIF4

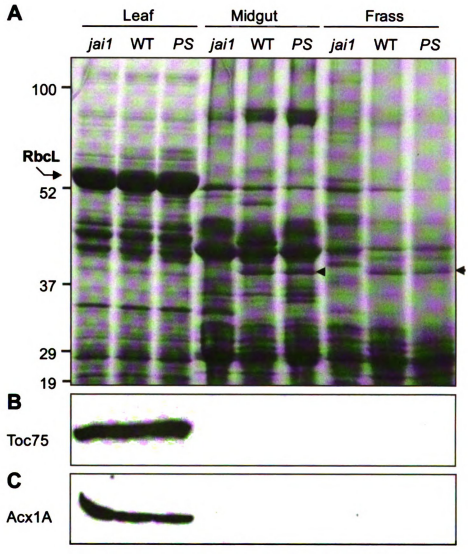


Figure 2.3. Digestion of tomato foliar protein during passage through the *M. sexta* digestive tract.

(A) *M. sexta* larvae were reared to the 4th instar on *jai1*, WT, or *35S::PS* (PS) tomato plants. Total protein was extracted from three different sources of material: tomato leaves that were heavily damaged by the herbivore (Leaf); the midgut content of actively feeding, 4th-instar larvae (Midgut); fecal droppings from actively feeding, 4th-instar larvae (Frass). Forty µg of protein from each sample was analyzed on a 10% to 18% polyacrylamide gradient gel, which was stained with Coomassie blue. The position of protein standards (kDa) is shown on the left, as is the major polypeptide corresponding to the large subunit of Rubisco (RbcL). The arrowhead denotes a 40-kDa polypeptide observed in midgut and frass of larvae reared on WT and *35S::PS* plants.

(B and C) Western blot analysis of the protein samples shown in (A) with polyclonal antibodies raised against the chloroplast outer envelope protein Toc75 (B) or the peroxisomal matrix protein Acx1A (C).

Figure 2.4. Proteolytic processing of TD2 in the digestive tract of *M. sexta* and *T. ni* larvae.

(A) MS-based identification of a truncated form (pTD2) of TD2. Underlined letters denote the amino acid sequence of TD2 that was identified by LC-MS/MS analysis of a gel slice containing the 40-kDa protein. The chloroplast-targeting peptide of TD2 is denoted by lowercase italicized letters. The "neck" region that connects the N-terminal catalytic and C-terminal regulatory domains is indicated by bold letters.

(B) Proteolytic processing of TD2 in *M. sexta*. Protein (10 μg per lane) isolated from tomato leaf, *M. sexta* midgut content, and *M. sexta* frass was separated by SDS-PAGE. The gel was subjected to Western blot analysis with an anti-TD2 antibody. See Figure 2. 3A for a description of the samples. The cross-reacting polypeptide labeled "TD2" corresponds to the 55-kDa mature form of the protein that accumulates in herbivore-damaged leaves. The polypeptide labeled "pTD2" is the proteolytically processed form of TD2 that lacks the C-terminal regulatory domain. Asterisks denote faint bands corresponding to incompletely processed TD2.

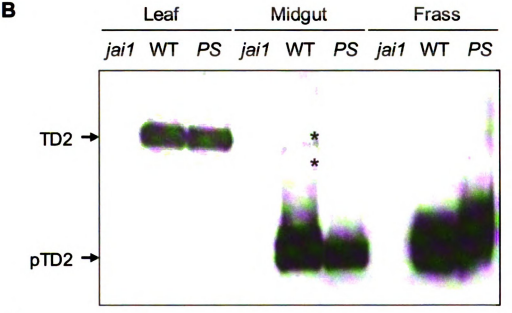
(C) Proteolytic processing of TD2 in the digestive tract of *T. ni* larvae. Protein was analyzed by Western blot analysis as described in (B). Protein was isolated from the following material: lane 1, herbivore-damaged WT leaves; lane 2, frass from *M. sexta* larvae reared on WT plants; lane 3, frass from *T. ni* larvae reared on WT plants; lane 4, frass from *T. ni* larvae reared on *jai1* plants.

(D) TD activity in frass from *T. ni* larvae reared on WT tomato plants. Frass extracts were assayed for TD activity in the absence or presence of 10 mM Ile. Data indicate the mean and SD of measurements from four different pools of frass.

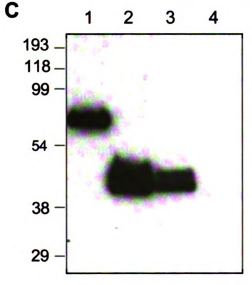
A

meflclaptrsfstnpkltksipsdhtsttsriftyqnmrgstmrplalp
*l*KMSPIVSVPDITAPVENVPAILPKVVPGELIVNKPTGGDSDELFQYLVD
ILASPVYDVAIESPLELAEKLSDRLGVNFYIKREDKQRVFSFKLRGAYNM
MSNLSREELDKGVITASAGNHAQGVALAGQRLNCVAKIVMPTTTPQIKID
AVRALGGDVVLYGKTFDEAQTHALELSEKDGLKYIPPFDDPGVIKGQGTI
GTEINRQLKDIHAVFIPVGGGGLIAGVATFFKQIAPNTKIIGVEPYGAAS
MTLSLHEGHRVKLSNVDTFADGVAVALVGEYTFAKCQELIDGMVLVANDG
ISAAIKDVYDEGRNILETSGAVAIAGAAAYCEFYKIKNENIVAIASGANM
DFS**KLHKVTELAGLG**SGKEALLATFMVEQQGSFKTFVGLVGSLNFTELTY
RFTSERKNALILYRVNVDKESDLEKMIEDMKSSNMTTLNLSHNELVVDHL
KHLVGGSANISDEIFGEFIVPEKAETLKTFLDAFSPRWNITLCRYRNQGD
INASLLMGFQVPQAEMDEFKNQADKLGYPYELDNYNEAFNLVVSE

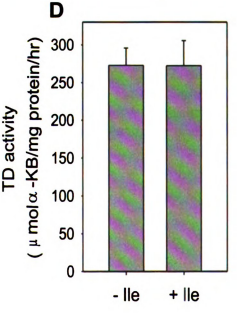
B



C



D



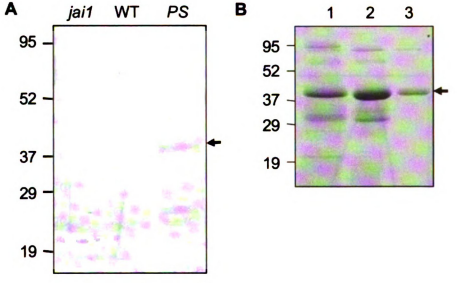


Figure 2.5. Purification of pTD2 from *M. sexta* frass.
(A) Frass pellets collected from *M. sexta* larvae grown on the indicated host plant genotype were extracted with an aqueous buffer to maintain TD activity. The resulting protein (~60 μg) was separated by SDS-PAGE, and the gel was stained with Coomassie blue. The arrow indicates pTD2, which accumulates in frass from larvae grown on WT and *35S::PS* plants, but not in frass from *jai1*-reared larvae. Migration position of molecular weight markers (kDa) are shown on the left.
(B) Frass from *M. sexta* larvae grown on WT tomato foliage was used as the starting material for purification of pTD2. The Coomassie-stained gel shows pTD2 at various steps of the purification procedure: Lane 1, 65% $(NH_4)_2SO_4$ cut; Lane 2, pooled TD-containing fractions from DEAE-cellulose chromatography; Lane 3, pooled TD-containing fractions from Superose-12 gel filtration chromatography. Migration position of molecular weight markers (kDa) are shown on the left. The arrow indicates the polypeptide corresponding to pTD2.

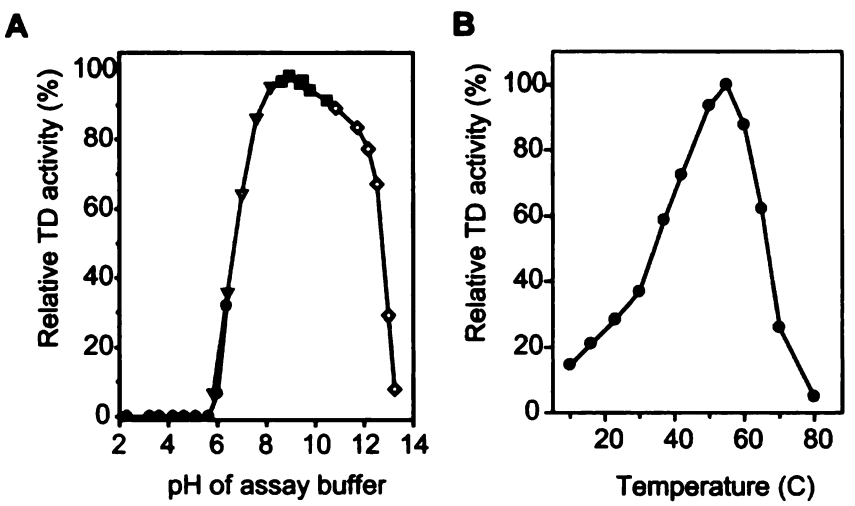


Figure 2.6. Biochemical features of purified pTD2.
(A) pH optimum of pTD2 activity assayed against L-Thr in the following buffer systems: Na-citrate (closed circles); $NaPO_4$ (inverted triangles); glycine (closed squares); KH_2PO_4 (open diamonds). The data are expressed relative to the activity at pH 9.0.
(B) Effect of temperature on pTD2 activity. Enzyme activity was assayed against L-Thr at the indicated temperature (°C). The data are expressed relative to the activity at 58°C.

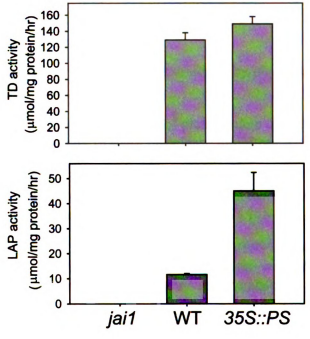


Figure 2.7. LAP-A is excreted from *M. sexta* as an active enzyme.
(A) TD activity was measured in frass collected from *M. sexta* larvae grown on the indicated host plant genotype. Data represent the mean and SD of three independent measurements.
(B) LAP activity in the same extracts used in (A). Data represent the mean and SD of three independent measurements.

LITERATURE CITED

Berenbaum MR (1995) Turnabout is fair play - Secondary roles for primary compounds. J Chem Ecol **21:** 925-940

Bergey DR, Howe GA, Ryan CA (1996) Polypeptide signaling for plant defensive genes exhibits analogies to defense signaling in animals. Proc Natl Acad Sci U S A **93:** 12053-12058

Bernays EA, Chapman RF (1994) Host-plant selection by phytophagous insects. New York: Chapman & Hall, New York

Broadway RM, Duffey SS (1988) The effect of plant protein quality on insect digestive physiology and the toxicity of plant proteinase inhibitors. J Insect Physiol **34:** 1111-1117

Casey TM (1976) Activity patterns, body temperature and thermal ecology in two desert caterpillars (Lepidoptera-Sphingidae). Ecology **57:** 485-497

Chao WS, Gu YQ, Pautot VV, Bray EA, Walling LL (1999) Leucine aminopeptidase RNAs, proteins, and activities increase in response to water deficit, salinity, and the wound signals systemin, methyl jasmonate, and abscisic acid. Plant Physiol **120:** 979-992

Chen H, McCaig BC, Melotto M, He SY, Howe GA (2004) Regulation of plant arginase by wounding, jasmonate, and the phytotoxin coronatine. J Biol Chem **279:** 45998-46007

Chen H, Wilkerson CG, Kuchar JA, Phinney BS, Howe GA (2005) Jasmonate-inducible plant enzymes degrade essential amino acids in the herbivore midgut. Proc Natl Acad Sci U S A **102:** 19237-19242

Coker JS, Vian A, Davies E (2005) Identification, accumulation, and functional prediction of novel tomato transcripts systemically upregulated after fire damage. Physiol Plant **124:** 311-322

Colau D, Negrutiu I, Vanmontagu M, Hernalsteens JP (1987) Complementation of a threonine dehydratase-deficient *Nicotiana plumbaginifolia* mutant after *Agrobacterium tumefaciens*-mediated transfer of the *Saccharomyces cerevisiae Ilv1* gene. Mol Cell Biol **7:** 2552-2557

Constabel CP, Bergey DR, Ryan CA (1995) Systemin activates synthesis of wound-inducible tomato leaf polyphenol oxidase via the octadecanoid defense signaling pathway. Proc Natl Acad Sci U S A **92:** 407-411

Craig R, Beavis RC (2003) A method for reducing the time required to match protein sequences with tandem mass spectra. Rapid Commun Mass Spectrom **17:** 2310-2316

Craig R, Beavis RC (2004) TANDEM: matching proteins with tandem mass spectra. Bioinformatics **20:** 1466-1467

Datta P, Goss TJ, Omnaas JR, Patil RV (1987) Covalent structure of biodegradative threonine dehydratase of *Escherichia coli* - Homology with other dehydratases. Proc Natl Acad Sci U S A **84:** 393-397

Domingo C, Conejero V, Vera P (1994) Genes encoding acidic and basic class III β-1,3-glucanases are expressed in tomato plants upon viroid infection. Plant Mol Biol **24:** 725-732

Duffey SS, Felton GW (1991) Enzymatic antinutritive defenses of the tomato plant against insects. ACS Symp Series **449:** 166-197

Duffey SS, Stout MJ (1996) Antinutritive and toxic components of plant defense against insects. Arch Insect Biochem Physiol **32:** 3-37

Eriksson J, Fenyo D (2004) Probity: a protein identification algorithm with accurate assignment of the statistical significance of the results. J Proteome Res **3:** 32-36

Feeny P (1976) Plant apparency in chemical defense. Recent Adv Phytochemistry **10:** 1-40

Felton GW (1996) Nutritive quality of plant protein: Sources of variation and insect herbivore responses. Arch Insect Biochem Physiol **32:** 107-130

Felton GW (2005) Indigestion is a plant's best defense. Proc Natl Acad Sci U S A **102:** 18771-18772

Felton GW, Bi JL, Summers CB, Mueller AJ, Duffey SS (1994) Potential role of lipoxygenases in defense against insect herbivory. J Chem Ecol **20:** 651-666

Ferreira-DaSilva CT, Gombarovits MEC, Masuda H, Oliveira CM, Carlini CR (2000) Proteolytic activation of canatoxin, a plant toxic protein, by insect cathepsin-like enzymes. Arch Insect Biochem Physiol **44:** 162-171

Ferreira RB, Picarra-Pereira MA, Monteiro S, Loureiro VB, Teixeira AR (2001) The wine proteins. Trends Food Sci & Technol **12:** 230-239

Flamini R, De Rosso M (2006) Mass spectrometry in the analysis of grape and wine proteins. Expert Rev Proteomics **3:** 321-331

Fraenkel GS (1959) The raison d'etre of secondary plant substances; these odd chemicals arose as a means of protecting plants from insects and now guide insects to food. Science **129:** 1466-1470

Francis F, Gerkens P, Harmel N, Mazzucchelli G, De Pauw E, Haubruge E (2006) Proteomics in *Myzus persicae*: Effect of aphid host plant switch. Insect Biochem Mol Biol **36:** 219-227

Gallagher DT, Gilliland GL, Xiao GY, Zondlo J, Fisher KE, Chinchilla D, Eisenstein E (1998) Structure and control of pyridoxal phosphate dependent allosteric threonine deaminase. Structure **6:** 465-475

Gangwere SK (1993) Feculae and defecation in phytophagous Orthoptera. Phytophaga **5:** 13-34

Gatehouse JA (2002) Plant resistance towards insect herbivores: a dynamic interaction. New Phytologist **156:** 145-169

Gilchrist A, Au CE, Hiding J, Bell AW, Fernandez-Rodriguez J, Lesimple S, Nagaya H, Roy L, Gosline SJ, Hallett M, Paiement J, Kearney RE, Nilsson T, Bergeron JJ (2006) Quantitative proteomics analysis of the secretory pathway. Cell **127:** 1265-1281

Giri AP, Wunsche H, Mitra S, Zavala JA, Muck A, Svatos A, Baldwin IT (2006) Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata*. VII. Changes in the plant's proteome. Plant Physiol **142:** 1621-1641

Green TR, Ryan CA (1972) Wound-induced proteinase inhibitor in plant leaves. Possible defense mechanism against insects. Science **175:** 776-777

Gu YQ, Holzer FM, Walling LL (1999) Overexpression, purification and biochemical characterization of the wound-induced leucine aminopeptidase of tomato. Eur J Biochem **263:** 726-735

Haukioja E, Ruohomaki K, Suomela J, Vuorisalo T (1991) Nutritional quality as a defense against herbivores. Forest Ecol Management **39:** 237-245

Hermsmeier D, Schittko U, Baldwin IT (2001) Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata*. I. Large-scale changes in the accumulation of growth- and defense-related plant mRNAs. Plant Physiol **125:** 683-700

Hildmann T, Ebneth M, Pena-Cortes H, Sanchez-Serrano JJ, Willmitzer L, Prat S (1992) General roles of abscisic and jasmonic acids in gene activation as a result of mechanical wounding. Plant Cell **4:** 1157-1170

Hough DW, Danson MJ (1999) Extremozymes. Curr Opin Chem Biol **3:** 39-46

Howe GA (2004) Jasmonates as signals in the wound response. J Plant Growth Regu **23:** 223-237

Howe GA, Lee GI, Itoh A, Li L, DeRocher AE (2000) Cytochrome P450-dependent metabolism of oxylipins in tomato. Cloning and expression of allene oxide synthase and fatty acid hydroperoxide lyase. Plant Physiol **123:** 711-724

Kang JH, Baldwin IT (2006) Isolation and characterization of the threonine deaminase promoter in *Nicotiana attenuata*. Plant Sci **171:** 435-440

Kang JH, Wang L, Giri AP, Baldwin IT (2006) Silencing threonine deaminase and the *JAR1* homologue in *Nicotiana attenuata* impairs JA-isoleucine-mediated defenses against the specialist herbivore, *Manduca sexta*. Plant Cell **18:** 3303-3320

Karowe DN, Martin MM (1989) The effects of quantity and quality of diet nitrogen on the growth, efficiency of food utilization, nitrogen budget, and metabolic rate of 5^{th} instar *Spodoptera eridania* larvae (Lepidoptera, Noctuidae). J Insect Physiol **35:** 699-708

Kessler A, Baldwin IT (2002) Plant responses to insect herbivory: The emerging molecular analysis. Annu Rev Plant Biol **53:** 299-328

Kim JM, Yoshikawa H, Shirahige K (2001) A member of the YER057c/yjgf/Uk114 family links isoleucine biosynthesis and intact mitochondria maintenance in *Saccharomyces cerevisiae*. Genes to Cells **6:** 507-517

Konno K, Hirayama C, Nakamura M, Tateishi K, Tamura Y, Hattori M, Kohno K (2004) Papain protects papaya trees from herbivorous insects: role of cysteine proteases in latex. Plant J **37:** 370-378

Kumar S, Tamura K, Nei M (2004) MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. Briefings Bioinformatics **5:** 150-163

Leitner-Dagan Y, Ovadis M, Zuker A, Shklarman E, Ohad I, Tzfira T, Vainstein A (2006) CHRD, a plant member of the evolutionarily conserved YjgF family, influences photosynthesis and chromoplastogenesis. Planta **225:** 89-102

Li L, Howe GA (2001) Alternative splicing of prosystemin pre-mRNA produces two isoforms that are active as signals in the wound response pathway. Plant Mol Biol **46:** 409-419

Li L, Zhao Y, McCaig BC, Wingerd BA, Wang J, Whalon ME, Pichersky E, Howe GA (2004) The tomato homolog of CORONATINE-INSENSITIVE1 is required for the maternal control of seed maturation, jasmonate-signaled defense responses, and glandular trichome development. Plant Cell **16:** 126-143

Linthorst HJM, Danhash N, Brederode FT, Vankan JAL, Dewit P, Bol JF (1991) Tobacco and tomato PR proteins homologous to Win and Pro-hevein lack the hevein domain. Mol Plant-Microbe Interact **4:** 586-592

Lippert D, Chowrira S, Ralph SG, Zhuang J, Aeschliman D, Ritland C, Ritland K, Bohlmann J (2007) Conifer defense against insects: Proteome analysis of Sitka spruce (*Picea sitchensis*) bark induced by mechanical wounding or feeding by white pine weevils (*Pissodes strobi*). Proteomics **7:** 248-270

Lison P RI, Conejero V. (2006) A novel function for the cathepsin D inhibitor in tomato. Plant Physiol. **142:** 1329-1339

Lison P, Rodrigo I, Conejero V (2006) A novel function for the cathepsin D inhibitor in tomato. Plant Physiol. In press.

Liu YL, Ahn JE, Datta S, Salzman RA, Moon J, Huyghues-Despointes B, Pittendrigh B, Murdock LL, Koiwa H, Zhu-Salzman K (2005) Arabidopsis vegetative storage protein is an anti-insect acid phosphatase. Plant Physiol **139:** 1545-1556

Lou YG, Baldwin IT (2006) Silencing of a germin-like gene in *Nicotiana attenuata* improves performance of native herbivores. Plant Physiol **140:** 1126-1136

Mattson WJ (1980) Herbivory in relation to plant nitrogen content. Annu Rev Ecol Systemat **11:** 119-161

McGurl B, Orozco-Cardenas M, Pearce G, Ryan CA (1994) Overexpression of the prosystemin gene in transgenic tomato plants generates a systemic signal that constitutively induces proteinase inhibitor synthesis. Proc Natl Acad Sci U S A **91:** 9799-9802

Mohan S, Ma PWK, Pechan T, Bassford ER, Williams WP, Luthe DS (2006) Degradation of the *S. frugiperda* peritrophic matrix by an inducible maize cysteine protease. J Insect Physiol **52:** 21-28

Moran N, Hamilton WD (1980) Low nutritive quality as defense against herbivores. J Theor Biol **86:** 247-254

Nampoothiri KM, Nagy V, Kovacs K, Szakacs G, Pandey A (2005) L-leucine aminopeptidase production by filamentous *Aspergillus fungi*. Lett Applied Microbiol **41:** 498-504

Naqvi SMS, Harper A, Carter C, Ren G, Guirgis A, York WS, Thornburg RW (2005) Nectarin IV, a potent endoglucanase inhibitor secreted into the nectar of ornamental tobacco plants. Isolation, cloning, and characterization. Plant Physiol **139:** 1389-1400

Old WM, Meyer-Arendt K, Aveline-Wolf L, Pierce KG, Mendoza A, Sevinsky JR, Resing KA, Ahn NG (2005) Comparison of label free methods for quantifying human proteins by shotgun proteomics. Mol Cell Proteomics **4:** 1487-1502

Parsons L, Bonander N, Eisenstein E, Gilson M, Kairys V, Orban J (2003) Solution structure and functional ligand screening of HI0719, a highly conserved protein from bacteria to humans in the YjgF/YER057c/UK114 family. Biochem **42:** 80-89

Pechan T, Cohen A, Williams WP, Luthe DS (2002) Insect feeding mobilizes a unique plant defense protease that disrupts the peritrophic matrix of caterpillars. Proc Natl Acad Sci U S A **99:** 13319-13323

Reumann S, Inoue K, Keegstra K (2005) Evolution of the general protein import pathway of plastids (Review). Molecular Membrane Biology **22:** 73-U20

Rhoades DF, Cates RG (1976) Toward a general theory of plant antiherbivore chemistry. Rec Adv Phytochemistry, pp. 168-213

Ryan CA (1990) Protease inhibitors in plants. Genes for improving defenses against insects and pathogens. Annu Rev Phytopathol **28:** 425-449

Samach A, Broday L, Hareven D, Lifschitz E (1995) Expression of an amino acid biosynthesis gene in tomato flowers: developmental upregulation and MeJa response are parenchyma-specific and mutually compatible. Plant J **8:** 391-406

Samach A, Hareven D, Gutfinger T, Ken-Dror S, Lifschitz E (1991) Biosynthetic threonine deaminase gene of tomato: isolation, structure, and upregulation in floral organs. Proc Natl Acad Sci U S A **88:** 2678-2682

Schaller A, Bergey DR, Ryan CA (1995) Induction of wound response genes in tomato leaves by bestatin, an inhibitor of aminopeptidases. Plant Cell **7:** 1893-1898

Schilmiller AL, Howe GA (2005) Systemic signaling in the wound response. Curr Opin Plant Biol **8:** 369-377

Schilmiller AL, Koo AJ, Howe GA (2006) Functional diversification of acyl-CoA oxidases in jasmonic acid biosynthesis and action. Plant Physiol, in press

Schmelz EA, Carroll MJ, LeClere S, Phipps SM, Meredith J, Chourey PS, Alborn HT, Teal PEA (2006) Fragments of ATP synthase mediate plant perception of insect attack. Proc Natl Acad Sci U S A **103:** 8894-8899

Sheen SJ (1991) Comparison of chemical and functional properties of soluble leaf proteins from four plant species. J Agric Food Chem **39:** 681-685

Sidorov V, Menczel L, Maliga P (1981) Isoleucine requiring *Nicotiana* plant deficient in threonine deaminase. Nature **294:** 87-88

Staswick PE, Yuen GY, Lehman CC (1998) Jasmonate signaling mutants of Arabidopsis are susceptible to the soil fungus *Pythium irregulare*. Plant J **15:** 747-754

Strassner J, Schaller F, Frick UB, Howe GA, Weiler EW, Amrhein N, Macheroux P, Schaller A (2002) Characterization and cDNA-microarray expression analysis of 12-oxophytodienoate reductases reveals differential roles for octadecanoid biosynthesis in the local versus the systemic wound response. Plant J **32:** 585-601

Szamosi I, Shaner DL, Singh BK (1993) Identification and characterization of a biodegradative form of threonine dehydratase in senescing tomato (*Lycopersicon esculentum*) leaf. Plant Physiol **101:** 999-1004

Tornero P, Conejero V, Vera P (1996) Primary structure and expression of a pathogen-induced protease (PR-P69) in tomato plants: Similarity of functional domains to subtilisin-like endoproteases. Proc Natl Acad Sci U S A **93:** 6332-6337

Tornero P, Conejero V, Vera P (1997) Identification of a new pathogen-induced member of the subtilisin-like processing protease family from plants. J Biol Chem **272:** 14412-14419

Tranel PJ, Froehlich J, Goyal A, Keegstra K (1995) A component of the chloroplastic protein import apparatus is targeted to the outer envelope membrane via a novel pathway. EMBO J **14:** 2436-2446

Utsugi S, Sakamoto W, Murata M, Motoyoshi F (1998) *Arabidopsis thaliana* vegetative storage protein (VSP) genes: gene organization and tissue-specific expression. Plant Mol Biol **38:** 565-576

van Loon LC, Rep M, Pieterse CM (2006) Significance of inducible defense-related proteins in infected plants. Annu Rev Phytopathol **44:** 135-162

Vera P, Conejero V (1988) Pathogenesis related proteins of tomato. P-69 as an alkaline endoproteinase. Plant Physiol **87:** 58-63

Vera P, Tornero P, Conejero V (1993) Cloning and expression analysis of a viroid-induced peroxidase from tomato plants. Mol Plant-Microbe Interact **6:** 790-794

Vinson SB (1976) Host selection by insect parasitoids. Annu Rev of Entomol **21:** 109-133

Walling LL (2000) The myriad plant responses to herbivores. J Plant Growth Regu **19:** 195-216

Walling LL (2006) Recycling or regulation? The role of amino-terminal modifying enzymes. Curr Opin Plant Biol **9:** 227-233

Wang JH, Constabel CP (2004) Polyphenol oxidase overexpression in transgenic *Populus* enhances resistance to herbivory by forest tent caterpillar (*Malacosoma disstria*). Planta **220:** 87-96

Weiss MR (2006) Defecation behavior and ecology of insects. Annu Rev Entomol **51:** 635-661

Wessel PM, Graciet E, Douce R, Dumas R (2000) Evidence for two distinct effector-binding sites in threonine deaminase by site-directed mutagenesis, kinetic, and binding experiments. Biochem **39:** 15136-15143

Zhao Y, Thilmony R, Bender CL, Schaller A, He SY, Howe GA (2003) Virulence systems of *Pseudomonas syringae* pv. tomato promote bacterial speck disease in tomato by targeting the jasmonate signaling pathway. Plant J **36:** 485-499

Chapter III

The defensive function of tomato threonine deaminase 2 is activated by an insect digestive protease

Abstract

Threonine deaminase (TD) is a pyridoxal phosphate-dependent enzyme that catalyzes the dehydration of threonine (Thr) to α-ketobutyrate and ammonia, which is the committed step in the biosynthesis of isoleucine (Ile). All plant and bacterial TDs consist of an N-terminal catalytic domain and a C-terminal regulatory domain that binds the allosteric inhibitor Ile. *Solanum lycopersicum* (cultivated tomato) has two TD-encoding genes, designated *TD1* and *TD2*. *TD1* likely serves a primary metabolic role in Ile synthesis because the gene is constitutively expressed in all tissues and is homologous to *TD*s from plants such as rice and Arabidopsis that contain a single *TD* gene. In contrast, *TD2* expression is strictly regulated in an inducible manner by the plant stress hormone jasmonate. Accumulation of TD2 protein in frass (feces) of insect herbivores fed on tomato leaves led to the hypothesis that TD2 is a defensive enzyme that acts post-ingestively to deplete Thr in the insect gut, thereby reducing the nutritional quality of ingested plant material. Interestingly, ingestion of tomato foliage by lepidopteran insects results in proteolytic removal of the TD2 regulatory domain, resulting in a TD2 variant (pTD2) that degrades Thr without being inhibited by Ile. Here, we employ *in vitro* TD2-cleavage assays to show that the protease responsible for removal of the regulatory

domain is most likely a chymotrypsin-like protease of insect origin. Incubation of TD2 with a crude mixture of lepidopteran digestive proteases resulted in production of pTD2 whereas the same protease mixture rapidly degraded and catalytically inactivated TD1. In addition to differential susceptibility to digestive proteases, TD2 exhibited high temperature stability in comparison to TD1. Lepidopteran larvae performed better on tomato TD2 antisense plants compared to wild-type plants, indicating that TD2 serves a role in anti-insect defense. These findings provide evidence for functional divergence of tomato TD isoforms, and further indicate that TD2 evolved structural and catalytic properties that facilitate its post-ingestive role in plant defense.

Introduction

The growth and development of insect herbivores depends on their ability to acquire essential amino acids from dietary protein (Nation, 2002). The essential amino acids for insects are arginine, threonine, isoleucine, leucine, lysine, histidine, methionine, phenylalanine, tryptophan, and valine (Nation, 2002; Chang, 2004). Insect herbivores are faced with the challenge of acquiring these amino acids from plant tissues that, unlike animal tissue, contain low protein levels (Mattson, 1980; Bernays and Chapman, 1994; Bernays et al., 2004). Plants have evolved several defensive strategies to impair the ability of insects to digest dietary protein. One strategy involves host plant secondary metabolites such as phenolics and tannins that act as digestibility reducers (Cates and Rhoades, 1977). Plants also synthesize various proteins that act postingestively to disrupt amino acid acquisition from dietary protein. These include proteinase inhibitors (PIs) that

impair insect digestive enzymes, polyphenol oxidases (PPOs) that covalently modify dietary protein, and enzymes such as threonine deamidase (TD) and arginase that degrade essential amino acids (Felton et al., 1994; Duffey and Stout, 1996; Bolter and Jongsma, 1997; Murdock and Shade, 2002; Pechan et al., 2002; Wang and Constabel, 2004; Chen et al., 2005), These plant defense proteins are thought to act coordinately and synergistically to starve herbivores of key nutrients (Felton, 2005; Zhu-Salzman et al., 2008).

The identification of an inducible isoform (TD2) of tomato TD as a highly abundant protein in the midgut and feces of tomato-reared lepidopteran larvae led to the suggestion that this enzyme has a post-ingestive role in depletion of Thr (Chen et al., 2005; Chen et al., 2007). In plants and microorganisms, TD (EC 4.2.1.16) catalyzes the dehydration/deamination of Thr to yield α-ketobutyrate and ammonia. This reaction constitutes the first step in the biosynthesis of isoleucine (Ile) and is tightly regulated by feedback inhibition (Hatfield and Umbarger, 1970). The enzyme consists of an N-terminal catalytic domain containing the cofactor pyridoxal phosphate and a C-terminal regulatory domain that binds Ile. The X-ray crystal structure of *E. coli* TD shows that the catalytic and regulatory domains are connected by a neck-like region, and that the holoenzyme assembles as a tetramer (Gallagher et al., 1998).

Tomato contains two *TD* genes that have presumably evolved under different selective pressures. *TD1* is constitutively expressed in all tissues, whereas *TD2* expression is dependent on the plant hormone jasmonate (JA) (Chen et al., 2007). Phylogenetic analysis of plant TDs showed that there are two distinct groups: the first group includes tomato TD1 and TDs from rice, Arabidopsis, and poplar, which serve a

primary metabolic role in Ile synthesis (Garcia and Mourad, 2004; Joshi et al., 2006; Chen et al., 2007). The second cluster is represented by TD2, the potato ortholog of TD2, and a TD isoform from chickpea (Chen et al., 2007). *Nicotiana attenuata*, which has one *TD* gene (Kang et al., 2006), occupies an intermediate position in the phylogenetic tree. The first clue that TD2 serves a role in plant defense was provided by Hildmann and coworkers (1992), who showed that potato *TD2* is highly expressed in response to mechanical wounding or treatment with ABA and methyl-JA (MeJA). Expression of the *TD2* ortholog in tomato, as well as *N. attenuata TD*, is also strongly induced by JA (Samach et al., 1995; Hermsmeier et al., 2001; Strassner et al., 2002; Li et al., 2004; Chen et al., 2007). The overall pattern of expression of TD2 in tomato is very similar to that of PIs and other JA-inducible proteins, and is indicative of a role in anti-insect defense.

The anti-nutritional role of TD2 appears to be enhanced by proteolytic cleavage of the enzyme's regulatory domain following ingestion of tomato leaves by insect larvae. Removal of the regulatory domain results in an enzyme (designated pTD2) that is insensitive to feedback inhibition by Ile and, as a consequence, highly efficient in degrading Thr in the Ile-rich environment of the lepidopteran gut (Chen et al., 2005). This finding indicates that whereas TD2 may function *in planta* as a biosynthetic enzyme that is regulated by Ile-mediated feedback inhibition, proteolytic processing within the insect gut converts TD2 to a degradative enzyme (pTD2) that depletes Thr (Chen et al., 2005; Chen et al., 2007).

The mechanism by which TD2 is converted to pTD2 during ingestion of plant tissue by lepidopteran insects is not known. It is possible that proteolytic cleavage is catalyzed by a plant protease that gains access to TD2, which is located in the chloroplast

(Samach et al., 1995), during ingestion of leaf tissue by the insect. An alternative hypothesis is that TD2 processing is catalyzed by an insect protease. Here, we report the results of experiments designed to discriminate between these two possibilities and to test the putative role of TD2 in anti-insect defense. First, we show that transgenic tomato lines silenced for the expression of TD2 are compromised in resistance to *Spodoptera exigua* larvae. Second, we demonstrate that the regulatory domain of TD2 is removed in the lepidopteran midgut in the absence of plant proteins by a chymotrypsin-like protease of insect origin. Finally, we show that TD1 and TD2 have unique biochemical features, including protease- resistance and thermostability. These findings suggest that these two isoforms are specialized for their respective roles in primary metabolism and plant defense.

Materials and Methods

Plant material and transformations

The full-length *TD2* cDNA was obtained by PCR with the primers 5'-ATCTCGAGATGGAATTCCTTTGTTTAGCCCCA-3' and 5'-ATGGATCCGCCATTACATTACATTGGATACAT-3' that contain restriction sites for cloning into the *XhoI* and *BamHI* sites of the binary vector pBI121 (Clontech). The resulting vector contains *TD2* in antisense orientation under the control of the CaMV 35S promoter, and was used to transform tomato (*Solanum lycopersicum* cv. Microtom) cotyledons with *Agrobacterium* strain AGL0 as previously described (Li et al., 2005). Kanamycin-resistant explants were screened by PCR with the primers 35S-G 5'-

CTATCCGCAAGACCC-3' and TD5 5'- ATGGAATTCCTTTGTTTAGCCCCA-3'to confirm the presence of the transgene. A secondary screen for TD2-deficient transformants took advantage of the fact that TD2 is constitutively expressed to very high levels in tomato flowers (Samach et al., 1995). Fully open flowers were ground in 200 μL of extraction buffer (100 mM Tris-HCl ,pH 6.8, 20 mM EDTA,100 mM KCl, 2% (v/v) 2-mercaptoethanol, 1 mM PMSF). The mixture was centrifuged for 20 min at 20,000*g* and the supernatant used for activity assays. Two antisense lines, TDAs7 and TDAs15, exhibited low levels of TD activity in flowers and thus were selected for subsequent experiments. Insect bioassays were conducted with T_3 generation plants obtained from a TDAs7 homozygous line, as well as with segregating progeny from a TDAs15 line that was hemizygous for the transgene; transgene-containing progeny from this line were identified by PCR screening. Conditions for plant growth have been described previously (Chen et al., 2005).

Insect rearing and bioassays

M. sexta (Linné) eggs were obtained from the Entomology Department at North Carolina State University. *T. ni* (Hübner) and *S. exigua* (Hübner) eggs were obtained from Benzon Research. All eggs were hatched at 30°C. Tobacco hornworm diet was obtained from Carolina Biological Supply. The diet for the noctuids was composed of enriched soybean fluor, wheat germ, sucrose, mineral salt and vitamin mix, agar, methyl paraben, sorbic acid, aureomycin, and calcium propionate (Southland Products Inc.). The diet was supplemented with raw linseed oil for rearing *T. ni*. For the bioassay of *S. exigua* on

antisense *TD2* plants, larvae were reared on diet for 72 h prior to transfer to plants. These larvae were pre-selected to have a uniform weight at the start of the feeding trial. ANOVA was used to test for significant differences in weight between larvae reared on the wild-type and transgenic plants, and larval weight data was log-transformed to meet ANOVA assumptions. Untransformed data were used in figures. Differences between treatments were assessed with the Least Significance Difference test. Statistical analysis was performed with SAS® software, Version 9.1.3 of the SAS System for Windows (Copyright © 2002-2003). For the experiments where TD was added to the food, the diet was prepared according to the manufacturer's instructions and allowed to cool to 55°C before adding recombinant TD2. The diet was poured on plates, cut into pieces and placed in plastic cups containing one insect each.

Expression of TD1 and TD2 in *E. coli*

The vector (pET30TD) for expression of *Arabidopsis thaliana* TD (AtTD) was kindly provided by R. Dumas (Wessel et al., 2000). Vectors for expression of tomato TDs were constructed by excising the *AtTD* cDNA from pET30TD with *NdeI* and *SalI*, with subsequent replacement with the tomato TD cDNAs. Prior to cloning of a *TD1* coding sequence lacking the N-terminal chloroplast targeting signal into these sites, the cDNA clone cTOD22M4 was site-directed mutagenized to remove two *NdeI* sites from the *TD1* coding region. This manipulation did not alter the amino acid sequence of TD1. The forward primer (5'-CGCATATGTCATCGCCAGCTACG-3') was designed to contain an *NdeI* restriction site and to replace the first amino acid (Leu-55) of the mature protein

with a Met (underlined). The reverse primer (5'-CGCTCGAGTCAATGCATTATGAGCTG-3') contains a stop codon (underlined) and an *XhoI* site that is compatible with the vector *SalI* site. Cloning and functional expression of TD2 was performed as previously described (Chen et al., 2007), except that Ile (1 mM) was added to the extraction buffer (50 mM Tris, pH 7.5, 1 mM EDTA) and all buffers used for purification except the final resuspension buffer. The purity of recombinant enzymes was determined by SDS-polyacrylamide gel electrophoresis and estimated to be above 95%.

TD enzyme assays

The protein concentration of purified TD1 and TD2 was calculated based on its amino acid composition and Beer's law. TD activity was determined by measuring the formation of keto acids as initially described by Hatfield and Umbarger (Hatfield and Umbarger, 1968). The reaction mixture (250 µl) contained 150 mM Tris-HCl, pH 9, 10 mM Thr, 12 mM KCl. Reactions were initiated by addition of enzyme, incubated at 30°C for 30 min, and terminated by the addition of TCA. Keto acid formation was monitored by absorbance at 505 nm on a Beckman Spectrophotometer. For K_m and V_{max} calculations, reactions were initiated by addition of a range (0 to 40 mM) of substrate (Thr) concentrations. Reactions were performed in triplicate and the data fitted with a non-linear regression model using Prism 5 for Windows, trial version 5.02 (GraphPad Software).

TD2 cleavage assays

For use as protease sources for the TD2 cleavage assay, total protein was extracted with an aqueous extraction buffer (250 mM Tris-HCl, pH 8, 2.5 M NaCl) from leaves wounded by *T. ni*, and frass collected from the same insect reared either on tomato or artificial diet. The cleavage test was performed by incubating 0.2-0.4 μg of TD2 at 37°C with 0.25 μg of the protease in assay buffer (150 mM Tris-HCl, pH 9.0, 2 mM $CaCl_2$, 0.5 mM DTT). The reaction product was loaded on a 10% SDS-polyacrylamide gel for visualization of TD2 cleavage by western blot analysis or Coomassie blue staining. Protease inhibitors were obtained from GBiosciences and used at 2X concentration, except for TPCK (obtained from Sigma) and used at 250 μM.

Protease fractionation

The TD2-cleaving protease was partially purified from *T. ni* frass as follows. Frass (2 grams fresh weight) was collected from first- to fifth-instar-*T. ni* larvae reared individually on artificial diet, and frozen at -20°C until further use. Frozen frass was ground in liquid nitrogen to a fine powder and homogenized in the extraction buffer described above. After centrifugation for 30 min at 3,210*g*, the supernatant was brought to 25% saturation with ammonium sulfate and stirred for 1 h at 4°C. The precipitated protein was collected by centrifugation and saved (0-25% fraction). The supernatant was adjusted to 50% ammonium sulfate, and the insoluble fraction collected as before (25-50% fraction). Once again, the supernatant was taken to 75% saturation and the pellet saved (50-75% fraction). The fractions were resuspended in extraction buffer and

dialyzed overnight against 500 volumes of extraction buffer at 4°C before being quantified with a Bradford assay.

Results

Silencing of TD2 expression improves *S. exigua* performance on tomato

To test the hypothesis that TD2 has a role in anti-insect defense, *Agrobacterium*-mediated transformation was used to generate transgenic tomato lines that express an antisense *TD2* cDNA under the control of the *Cauliflower mosaic virus* (CaMV) 35S promoter. Regenerated plants expressing the *35S-TD2-As* transgene were screened for TD2 deficiency by measuring TD activity in flowers, which constitutively express TD2 to very high levels (Samach et al., 1995). *35S-TD2-As* lines TDAs7and TDAs15 were identified among several transgenic lines with reduced TD2 expression (Figure 3.1). The severely reduced expression of TD2 in these lines did not result in any morphological or developmental phenotypes related to Ile deficiency, in contrast to the observations in *Nicotiana* species (Sidorov et al., 1981; Colau et al., 1987; Kang et al., 2006). This observation is consistent with the idea that tomato TD1 is responsible for synthesis of Ile for use in general metabolism, whereas TD2 serves a specialized role in defense (Chen et al., 2005; 2007).

The TDAs7and TDAs15 lines were used for their low levels of enzyme activity for bioassays with *S. exigua*. After 4 days of feeding, larvae growing on TDAs7 plants were significantly heavier than larvae on growing on wild-type plants (*t*-test: 5.26, *p*-value < 0.0001). After 7 days of feeding, the difference in larval weights was more

pronounced (*t*-test: 6.58, *p*-value < 0.0001) (Figure 3.2.A and B). A significant difference in the weight of larvae grown on TDAs15 and wild-type plants was observed at the 7-day time point (*t*-test: 3.14, *p*-value: 0.0021) (Figure 3.2.B). Increased caterpillar performance on TDAs7 and TDAs15 lines was inversely correlated with TD2 protein levels in *S. exigua*-challenged leaves (Figure 3.2.C). The accelerated growth of *S. exigua* on both lines was also correlated with increased leaf consumption (data not shown). The experiments were repeated three times with similar results. We conclude that tomato TD2 has a role in defense against attack by *S. exigua*.

Previous studies have shown that TD2 is proteolytically processed to a lower-molecular-weight, Ile-insensitive form of the enzyme (pTD2) during digestion of tomato leaves by *M. sexta* (Chen et al., 2005), and that pTD2 is excreted as a stable and active enzyme in the frass of tomato-reared *M. sexta* and *T. ni* (Chen et al., 2007). To determine whether this is also the case for *S. exigua*, we used immunoblot analysis to determine the TD2 content of frass collected from *S. exigua* grown either on wild-type or TDAs7 plants. The results in Figure 3.2.D show that TD2 is processed to pTD2 during passage of tomato leaves through *S. exigua*. The absence of immunodetecable pTD2 in frass from larvae reared on TDAs7 plants indicates that the anti-TD2 antibody does not cross-react with proteins of insect origin.

Proteolytic cleavage of TD2 by a lepidopteran protease

We developed an *in vitro* processing assay to investigate the identity of the protease responsible for removal of TD2's regulatory domain in the lepidopteran gut. This assay

used recombinant TD2 as a substrate for processing by candidate proteases in plant and insect protein extracts. Formation of the ~40 kDa pTD2 product from the higher molecular weight (~55 kDa) TD2 precursor was assessed by separation of reaction products by SDS-PAGE and visualization of the cleavage product by Coomassie staining or immunoblotting. As an initial test to determine whether TD2 processing results from the action of a host plant- or insect-derived protease, recombinant TD2 was incubated at pH 9 (to simulate conditions in the lepidopteran gut) with crude protein extract from insect-damaged tomato leaves or frass collected from larvae (*Trichoplusia. ni*) grown on tomato leaves. As shown in Figure 3.3, TD2 was efficiently processed to a ~40 kDa product in the presence of the frass extract but not upon addition of either the leaf extract or a mock control. The identity of the 40 kDa protein as pTD2 was verified by LC-MS/MS (Figure 3.4), as well as by measurement of Ile-insensitive TD activity (see below). To exclude the possibility that TD2 processing results from the presence of a tomato protease excreted in the frass, we conducted processing assays with frass collected from larvae grown on artificial diet. The results showed that frass from artificial diet-reared larvae, which had not encountered tomato proteins, contained a protease that efficiently processes TD2 to pTD2 (Figure 3.3).

We next addressed the question of whether TD2 is processed by an insect protease during passage of food through the gut. Artificial diet containing recombinant TD2 was fed to *T. ni* larvae that had previously been reared on diet lacking TD2. Proteolytic cleavage of TD2 was then assessed by immunoblot analysis of frass protein. The appearance of pTD2 was detected in frass from diet-reared larvae (Figure 3.5.A), thus demonstrating that TD2 is processed in the *T. ni* digestive system in the absence of

any other tomato protein. Repetition of this experiment with *M. sexta* larvae, whose relatively large size facilitates dissection of the gut into its component foregut, midgut, and hindgut compartments, showed that dietary TD2 was completely processed in the midgut, hindgut, and frass, whereas the predominant form of TD2 was the 55 kDa protein and only trace amounts of pTD2 were found in the foregut (Figure 3.5.B). These findings indicate that the midgut is the major site of TD2 processing.

TD2 is cleaved by a chymotrypsin-like protease

To further investigate the nature of the excreted insect protease that cleaves TD2, frass protein obtained from *T. ni* larvae reared on artificial diet was fractionated by ammonium sulfate precipitation (Figure 3.6.A and B). The protein fraction precipitating between 50-75% ammonium sulfate saturation contained a protease activity that cleaves the regulatory domain from TD2. This protein fraction, referred to hereafter as the frass protease, was largely deficient in the protease responsible for generating a slightly lower molecular weight cleavage product (pTD2*) that presumably results from further processing of pTD2. The partially purified frass protease was insensitive to inhibitors of aspartic (i.e., pepstatin), metallo (i.e., EDTA), cysteine (i.e., E-64) and amino (i.e., bestatin) peptidases (Figure 3.7.A). Among several serine protease inhibitors tested, the frass protease was insensitive to inhibition by aprotinin but sensitive to PMSF. We also tested N-*p*-Tosyl-L-phenylalanine chloromethyl ketone (TPCK) and chymostatin, which are specific inhibitors of chymotrypsin, to determine whether the protease might be a trypsin- or chymotrypsin-like serine protease. The results showed that cleavage of TD2

was partially inhibited by TPCK and strongly inhibited by chymostatin. Chymostatin inhibited TD2 processing by the frass protease in a dose-dependent manner (Figure 3.7.B). These results indicate that TD2 is cleaved by a chymotrypsin-like protease that is excreted from *T. ni* larvae.

To further test the hypothesis that TD2 is cleaved in the insect by a chymotrypsin-like protease, we used LC-MS/MS to compare the TD2 proteolytic fragments produced by the frass protease to those generated by digestion of the substrate with commercial chymotrypsin (Figure 3.4.B). For this purpose, the pTD2 product generated by the two proteases was excised from an SDS-PAGE gel and subjected to in-gel digestion with trypsin and subsequent analysis by LC-MS/MS. As shown in Figure 3.4.A, peptide fragments identified after cleavage of TD2 with the *T. ni* protease completely overlapped with the peptides identified from TD2 cleavage by commercial chymotrypsin. Taken together, these results provide evidence that removal of the TD2 regulatory domain during passage of the enzyme through the lepidopteran gut is catalyzed by a chymotrypsin-like protease of insect origin.

TD1 and TD2 exhibit differential stability to insect digestive proteases

The existence in tomato of separate genes encoding "housekeeping" (TD1) and defense-related (TD2) TD isoforms provides an opportunity to study how enzymes involved in primary metabolism have adopted novel roles in defense. We first addressed this question by comparing the effect of *T. ni* digestive (excreted) proteases on TD1 and TD2. As expected, incubation of TD2 with *T. ni* digestive proteases resulted in accumulation of

the pTD2 fragment that is highly resistant to proteases (Figure 3.8). That the pTD2 product formed in this reaction corresponds to the enzyme's catalytic domain was confirmed by enzymatic assays showing that processing is accompanied by an increase in Ile-insensitive TD activity (Figure 3.8.A and B). Incubation of TD1 with the frass protease fraction resulted in rapid disappearance of the substrate (Figure 3.8.A) and a corresponding disappearance in TD activity (Figure 3.8.C). In the absence of frass proteases, TD1 activity was completely inhibited by the addition of 10 mM Ile, and formation of an Ile-insensitive form of TD1 was not observed. We conclude that TD1 and TD2 are highly susceptible and resistance, respectively, to degradation by insect digestive proteases.

pH optimum and temperature stability of TD1 and TD2

Among the biochemical properties that likely facilitate TD2's ability to degrade Thr in the insect gut are activity at alkaline pH and resistance of the catalytic domain to insect digestive proteases. Previous experiments have shown that pTD2 is active at high pH and has a high temperature optimum (Chen et al., 2007). We performed experiments to determine whether these features are shared by TD1. Both recombinant enzymes exhibited a pH optimum between 8 and 10 (data not shown), indicating that the alkaliphilic nature of TD2 is not specific for this isoform. The temperature stability of the two proteins was assessed by measuring TD activity over a range of temperatures between 16 and 80°C (Figure 3.9.A). TD2 was active over a broad range of temperatures, with optimal activity observed around 60°C. This finding is consistent with the

temperature optimum of 58°C reported for pTD2 (Chen et al., 2007). In contrast to TD2, TD1 was active over a range of lower temperatures. Maximal TD1 activity was observed at 16°C and no activity was detected at temperatures above 55°C. The heat lability of TD1 was confirmed by incubating the enzyme at 55°C for various times prior to measuring enzyme activity (Figure 3.9.B). Incubation of TD1 at 55°C for 1 min was sufficient to completely inactivate the enzyme. The same incubation condition (i.e., 1 min at 55°C) resulted in a 7% decrease in TD2 activity. Following 30 min incubation at 55°C, the activity of TD2 was 76% of that observed in a control reaction maintained at 30°C (Figure 3.7A). These results demonstrate that the thermostability of TD2 is unique to this isoform.

Kinetic parameters of TD2 and TD1

Purified recombinant enzymes were used to compare the kinetic parameters of the tomato TDs. Using Thr as a substrate, the apparent K_m of TD1 and TD2 was 5.7 ± 0.6 and 1.0 ± 0.1 mM, respectively. The V_{max} of TD1 (18,759 ± 746 μmoles α-KB/mg protein/h), however, was 7.5-fold greater than the V_{max} of TD2 (2,494 ± 61 μmoles α-KB/mg protein/h). The turnover number (k_{cat}) for TD1 was approximately 8-fold higher than for TD2. The k_{cat}/K_m ratio was 36% larger for TD1, indicating that TD1 is more efficient in catalysis. We also determined the sensitivity of the enzymes to various concentrations of Ile (Figure 3.10). The activity of TD1 was completely inhibited by 1 mM and higher concentrations of Ile. TD2 activity was strongly but not completely inhibited by 1 mM

Ile. Even in the presence of 10 mM Ile, a low level TD2 activity (~5% of the control) still remained.

Discussion

Defensive function of tomato TD2

Several lines of evidence indicate that tomato TD2 performs a role in plant defense against lepidopteran insects. First, the *TD2* gene is strongly upregulated in tomato, potato and *N. attenuata* in response to leaf wounding (Hildmann et al., 1992; Samach et al., 1995; Dammann et al., 1997; Hermsmeier et al., 2001; Schittko et al., 2001; Kang et al., 2006). Second, the protein is enzymatically active in the frass of lepidopteran larvae reared on tomato leaves (Chen et al., 2005; Chen et al., 2007). And third, previous studies with JA mutants of tomato established a correlation between loss of TD2 expression and reduced resistance to arthropod herbivores (Li et al., 2002; Li et al., 2004). The latter genetic evidence was inconclusive because these mutants are compromised in many JA-regulated defensive traits. Direct evidence for an anti-insect role of TD was provided by Kang and coworkers (2006), who showed that silencing TD expression in *N. attenuata* increased the growth rate of *M. sexta* larvae. This study also showed that supplementation of *N. attenuata* leaves with exogenous Thr improved insect performance, indicating that Thr is likely a limiting nutrient for caterpillars feeding on wild-type plants. Silencing of *NaTD* also caused reduced accumulation of Ile and, as a consequence, decreased production of the bioactive jasmonate jasmonoyl-L-isoleucine (JA-Ile) (Staswick et al., 1998; Kang et al., 2006; Katsir et al., 2008). The increased susceptibility of *NaTD*-

silenced plants to insects was thus attributed mainly to reduced signaling through the JA pathway, although a post-ingestive function in Thr degradation is also possible (Kang et al., 2006). Because TD is encoded by a single gene in *N. attenuata*, it is likely that the essential function of this enzyme in branched-chain amino acid synthesis has constrained its ability to evolve as an anti-nutritional enzyme.

In contrast to *N. attenuata*, our results provide evidence that two distinct TD isoforms in tomato, TD1 and TD2, serve separate roles in Ile synthesis and post-ingestive Thr degradation, respectively. This conclusion is supported by the finding that *TD2*-silenced transgenic plants are compromised in resistance to the generalist insect *S. exigua*. In the most strongly silenced lines (TDAs7), TD2 activity was severely reduced (e.g. ~30-fold in flowers) without obvious negative effects on plant growth and development. This observation, together with the normal vegetative growth habit of *jai1* plants that fail to express detectable levels of *TD2* (Li et al., 2004), implies that TD1 provides sufficient amounts of Ile for growth and development. The fact that other JA-regulated defenses (e.g. PIs) are expressed normally in TD2-silenced lines also suggests that TD2 is not required for the synthesis of JA-Ile. Previous studies suggested that TD2 is one of multiple JA-inducible proteins in tomato that act synergistically to reduce the nutritional quality of the plant food (Chen et al., 2005; Felton, 2005; Chen et al., 2007). The measurable effect of silencing *TD2* expression on *S. exigua* growth provides direct evidence that TD2 is an important component of this induced defense response.

Biodegradative TD in tomato

Szamosi and coworkers (1993) described a biodegradative TD activity in senescing tomato leaves that is insensitive to feedback inhibition by Ile. Enzymatic studies differentiated this biodegradative isoform from an Ile-sensitive TD activity that decreased with leaf age. Interestingly, the Ile-insensitive enzyme had a lower molecular weight and higher affinity for Thr than did the Ile-sensitive enzyme. The authors concluded that tomato contains distinct biodegradative (Ile-insensitive) and biosynthetic (Ile-sensitive) TDs that are likely encoded by two different genes. Our results are consistent with the hypothesis that the biosynthetic isoform reported by Szamosi et al (1993) corresponds to TD1, whereas the biodegradative isoform corresponds to an active proteolytic fragment of TD2 that has lost the regulatory domain. Among the many metabolic changes that occur during leaf senescence is disintegration of chloroplasts and the upregulation of proteases (BuchananWollaston, 1997). Such proteases may be capable of processing TD2 upon its release from plastids in senescing tissues. We did not observe processing of endogenous TD2 in insect-damaged tomato leaves, nor did we obtain evidence for cleavage of recombinant TD2 by proteases in crude protein extracts from damaged (but non-senescing) tomato leaves. Future studies aimed at determining whether TD2 is processed during tomato leaf senescence are warranted.

Evolution of TD2

As is the case for plant secondary metabolites (Fraenkel, 1959), the restricted occurrence of *TD2* to a subgroup of *Solanum* spp (Chen et al., 2007) suggests that this gene evolved from *TD1*, which serves an essential role in plant primary metabolism. One obvious

hypothesis is that duplication of *TD1* and subsequent neofunctionalization produced a novel isoform (TD2) that functions in plant anti-insect defense. If this idea is correct, several key features that distinguish TD2 from TD1 must have evolved over time, presumably in response to selection pressure imposed by insect herbivores whose growth is limited by Thr availability. One of these features is the regulation of *TD2* expression by the JA signaling pathway (Chen et al., 2007). *cis*-acting elements responsible for JA-induced *TD* expression have been identified (Samach et al., 1995; Kang and Baldwin, 2006). It is possible that a gene duplication event placed *TD2* under the control of such *cis*-acting elements that pre-existed in the tomato genome. Gene duplication may be a common strategy in the Solanaceae for recruitment of anti-nutritional defenses from enzymes involved in primary metabolism. In support of this idea, tomato contains two genes (designated *ARG1* and *ARG2*) encoding the arginine-degrading enzyme arginase. Whereas *ARG1* is constitutively expressed in all tissues, *ARG2* is co-expressed with *TD2* in response to wounding and JA treatment (Chen et al., 2004). ARG2 was also shown to have a post-ingestive role depleting Arg in the lepidopteran midgut (Chen et al., 2005). Another example is the enzyme leucine aminopeptidase-N (LAP-N). While all plants appear to contain a constitutively expressed *LAP-N* gene, a wound- and JA-inducible isoform (LAP-A) is found only in the Solanaceae family (Chao et al., 2000). The high stability and activity of LAP-A in *M. sexta* larvae reared on tomato suggests a role for this peptidase in anti-insect defense (Chen et al., 2007). A recent study indicates that tomato LAP-A also plays a role in modulating the JA response pathway (Fowler et al., 2009).

The post-ingestive defensive function of TD2 depends on the ability of the enzyme to efficiently degrade Thr in the insect gut. Accumulation of the protein in the digestive tract is facilitated by wound-induced expression of *TD2* in insect-damaged leaves, as well as the inherent stability of the enzyme. Amino acid sequence determinants that confer resistance of pTD2 to digestive proteases remain to be identified. In this context, it is noteworthy that tomato TD1 and TD2 are 51% identical (74.5% similar) at the amino acid sequence level (Chen et al., 2007). Major blocks of sequence conservation in TDs from *E. coli*, *Saccharomyces cerevisiae*, and several higher plants are also conserved in the two tomato TDs (Taillon et al., 1988; Garcia and Mourad, 2004). That both tomato enzymes are active at high pH suggests that the alkaliphilic property of TD2 did not evolve in response to the environment of the lepidopteran gut. Rather, the inherent ability of TD1, as well as other TDs, to catabolize Thr at alkaline pH may have provided a starting point for the evolutionary specialization of TD2 as a defense protein.

Another important biochemical feature of TD2 as an anti-insect protein is its high stability. Our results show that TD2 has a temperature optimum around 60°C and is active over a wide range of temperatures. This property would presumably allow the enzyme to function at the elevated body temperatures experienced by insect larvae in natural conditions (Casey, 1976). In addition to thermostability, the excretion of catalytically active pTD2 from tomato-reared larvae indicates that this activated form of the enzyme is remarkably resistant to digestive proteases (Chen et al., 2007). In contrast to TD2, TD1 is inactivated by elevated temperatures and readily degraded by lepidopteran digestive proteases. It thus appears that TD2 has acquired unique biochemical features that are consistent with its role in defense. Because stable proteins better tolerate mutations than

unstable proteins, protein stability can promote the evolution of novel biochemical functions (Bloom et al., 2006). It has also been shown that proteins tend to be only marginally more stable than is required by their environment (Bloom et al., 2006). These considerations lead us to speculate that after duplication of *TD1*, subsequent mutations conferred increased stability to TD2 and increased capacity of the enzyme to degrade Thr in the insect gut. Strong selection pressure imposed by insect herbivores presumably facilitated this process of functional specialization. Structural biological approaches aimed at comparing TD1 and TD2 may help to elucidate sequence determinants that confer protease resistance and thermostability to TD2, and provide insight into how these determinants evolved.

Plant TDs, as their counterparts in bacteria, are feedback inhibited upon binding of Ile to allosteric sites in the regulatory domain (Sharma and Mazumder, 1970; Wessel et al., 2000; Halgand et al., 2002; Garcia and Mourad, 2004). Amino acid residues that mediate Ile binding to the regulatory domain of Arabidopsis TD have been identified and found to be highly conserved among TDs from monocots and dicots, including tomato TD1 (Wessel et al., 2000; Garcia and Mourad, 2004). Interestingly, two of these conserved residues (R499 and H542 in Arabidopsis TD) are not conserved in TD2 from tomato and potato. These changes may explain the residual activity of recombinant TD2 in the presence of high levels of Ile (Figure 3.8). Relaxed selection pressure on these residues in the TD2 regulatory domain would be consistent with the hypothesis that feedback inhibition by Ile is less important for the function of TD2 than it is for the function of the TD1 biosynthetic isoform.

In fact, a key biochemical feature of TD2 as an antinutritional protein is that proteolytic removal of the entire regulatory domain (~150 amino acids) allows the enzyme to efficiently degrade Thr in the presence of high concentrations of Ile (2.5 mM) present in the midgut (Chen et al., 2005; 2007). In this study, we demonstrate that conversion of TD2 into the biodegradative pTD2 variant occurs in the midgut of lepidopteran herbivores. This proteolytic processing event can be catalyzed by a chymotrypsin-like protease of insect origin. TD2 can thus be viewed as a proenzyme that is activated by proteolytic removal of the C-terminal regulatory domain. Other examples in which plant defense proteins are activated by insect digestive proteases have been described (Carlini et al., 1997; Wang and Constabel, 2003, 2004; Schmelz et al., 2006).

Our results support a scenario in which TD2 accumulates in the chloroplast in response to wound stress or other conditions that activate the JA signaling pathway. This unprocessed form of enzyme is most likely subject to feedback inhibition by Ile pools in the chloroplast. Although it remains to be determined whether TD2 performs a role in amino acid biosynthesis *in planta*, the complete lack of TD2 expression in the *jai1-1* mutant (Li et al., 2004) indicates that the enzyme is not required for normal plant growth and development. Upon ingestion of induced leaf tissue by a lepidopteran insect, TD2 is cleaved by a chymotrypsin-like digestive protease, presumably after release of the substrate from the chloroplast. Because lepidopterans depend on chymotrypsin for food digestion, it would appear that tomato has exploited this fundamental aspect of insect digestive physiology as part of a complex antinutritional defense strategy that includes protease inhibitors and amino acid-degrading enzymes. Chymotrypsin-like proteases in lepidopteran insects are encoded by a large gene family (Srinivasan et al., 2006; Broehan

et al., 2008). Our results indicate that numerous chymotrypsin-like proteases from *T. ni* are excreted in the frass (E Gonzales-Vigil and G Howe, unpublished results). The broad substrate specificity of chymotrypsin (Kraut, 1977; Peterson et al., 1995) suggests that more than one of these enzymes is capable of cleaving TD2. If this is the case, it would be difficult for *T. ni* or other lepidopterans to adapt to TD2-mediated defense by blocking the processing of TD2. Other mechanisms by which insects may adapt to TD2-mediated Thr degradation include activation of transport systems that efficiently sequester dietary Thr and proteolytic destruction of pTD2. X-ray crystallography studies promise to provide insight into the structural basis of pTD2 resistance to digestive proteases.

Acknowledgements

The experiments described here would not have been possible without the technical assistance of Christopher Bergum.

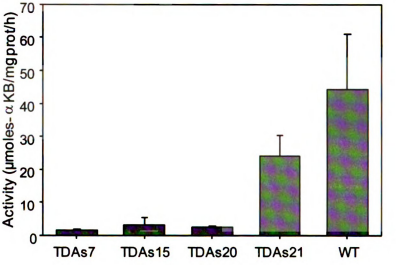


Figure 3.1. TD activity in flowers of TD2 antisense lines.

Open flowers from individual (T_1 generation) transgenic plants were collected and frozen in liquid nitrogen. The tissue was ground in liquid nitrogen and 100 mg of the resulting powder was homogenized in 600 µL aqueous extraction buffer (see Methods). Following centrifugation, the supernatant was used for TD assays. The bars represent the mean ± SE (n=10 for TDAs7; n= 12 for TDAs15; n=15 for TDAs20; n=8 for TDAs21; n=7 for wild-type).

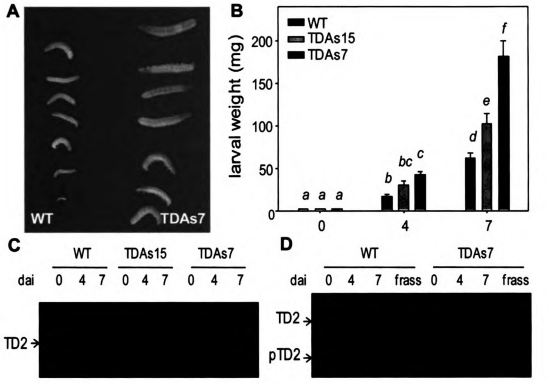


Figure 3.2. TD2 reduces the growth rate of *Spodoptera exigua* larvae.

Three-day old larvae were transferred from artificial diet to 4-week old wild-type or *35S-TD2As* transgenic lines (TDAs15 or TDAs7). One larva was caged per plant as described in the Methods section. At the indicated time points after challenge (days after infestation), larvae were weighted and returned to their plant of origin.

(A) Representative picture of larvae recovered 7 days after infestation (dai) of wild-type and TDAs7 plants.
(B) Weight gain of *S. exigua* on wild-type and two independent *35S-TD2As* transgenic lines. Values indicate the mean ± SE before infestation (0), and at 4 and 7 dai. Means with the same italicized letter are not significantly different at a *p*-value of 0.01 (Differences of Least Squares Means adjusted with the Bonferrroni inequality).
(C) Immunoblot analysis of TD2 protein accumulation in undamaged control (before infestation, 0 in figure) and damaged tomato leaves at 4 and 7 dai.
(D) Immunoblot analysis of TD2 accumulation in the damaged leaves from wild-type and TDAs7 plants. The lane marked "frass" represents protein extracted from frass of *S. exigua* larvae reared on wild-type and TDAs7 transgenic plants.

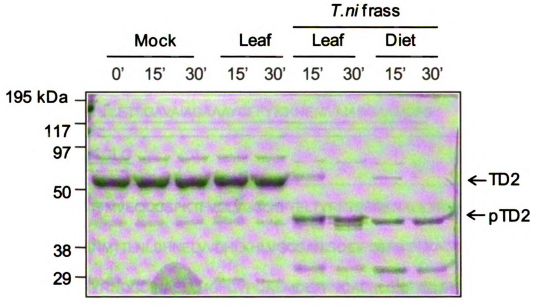


Figure 3.3. TD2 is cleaved by an insect protease.

Recombinant TD2 (40 μg) was incubated at 37°C (pH 9.5) for the indicated time (min) with crude protein extract (0.25 μg protein) from *T. ni*-damaged tomato leaves (Leaf), frass from *T. ni* reared on tomato (*T. ni* frass, Leaf), or frass from *T. ni* reared on artificial diet (*T. ni* frass, Diet). As a control, TD2 was incubated at 37°C with extraction buffer only (Mock). Arrows denote the unprocessed form of recombinant TD2 (~55 kDa) and the pTD2 processed form (~40 kDa) lacking the C-terminal regulatory domain.

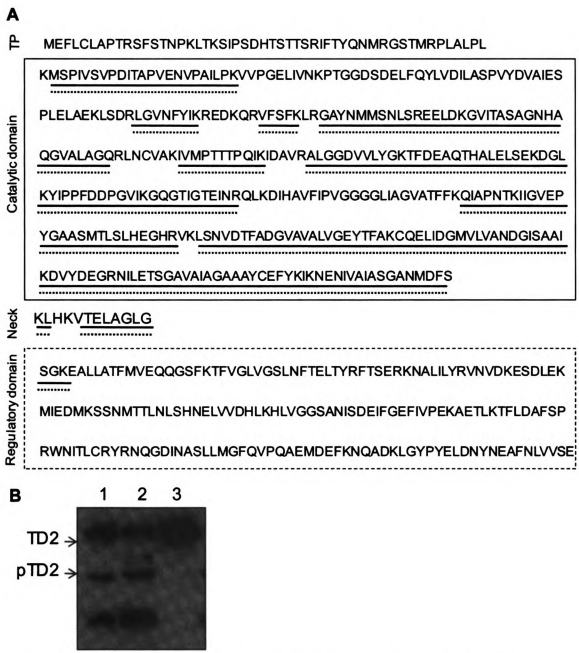


Figure 3.4. Comparison of TD2 peptide sequence identified after TD2 processing by frass proteases and commercial chymotrypsin.

(A) Sequencing of proteolytic processing products obtained after incubation of TD2 with an extract from *T. ni* frass (dotted line) or commercial chymotrypsin (solid line). Processing reactions were conducted as described in the legend to Figure 3.2. Cleavage products (~40 kDa) were excised from polyacrylamide gels and subjected to in-gel trypsin digestion and LC-MS/MS. Peptides identified were mapped to the various domains of TD2: TP (chloroplast transit peptide), Catalytic domain, Regulatory domain.

(B) Western blot analysis of TD2 proteolysis products obtained after incubation of recombinant TD2 with partially purified proteases excreted from *T. ni* (lane 1), and commercial bovine chymotrypsin (lane 2). As a control, TD2 was incubated without addition of proteases (lane 3).

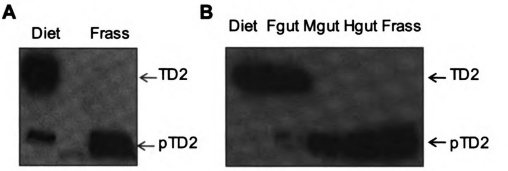


Figure 3.5. *In vivo* processing of TD2.

Insects were reared on artificial diet until they reached the 3rd (*M. sexta*) or 4th instar (*T. ni*), at which time larvae were starved for 16 h before being transferred to TD2-containing artificial diet. Following a 24 h period of feeding on TD2-supplemented diet, insect frass and the remaining diet were collected. The actively feeding larvae were frozen at -80°C for 10 min and then dissected. Protein extracts from the various dissected gut regions were analyzed by Western blotting for the presence of TD2.

(A) *T. ni* larvae were fed with artificial diet containing 0.01% TD2. Each lane was loaded with the following amount of total protein: diet, 0.5 μg; frass, 1 μg.

(B) *M. sexta* fed larvae were fed with artificial diet containing 0.0075% TD2. Each lane was loaded with the following amount of total protein: diet, 1 μg; foregut (Fgut in the figure), 60 μg; midgut (Mgut); 120 μg; hindgut (Hgut), 30 μg; frass, 30 μg.

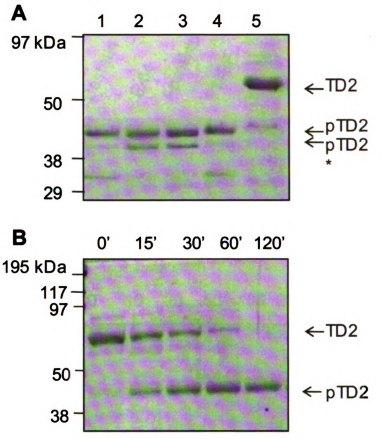


Figure 3.6. Fractionation of digestive proteases excreted in the frass of *T. ni* reared on artificial diet.

(A) *T. ni* larvae were grown on artificial diet until fifth instar. At this stage, feces were collected and stored at -20°C until further use for protein extraction. Protein was extracted in an aqueous buffer (see Methods) and subsequently fractionated by ammonium sulfate precipitation. Fractions were dialyzed against extraction buffer prior to use in the TD2 processing assay. The cleavage assay was performed with recombinant TD2 (0.2 µg) and one of the following protein fractions (0.25 µg frass protein): Lane 1, total soluble protein extracted from frass; lane 2, 0-25% ammonium sulfate cut; lane 3, 25-50% ammonium sulfate cut; lane 4, 50-75% ammonium sulfate cut; lane5, soluble protein after precipitation with 75% ammonium sulfate. TD2 cleavage products were separated by SDS-PAGE and the gel was stained with Coomassie Blue.

(B) Coomassie Blue-stained gel showing the TD2 cleavage products generated at various times after incubation of TD2 with the 50-75% ammonium sulfate fraction described in panel (A).

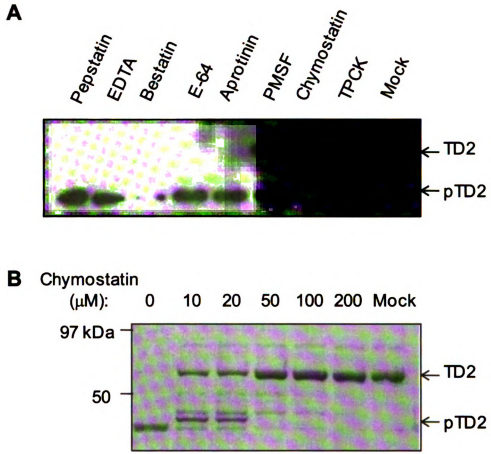


Figure 3.7. TD2 is cleaved by an excreted chymotrypsin-like protease from *T. ni.*

(A) Effect of protease inhibitors on TD2 processing. The indicated protease inhibitors were incubated with a protein fraction containing the *T. ni* frass protease for 15 min at 25°C, at which time 0.2 μg TD2 substrate was added. Cleavage reactions were incubated at 37°C for 1 h and then stopped by addition of SDS-containing loading buffer and incubation for 10 min at 100C. Formation of pTD2 was assessed by Western blot analysis with an anti-TD2 antibody.

(B) Dose-dependent effect of chymostatin on TD2 processing by an excreted protease from *T. ni*. Chymostatin (at the indicated concentration) was incubated with the frass protease for 15 min prior to addition of 0.4 μg TD2 substrate. Reactions were incubated and processed as described in panel (A). Reaction products were separated by SDS-PAGE and the resulting gel was stained with Coomassie Blue. A reaction containing TD2 without the frass protease or chymostatin was included as a control (Mock).

Figure 3.8. Degradation of tomato TDs by insect digestive proteases.

Recombinant TD substrates (0.2 μg) were incubated with the frass protease fraction (0.25 μg) for the indicated time at 37°C. As a control, the substrates were incubated at 37°C in the absence of frass protease (Mock).

(A) Analysis of cleavage reactions by SDS-PAGE and Coomassie staining of the gel.

(B) TD2 enzymatic activity assayed after the cleavage reaction. Activity was measured either in the absence (black bars) or presence (gray bars) of 10 mM Ile.

(C) TD1 enzymatic activity assayed after the cleavage reaction. Activity was measured either in the absence (black bars) or presence (gray bars) of 10 mM Ile.

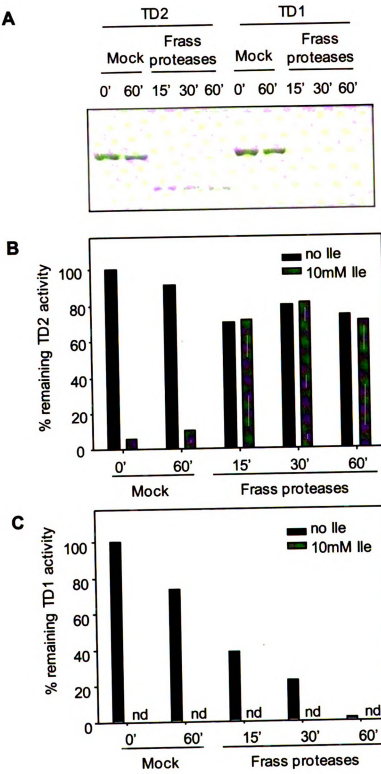
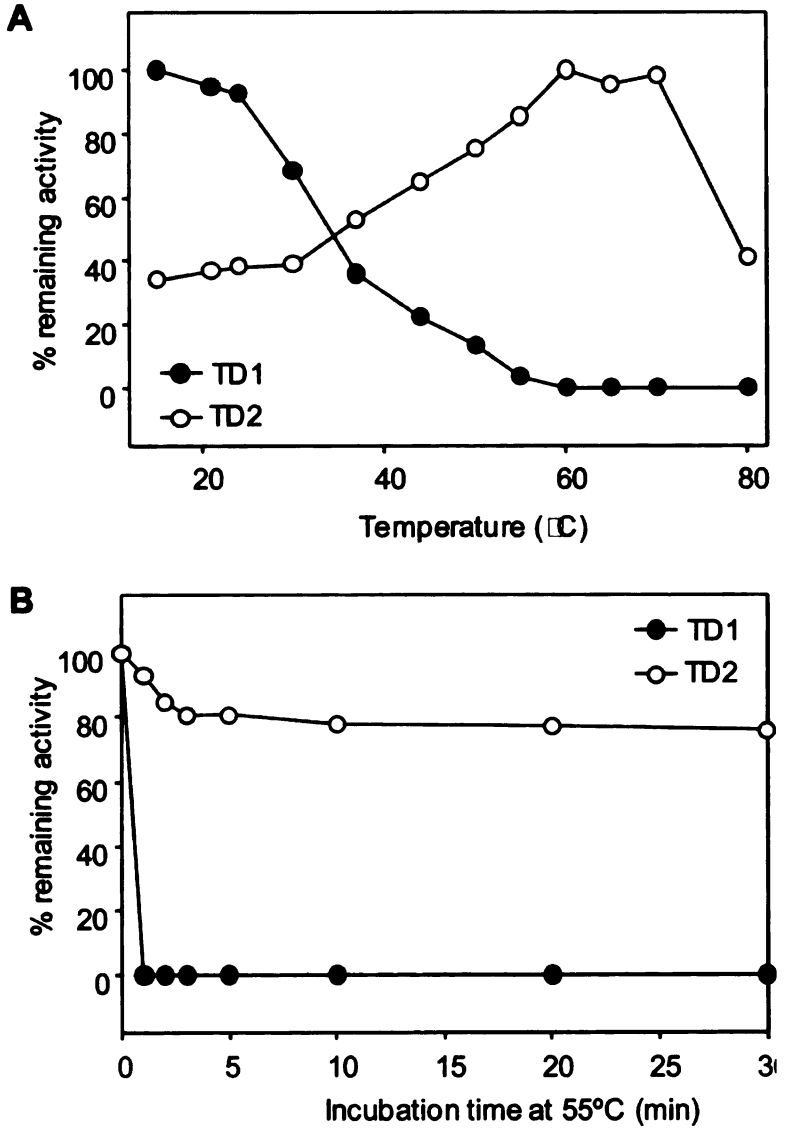

A
TD2
TD1
Mock
Frass proteases
Mock
Frass proteases
0' 60' 15' 30' 60' 0' 60' 15' 30' 60'
B
% remaining TD2 activity
no Ile
10mM Ile
0
20
40
60
80
100
0' 60' 15' 30' 60'
Mock
Frass proteases
C
% remaining TD1 activity
no Ile
10mM Ile
0
20
40
60
80
100
nd nd nd nd nd
0' 60' 15' 30' 60'
Mock
Frass proteases

Figure 3.9. Temperature stability of tomato TD1 and TD2.

(A) Differential temperature optimum of tomato TD1 and TD2. Reaction mixtures containing 0.20 μg recombinant TD1 (closed circles) or TD2 (open circles) were incubated at the indicated temperature for 30 min prior to measuring TD activity. Activity is expressed relative to the activity observed at the temperature optimum 16°C and 60°C for TD1 and TD2, respectively.

(B) Differential heat inactivation of TD1 and TD2. Recombinant proteins (0.20 μg) were incubated at 55°C for the indicated time (1 min to 30 min) prior to measuring TD activity at 30°C for 30 min. Activity is expressed relative to a control reaction that was not pre-incubated at 55°C.


A
100
80
60
40
20
0
% remaining activity
TD1
TD2
20
40
60
80
Temperature (□C)
B
TD1
TD2
100
80
60
40
20
0
% remaining activity
0
5
10
15
20
25
Incubation time at 55°C (min)

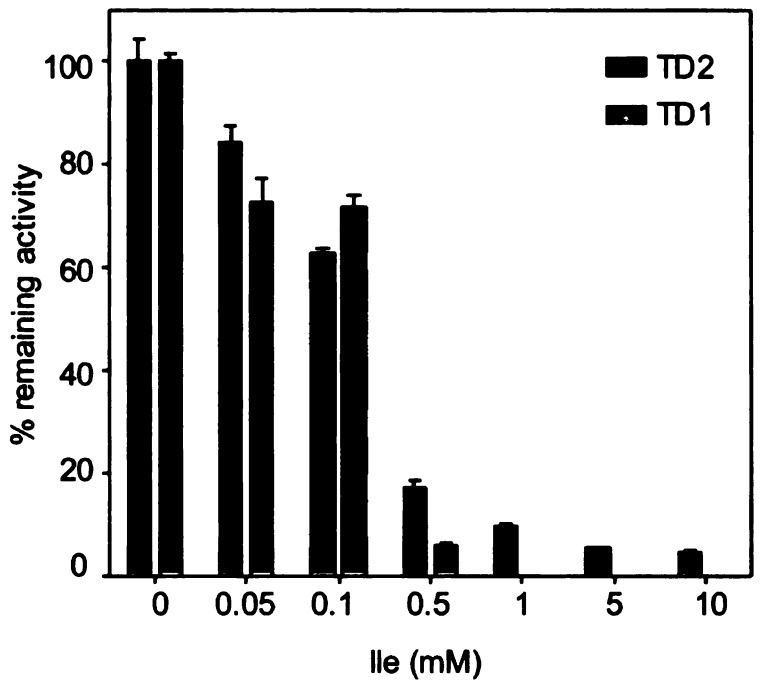


Figure 3.10. Sensitivity of tomato TDs to Ile.

Enzyme activity assays were performed in the presence of various concentrations (0 to 10 mM) of the allosteric inhibitor Ile. Enzyme activity is expressed as a percentage of the activity measured in a control reaction that did not contain the inhibitor.

LITERATURE CITED

Bernays EA, Chapman RF (1994) Host-plant selection by phytophagous insects. Chapman & Hall, New York

Bernays EA, Chapman RF, Singer MS (2004) Changes in taste receptor cell sensitivity in a polyphagous caterpillar reflect carbohydrate but not protein imbalance. J Comp Physiol A Neuroethol Sens Neural Behav Physiol **190:** 39-48

Bloom JD, Labthavikul ST, Otey CR, Arnold FH (2006) Protein stability promotes evolvability. Proceedings of the National Academy of Sciences of the United States of America **103:** 5869-5874

Bolter C, Jongsma MA (1997) The adaptation of insects to plant protease inhibitors. J Insect Physiol **43:** 885-895

Broehan G, Kemper M, Driemeier D, Vogelpohl I, Merzendorfer H (2008) Cloning and expression analysis of midgut chymotrypsin-like proteinases in the tobacco hornworm. J Insect Physiol **54:** 1243-1252

BuchananWollaston V (1997) The molecular biology of leaf senescence. JOURNAL OF EXPERIMENTAL BOTANY **48:** 181-199

Carlini CR, Oliveira AE, Azambuja P, Xavier-Filho J, Wells MA (1997) Biological effects of canatoxin in different insect models: evidence for a proteolytic activation of the toxin by insect cathepsinlike enzymes. J Econ Entomol **90:** 340-348

Casey TM (1976) Activity Patterns, Body-Temperature and Thermal Ecology in 2 Desert Caterpillars (Lepidoptera-Sphingidae). Ecology **57:** 485-497

Cates RG, Rhoades DF (1977) Patterns in Production of Anti-Herbivore Chemical Defenses in Plant Communities. Biochemical Systematics and Ecology **5:** 185-193

Chang CL (2004) Effect of amino acids on larvae and adults of Ceratitis capitata (Diptera : Tephritidae). Annals of the Entomological Society of America **97:** 529-535

Chao WS, Pautot V, Holzer FM, Walling LL (2000) Leucine aminopeptidases: the ubiquity of LAP-N and the specificity of LAP-A. Planta **210:** 563-573

Chen H, Gonzales-Vigil E, Wilkerson CG, Howe GA (2007) Stability of plant defense proteins in the gut of insect herbivores. Plant Physiol **143:** 1954-1967

Chen H, McCaig BC, Melotto M, He SY, Howe GA (2004) Regulation of plant arginase by wounding, jasmonate, and the phytotoxin coronatine. J Biol Chem **279:** 45998-46007

Chen H, Wilkerson C, Kuchar J, Phinney B, Howe G (2005) Jasmonate-inducible plant enzymes degrade essential amino acids in the herbivore midgut. Proc Natl Acad Sci U S A **102:** 19237-19242

Chen H, Wilkerson CG, Kuchar JA, Phinney BS, Howe GA (2005) Jasmonate-inducible plant enzymes degrade essential amino acids in the herbivore midgut. Proc Natl Acad Sci U S A **102:** 19237-19242

Colau D, Negrutiu I, Van Montagu M, Hernalsteens JP (1987) Complementation of a threonine dehydratase-deficient Nicotiana plumbaginifolia mutant after Agrobacterium tumefaciens-mediated transfer of the Saccharomyces cerevisiae ILV1 gene. Molecular and Cellular Biology **7:** 2552-2557

Dammann C, Rojo E, Sánchez-Serrano JJ (1997) Abscisic acid and jasmonic acid activate wound-inducible genes in potato through separate, organ-specific signal transduction pathways. Plant J **11:** 773-782

Duffey SS, Stout MJ (1996) Antinutritive and toxic components of plant defense against insects. Archives of Insect Biochemistry and Physiology **32:** 3-37

Felton GW (2005) Indigestion is a plant's best defense. Proc Natl Acad Sci U S A **102:** 18771-18772

Felton GW, Bi JL, Summers CB, Mueller AJ, Duffey SS (1994) POTENTIAL ROLE OF LIPOXYGENASES IN DEFENSE AGAINST INSECT HERBIVORY. Journal of Chemical Ecology **20:** 651-666

Fowler JH, Narvaez-Vasquez J, Aromdee DN, Pautot V, Holzer FM, Walling LL (2009) Leucine Aminopeptidase Regulates Defense and Wound Signaling in Tomato Downstream of Jasmonic Acid. PLANT CELL

Fraenkel GS (1959) The raison d'etre of secondary plant substances; these odd chemicals arose as a means of protecting plants from insects and now guide insects to food. Science **129:** 1466-1470

Gallagher DT, Gilliland GL, Xiao G, Zondlo J, Fisher KE, Chinchilla D, Eisenstein E (1998) Structure and control of pyridoxal phosphate dependent allosteric threonine deaminase. Structure **6:** 465-475

Garcia EL, Mourad GS (2004) A site-directed mutagenesis interrogation of the carboxy-terminal end of Arabidopsis thaliana threonine dehydratase/deaminase reveals a synergistic interaction between two effector-binding sites and contributes to the development of a novel selectable marker. Plant Mol Biol **55:** 121-134

Halgand F, Wessel PM, Laprévote O, Dumas R (2002) Biochemical and mass spectrometric evidence for quaternary structure modifications of plant threonine deaminase induced by isoleucine. Biochemistry **41:** 13767-13773

Hatfield GW, Umbarger HE (1968) A time-dependent activation of threonine deaminase. Biochem Biophys Res Commun **33:** 397-401

Hatfield GW, Umbarger HE (1970) Threonine deaminase from Bacillus subtilis. II. The steady state kinetic properties. J Biol Chem **245:** 1742-1747

Hermsmeier D, Schittko U, Baldwin IT (2001) Molecular interactions between the specialist herbivore Manduca sexta (Lepidoptera, Sphingidae) and its natural host Nicotiana attenuata. I. Large-scale changes in the accumulation of growth- and defense-related plant mRNAs. Plant Physiol **125:** 683-700

Hildmann T, Ebneth M, Peña-Cortés H, Sánchez-Serrano JJ, Willmitzer L, Prat S (1992) General roles of abscisic and jasmonic acids in gene activation as a result of mechanical wounding. Plant Cell **4:** 1157-1170

Joshi V, Laubengayer KM, Schauer N, Fernie AR, Jander G (2006) Two Arabidopsis Threonine Aldolases Are Nonredundant and Compete with Threonine Deaminase for a Common Substrate Pool. Plant Cell

Kang J-H, Baldwin IT (2006) Isolation and characterization of the threonine deaminase promoter in Nicotiana attenuata. Plant Science **171:** 435-440

Kang J, Wang L, Giri A, Baldwin I (2006) Silencing threonine deaminase and JAR4 in Nicotiana attenuata impairs jasmonic acid-isoleucine-mediated defenses against manduca sexta. Plant Cell **18:** 3303-3320

Kang JH, Wang L, Giri A, Baldwin IT (2006) Silencing threonine deaminase and JAR4 in Nicotiana attenuata impairs jasmonic acid-isoleucine-mediated defenses against manduca sexta. Plant Cell **18:** 3303-3320

Katsir L, Schilmiller AL, Staswick PE, He SY, Howe GA (2008) COI1 is a critical component of a receptor for jasmonate and the bacterial virulence factor coronatine. Proceedings of the National Academy of Sciences of the United States of America **105:** 7100-7105

Kraut J (1977) Serine proteases: structure and mechanism of catalysis. Annu Rev Biochem **46:** 331-358

Li C, Schilmiller AL, Liu G, Lee GI, Jayanty S, Sageman C, Vrebalov J, Giovannoni JJ, Yagi K, Kobayashi Y, Howe GA (2005) Role of beta-oxidation in jasmonate biosynthesis and systemic wound signaling in tomato. Plant Cell **17:** 971-986

Li C, Williams MM, Loh YT, Lee GI, Howe GA (2002) Resistance of cultivated tomato to cell content-feeding herbivores is regulated by the octadecanoid-signaling pathway. Plant Physiol **130:** 494-503

Li L, Zhao Y, McCaig BC, Wingerd BA, Wang J, Whalon ME, Pichersky E, Howe GA (2004) The tomato homolog of CORONATINE-INSENSITIVE1 is required for the maternal control of seed maturation, jasmonate-signaled defense responses, and glandular trichome development. Plant Cell **16:** 126-143

Mattson W (1980) Herbivory in Relation to Plant Nitrogen Content. Annual Review of Ecology and Systematics **11:** 119-161

Murdock LL, Shade RE (2002) Lectins and protease inhibitors as plant defenses against insects. Journal of Agricultural and Food Chemistry **50:** 6605-6611

Nation JL (2002) Insect physiology and biochemistry. CRC Press, Boca Raton, Fla.

Pechan T, Cohen A, Williams WP, Luthe DS (2002) Insect feeding mobilizes a unique plant defense protease that disrupts the peritrophic matrix of caterpillars. Proceedings of the National Academy of Sciences of the United States of America **99:** 13319-13323

Peterson AM, Fernando GJ, Wells MA (1995) Purification, characterization and cDNA sequence of an alkaline chymotrypsin from the midgut of Manduca sexta. Insect Biochem Mol Biol **25:** 765-774

Samach A, Broday L, Hareven D, Lifschitz E (1995) Expression of an amino acid biosynthesis gene in tomato flowers: developmental upregulation and MeJa response are parenchyma-specific and mutually compatible. Plant J **8:** 391-406

Schittko U, Hermsmeier D, Baldwin IT (2001) Molecular interactions between the specialist herbivore Manduca sexta (Lepidoptera, Sphingidae) and its natural host Nicotiana attenuata. II. Accumulation of plant mRNAs in response to insect-derived cues. Plant Physiol **125:** 701-710

Schmelz EA, Carroll MJ, LeClere S, Phipps SM, Meredith J, Chourey PS, Alborn HT, Teal PE (2006) Fragments of ATP synthase mediate plant perception of insect attack. Proc Natl Acad Sci U S A **103:** 8894-8899

Sharma RJ, Mazumder R (1970) Purification, properties, and feedback control of L-threonine dehydratase from spinach. J Biol Chem **245:** 3008-3014

Sidorov V, Menczel L, Maliga P (1981) Isoleucine-Requiring Nicotiana Plant Deficient in Threonine Deaminase. Nature **294:** 87-88

Srinivasan A, Giri AP, Gupta VS (2006) Structural and functional diversities in lepidopteran serine proteases. Cell Mol Biol Lett **11:** 132-154

Staswick PE, Yuen GY, Lehman CC (1998) Jasmonate signaling mutants of Arabidopsis are susceptible to the soil fungus Pythium irregulare. PLANT JOURNAL **15:** 747-754

Strassner J, Schaller F, Frick UB, Howe GA, Weiler EW, Amrhein N, Macheroux P, Schaller A (2002) Characterization and cDNA-microarray expression analysis of 12-oxophytodienoate reductases reveals differential roles for octadecanoid biosynthesis in the local versus the systemic wound response. Plant J **32:** 585-601

Szamosi I, Shaner DL, Singh BK (1993) Identification and Characterization of a Biodegradative Form of Threonine Dehydratase in Senescing Tomato (Lycopersicon esculentum) Leaf. Plant Physiol **101:** 999-1004

Taillon BE, Little R, Lawther RP (1988) Analysis of the Functional Domains of Biosynthetic Threonine Deaminase by Comparison of the Amino-Acid Sequences of 3 Wild-Type Alleles to the Amino-Acid Sequence of Biodegradative Threonine Deaminase. Gene **63:** 245-252

Wang J, Constabel CP (2003) Biochemical characterization of two differentially expressed polyphenol oxidases from hybrid poplar. Phytochemistry **64:** 115-121

Wang J, Constabel CP (2004) Polyphenol oxidase overexpression in transgenic Populus enhances resistance to herbivory by forest tent caterpillar (Malacosoma disstria). Planta **220:** 87-96

Wessel PM, Graciet E, Douce R, Dumas R (2000) Evidence for two distinct effector-binding sites in threonine deaminase by site-directed mutagenesis, kinetic, and binding experiments. Biochemistry **39:** 15136-15143

Zhu-Salzman K, Luthe DS, Felton GW (2008) Arthropod-inducible proteins: broad spectrum defenses against multiple herbivores. Plant Physiol **146:** 852-858

Chapter IV

Differential digestion of the *Solanum lycopersicum* leaf proteome by lepidopteran and coleopteran insect herbivores*

Abstract

The growth of phytophagous insects is constrained by the low protein content of plant tissue and by the plant defensive compounds, including proteins, which impair the ability of herbivores to acquire amino acids from the diet. In *Solanum lycopersicum* (cultivated tomato) and many other plants, anti-nutritional defense responses are regulated in an inducible manner by the jasmonate (JA) signaling pathway. Here, we used LC-MS/MS to investigate the fate of tomato leaf proteins during passage through the gut of three insect herbivores whose growth is negatively affected by the JA signaling pathway: the lepidopteran generalist *Trichoplusia ni* that consumes a wide variety of plant species, the lepidopteran specialist *Manduca sexta*, and the coleopteran specialist *Leptinotarsa decemlineata* that only thrive on Solanaceae species. Over 200 tomato proteins were identified in the feces from one or more of the three herbivores. Comparative analysis of the proteomic data showed that JA-inducible and other defense-related proteins were highly stable in all three insects, and also revealed insect order-specific differences in the enrichment of certain classes of dietary proteins. One of the most abundant tomato

* In chapter IV, the experiments were conducted by the author of this thesis and Dr. Youfu Zhao. Dr. Zhao was responsible for the microarray data and analysis.

proteins excreted by all three insects was an isoformof threonine deaminase (TD2) that efficiently degrades Threonine following proteolytic removal of the enzyme's C-terminal regulatory domain. TD2 was proteolytically activated in the gut of both lepidopteran insects but not in the gut of *L. decemlineata*. The results show that the protein content in plant food is differentially digested by different herbivore species, and suggest that these differences contribute to the outcome of plant-insect interactions.

Introduction

Herbivorous insects pose a significant threat to the reproductive fitness of plants in natural and agricultural ecosystems. Plants have evolved myriad constitutive and inducible self-protection mechanisms to thwart these attackers. Inducible defenses are initiated upon generation of signals at the plant-insect interface that trigger *de novo* synthesis of the plant defense hormone jasmonic acid (JA) (Schilmiller and Howe, 2005; 2005). JA is conjugated to isoleucine (Ile) to form the bioactive jasmonoyl-isoleucine, which is recognized by the Coronatine Insensitive 1-JAZ receptor system that orchestrates the expression of anti-insect defenses (Browse and Howe, 2008; Howe and Jander, 2008; Katsir et al., 2008; Yoshida et al., 2009). Mutants defective in the COI1 component of the JA receptor have been described in Arabidopsis, *Solanum lycopersicum* (cultivated tomato), and *Nicotiana attenuata* (Feys et al., 1994; Li et al., 2004; Paschold et al., 2007). These mutants have been invaluable for studies aimed at defining the global contribution of the JA pathway to plant defense against arthropod herbivores and other plant aggressors (Browse and Howe, 2008; Howe and Jander, 2008; Browse, 2009).

Gene expression profiling experiments have shown that tissue damage inflicted by wounding or herbivore attack results in large-scale, JA-mediated changes in gene expression (Hildmann et al., 1992; Reymond et al., 2000; Halitschke et al., 2001; Hermsmeier et al., 2001; Strassner et al., 2002; Reymond et al., 2004; Devoto et al., 2005; Sasaki-Sekimoto et al., 2005; Uppalapati et al., 2005). These studies provide insight into how plant defense responses are spatially and temporally regulated, but have been less useful for identifying specific proteins or other plant compounds that directly deter insect feeding. LC-MS/MS analysis of plant proteins that accumulate in the insect gut provides an alternative approach for identifying proteins that impair insect performance (Chen et al., 2005; Chen et al., 2007). This idea is based on the fact that many plant defense proteins are highly expressed in response to herbivory and, in addition, are highly resistant to insect digestive proteases. Proteinase inhibitors (PIs), polyphenol oxidases (PPOs), lectins, protease/peptidase, and enzymes involved in amino acid degradation are among the plant proteins that have a known or proposed role in anti-insect defense and that are stable after passage through the lepidopteran gut (Green and Ryan, 1972; Felton et al., 1992; Felton et al., 1994; Duffey and Stout, 1996; Murdock and Shade, 2002; Pechan et al., 2002; Chen et al., 2005).

The success of shotgun proteomic analysis depends in part on the availability of samples that are enriched for the target proteins. Several considerations indicate that insect frass provides an excellent source of protein for these analyses. Protease-resistant plant proteins that escape digestion in the insect gut are highly enriched in the frass, thus facilitating their identification by LC-MS/MS. Whereas dietary protein extract from the insect gut may be partially digested because of non-uniform exposure to digestive

proteases, host proteins excreted in the frass tend to be subject to homogeneous digestive conditions by virtue of their complete passage through the animal. Chen et al (2007) found that most of the tomato proteins that accumulate in the *M. sexta* midgut are also excreted in the frass, thus validating the choice of this material for proteomic analysis.

The low level of protein in plant tissue (Mattson, 1980) poses a major nutritional challenge to phytophagous insects that must acquire essential amino acids from dietary protein. The physicochemical properties of the insect digestive tract play a major role in determining how protein-derived amino acids and other nutrients are obtained from plant tissue. Perhaps not surprisingly, insects have evolved feeding and digestive strategies to optimize nutrient acquisition from diverse diets. Insight into the mechanisms by which insect herbivores cope with variation in dietary protein has come mainly from studies performed with "model" proteins, such as casein or ribulose bisphosphate carboxylase, incorporated into artificial diets (Martin et al., 1987; Simpson and Simpson, 1989; Bernays and Chapman, 1994). These studies have shown that herbivores can respond to low protein diet by increasing their rate of food ingestion or retention, a process known as compensatory feeding (Lundberg and Palo, 1993; Yang and Joern, 1994, 1994; Lee et al., 2004). However, because artificial diets do not accurately reflect the protein composition of plant tissue, it is unclear whether compensatory feeding is a widespread phenomenon in plant-insect interactions. It was recently shown, for example, that induction of compensatory feeding by *Manduca sexta* in response to high PI levels in *N. attenuata* was reduced by the presence of a toxic metabolite (nicotine) in the leaf diet (Steppuhn and Baldwin, 2007). The availability of modern proteomic technologies to monitor

hundreds of proteins in a single sample provides an attractive opportunity to better understand the dual role of the plant proteome in host defense and insect nutrition.

Protein digestion by insect herbivores is influenced not only by the chemical composition of the diet, but also by differences in the gut environment of different insect herbivores. The physicochemical properties of the coleoptera and lepidoptera digestive system, for example, differ in pH, redox potential, ionic strength, as well as in the type of proteases used for dietary protein digestion (Dow, 1984; Johnson and Felton, 1996; Bolter and Jongsma, 1997; Krishnan et al., 2007). Whether or not these order-specific differences in gut physiology affect protein digestion in natural diets remains unclear. In this study, we used LC-MS/MS to investigate the extent to which the protein content in tomato leaves is digested by insect herbivores that have different host ranges and gut physiologies. *L. decemlineata* (Colorado potato beetle), which belongs to the order coleoptera, specializes in the nightshade family and is the most destructive potato pest in North America, Europe and Asia (Franca et al., 1994; Hitchner et al., 2008). *M. sexta* is also highly specialized for feeding on solanaceous plants but belongs to the lepidoptera. We also studied the interaction of tomato with a second lepidopteran, *Trichoplusia ni,* which has a wider host range that includes tomato, potato, lettuce, beans, maize, and cotton (Vogel et al., 2007). Our results show that proteins involved in defense (i.e. JA-inducible proteins, proteases and protease inhibitors) are enriched in the frass of the three insects studied here. However, there were some insect order specific differences. Additionally, we found a correlation between the levels of expression in the leaf and the abundance of the protein in the excreta of the insects. We also show that JA-regulated defenses confer resistance of tomato to the beetle specialist *L. decemlineata* and the

generalist caterpillar *T. ni*, and that this form of induced defense affects different fitness components of the two insect species.

Results

Performance of *Leptinotarsa decemlineata* and *Trichoplusia ni* on tomato is reduced by COI1-dependent defenses

To assess the effect of JA-regulated defenses on the solanaceous specialist *Leptinotarsa decemlineata* (Colorado Potato Beetle) and the generalist *Trichoplusia ni* (cabbage looper), first-instar larvae were reared on either wild-type tomato plants or on the *jai1-1* mutant that fails to perceive JA (Li et al., 2004). *T. ni* larvae gained significantly more weight on *jai1-1* plants than on the wild-type host ($F_{1,180}$=842.08, P<0.0001) (Figure 4.1a and b). The weight of surviving larvae was increasingly affected with feeding time ($F_{2,180}$=78.78,P<0.0001); three days after initiating the feeding trial *jai1-1*-reared larvae were 3.4-fold heavier than larvae grown on wild-type plants, and this difference increased to 11.6- and 17.4-fold at the 6- and 8-day time points, respectively. At the end of the feeding trial, the survival rate of *T. ni* reared on *jai1-1* plants was significantly increased in comparison to caterpillars grown on wild-type plants ($F_{1,28}$=15.05,P=0.0006) (Figure 4.1c).

L. decemlineata larvae also gained more weight on *jai1-1* than on wild-type plants ($F_{1,160}$=13.0, P=0.0004) (Figure 4.2a and b). In contrast to *T. ni*, however, the largest

difference (1.9-fold) in *L. decemlineata* larval weight gain was observed 2 days after feeding. This difference was generally maintained throughout the time course of the experiment (Figure 4.2a). The number of *L. decemlineata* larvae recovered from wild-type plants at the end of the feeding trial was only 50% of that recovered from *jai1-1* plants ($F_{1,18}$=54.19,P<0.0001) (Figure 4.2c), indicating that COI1-dependent defenses have a major effect decreasing the survival of *L. decemlineata*. The experiments to assess the effect of jasmonate defenses on larval weight were repeated twice with similar results. These results demonstrate that the JA signaling pathway confers resistance of tomato to both *T. ni* and *L. decemlineata*, and provide evidence that the major effect of this defense pathway on *T. ni* and *L. decemlineata* fitness is related to larval growth rate and survivorship, respectively.

Fate of the tomato leaf proteome in the digestive tract of *L. decemlineata*

Induced resistance of tomato to lepidopteran insects results, at least in part, from the action of JA-regulated proteins that accumulate in and interfere with the insect's digestive system (Green and Ryan, 1972; Felton, 2005; Browse and Howe, 2008). In contrast to the wealth of information concerning the biochemical basis of JA-mediated resistance to caterpillars, very little is known about the nature of induced traits that confer resistance to coleopteran pests such as *L. decemlineata* (Felton et al., 1992). To investigate this question, we used LC-MS/MS to identify tomato leaf proteins that are excreted in the frass of tomato-reared *L. decemlineata* larvae. Discovery of these stable host proteins was facilitated by comparison of the most abundant proteins in *L. decemlineata*-challanged

tomato leaves to the most abundant proteins recovered in frass from larvae fed on these leaves (Figure 4.3a).

A total of 319 tomato proteins were identified in at least one of the two (i.e., leaf and frass) samples. As shown in 4.3a, 216 proteins were leaf-specific (group A), whereas 52 proteins were found in both the leaf and frass (group B). To gain insight into why group B proteins accumulate in the frass, proteins were assigned to a functional category according to the FunCat classification scheme (Ruepp et al., 2004) (Figure 4.3b). The larger classes of group B proteins were "perception/response to stimuli", which was the largest protein class among the leaf-specific proteins (group A), or as proteins involved in energy-generating processes. The latter group included highly abundant chloroplast proteins such as the large subunit of ribulose-1,5-bisphosphate carboxylase (RbcL) and the α and β subunits of the CF1 portion of ATP synthase. These results suggest that the occurrence of group B proteins in the frass may be explained by their high level of accumulation in leaves, and not necessarily by the resistance of the protein to insect digestive proteases. The third group (group C) of tomato proteins identified in this experiment was exclusive to the *L. decemlineata* frass (Figure 4.3; Table 4.1). The two most prominent functional categories represented by group C proteins was perception/response to stimuli (45%), which includes proteins involved in defensive processes; and protein degradation (11%). Tomato proteins having a defense role are enriched relative to less stable dietary proteins during passage of the leaf diet through *L. decemlineata.*

Effect of insect gut environment on digestion of tomato leaf proteins

The ability of phytophagous insects to digest dietary proteins may be influenced by the physiochemical features of the insect's digestive system (Terra, 1987, 1990; Felton et al., 1992; Johnson and Felton, 1996, 1996) or by the degree of host specialization of the insect. To test these hypotheses, we used LC-MS/MS to compare the composition of tomato leaf proteins excreted by larvae of *L. decemlineata* (coleopteran; solanaceous specialist), *M. sexta* (lepidopteran; solanaceous specialist), and *T. ni* (lepidopteran; generalist) reared on tomato. A total of 212 unique proteins were identified in at least one of the three frass samples (Appendix I). As shown in Figure 4.4a, 103 proteins were catalogued in both the *L. decemlineata* and *T. ni* frass samples and 141 proteins were identified in *M. sexta* frass. One hundred eighteen (56% of all proteins) proteins were specific to a single frass sample. The remaining proteins were identified either in all three samples (41 proteins) or in two of the samples (53 proteins). Inspection of the latter group of 53 proteins indicated that the frass proteomes of the two lepidopteran species are more similar to each other than they are to the *L. decemlineata* sample, and that the *L. decemlineata* proteome shared more proteins with the specialist *M. sexta* than it did with the generalist *T. ni*. These observations were supported by the distribution of proteins within various functional categories (Figure 4.4b). The general distribution of proteins identified in the frass of *T. ni* and *M. sexta* were very similar to each other, and distinguishable from that of the *L. decemlineata* frass.

The list of proteins identified in frass from the three insect herbivores revealed several noteworthy trends. For example, proteins involved in photosynthesis, protein degradation, protease inhibition and other defense-related processes were highly

represented in the group of 41 proteins that were common to all three frass samples (Table 4.1). Among these were several well characterized wound-inducible proteins implicated in plant anti-insect defense, including leucine amino peptidase-A (LAP-A), arginase 2 (ARG2), Thr deaminase 2 (TD2), TCI21 and other PIs, and polyphenol oxidase-F (PPO-F) (Green and Ryan, 1972; Chao et al., 1999; Wang and Constabel, 2004; Chen et al., 2005; Lison et al., 2006; Chen et al., 2007). Pathogenesis-related proteins (PR proteins) were also found in the frass. Several PR proteins were identified exclusively in the *L. decemlineata* sample. Host plant proteases, including several types of endoproteinases (metallo-, serine-, aspartic- and cysteine-proteases) and exoproteases (carboxypeptidases and aminopeptidase) were also highly represented in frass from all three insects. Members of the subtilisin-like protease family (Vera and Conejero, 1988; Tornero et al., 1996; Meichtry et al., 1999; Rautengarten et al., 2005) comprised the most abundant class of proteases as determined by number of spectral counts (Table 4.1). Based on the observation that peptides corresponding to only one subtilisin-like protease were identified in the leaf sample, it would appear that these proteins are highly stable and therefore enriched during passage of leaf tissue through the insect. The P69 members of the tomato subtilisin-like protease family have been classified as PR proteins (PR-7) (van Loon et al., 2006).

Overrepresentation of energy-related proteins was observed in the *L. decemlineata* frass sample (Figure 4.4.b). RbcL, the β subunit of ATPase and other proteins involved in the production of energy contribute to 29% of the total spectral counts identified in this sample. This is 2 to 3 times higher than the spectral counts from the same class of proteins in *T. ni* and *M. sexta* (14 and 10%, respectively), and more

similar to the ratio observed in the tomato leaf (36%). This could indicate differential processing of bulk leaf protein in *L. decemlineata* compared to the lepidopterans.

Differential digestion of TD2 in lepidopteran and coleopteran herbivores

Proteolytic removal of the C-terminal regulatory domain of TD2 produces an enzyme variant (called pTD2) whose ability to degrade Thr in the insect gut is not impaired by Ile, a negative allosteric regulator of TD catalytic activity (Chen et al., 2007). To further test the hypothesis that lepidopteran and coleopteran insects differentially digest tomato leaf protein, we investigated the fate of TD2 after passage of leaf tissue through *M. sexta, T. ni,* and *L. decemlineata*. Analysis of LC-MS/MS data showed that the peptide coverage of TD2 in *M. sexta* and *T. ni* frass was limited to the catalytic domain. In contrast, the peptide coverage observed for tomato leaf and *L. decemlineata* frass samples included both the catalytic and regulatory domains (Figure 4.5a). This observation suggested that TD2 processing does not occur, or occurs inefficiently, in *L. decemlineata*. To test this idea further, we used immunoblotting to analyze the extent of TD2 processing in frass samples obtained from the three insects reared either on wild-type plants or, as a control for antibody specificity, *jai1-1* plants. The results showed that TD2 is efficiently processed in the two lepidopteran insects but, interestingly, remained unprocessed in *L. decemlineata* frass (Figure 4.5b). This observation was extended to the potato TD2 (Figure 4.6).

RbcL, which is one of the most abundant soluble proteins in tomato leaves, is a major source of amino acids for insect herbivores and a suitable marker for bulk leaf

protein (Bernays and Chapman, 1994; Felton, 1996; Chen et al., 2007). We performed immunoblot analysis with an anti-RbcL antibody to determine the extent to which bulk tomato leaf protein was digested in the same protein samples used for analysis of TD2 processing. Cross-reactive RbcL polypeptides were not detected in frass from either of the two lepidopteran insects, whereas a lower molecular-weight form of RbcL (presumably a stable degradation product) was detected in *L. decemlineata* frass (Figure 4.5b). This finding is consistent with the proteomic analysis showing that RbcL-derived peptides are present in the beetle frass. We conclude that both nutritional (e.g., RbcL) and defense-related (TD2) host proteins are digested less efficiently by *L. decemlineata* in comparison to the lepidopteran insects.

Stability of JA-inducible proteins in the insect gut

We previously reported that several tomato JIPs, including TD2 and various PIs, accumulate in the midgut and frass of *M. sexta* larvae reared on tomato plants (Chen et al., 2005; 2007). To systematically investigate the fate of JIPs during passage of leaf tissue through different tomato-reared insects, we searched the list of tomato proteins identified in each frass sample against a list of tomato genes whose expression in leaves is differentially regulated in response to methyl-JA (MeJA) treatment. JA-regulated genes were identified by hybridizing mRNA from MeJA-treated wild-type and *jai1-1* leaves to the TOM1 cDNA array that contains 12,899 ESTs corresponding to ~8500 unique genes (Van der Hoeven et al., 2002; Alba et al., 2004). We identified 292 genes that are differentially regulated at least three-fold by the JA/COI1 pathway. Of these, 239 genes

were upregulated (and thus potentially encode a JIP), whereas 53 genes were down-regulated.

Merging of the transcriptomics and proteomics data showed that 45 proteins in either the tomato leaf or insect frass samples are encoded by genes whose expression was determined to be differentially regulated in the microarray experiment (Table 4.2). Genes encoding 33 of these proteins were upregulated by the JA/COI1 pathway and could thus be classified as putative JIPs. We used spectral count data, which is a reliable indicator of protein abundance in label-free shotgun proteomic analyses (Zhang et al., 2006), to estimate the abundance of each putative JIP in the various protein samples and the proportion of total spectra represented by JIPs. As shown in Figure 4.6a (and Table 4.2), JIPs accounted for 12% of the total spectral counts obtained for the herbivore-induced tomato leaf sample. In frass samples obtained from *T. ni*, *M. sexta*, and *L. decemlineata*, spectra corresponding to JIPs accounted for 43, 31, and 23%, respectively, of the total spectra. Therefore, the abundance of proteins annotated as JIPs (on the basis of gene expression data) appeared to be enriched in frass.

A breakdown of the most abundant JIPs in the leaf and frass samples is shown in Figure 4.7. These results are consistent with previous studies showing that LAP-A, TD2, and various serine PIs are among the most abundant tomato proteins in midgut and frass of tomato-reared *M. sexta* (Chen et al., 2005; Chen et al., 2007). The silver leaf whitefly1 (SLW-1) and YjgF proteins, which were previously described as JA-inducible inducible (van de Ven et al., 2000; Li et al., 2004), were also found in all frass and leaf samples. As shown in Table 4.2 and in previous studies (Hildmann et al., 1992; Pautot et al., 1993; Chen et al., 2004; Li et al., 2004; Uppalapati et al., 2008; Ishiga et al., 2009), the

expression of genes encoding the high abundance JIPs is strongly induced by the JA pathway. Enrichment of these JIPs in insect frass likely reflects the combined effects of high expression in insect-damaged leaves and protein stability in the insect gut.

Discussion

Adaptability of *L. decemlineata* to tomato defenses

Our results indicate that JA-regulated defenses impair the growth of both *T. ni* and *L. decemlineata*, but that different components of insect fitness are affected by COI1-dependent defenses. These defenses affected the development of *T. ni* larvae more than that of *L. decemlineata*, which might be expected for a generalist insect that is not well adapted to tomato defenses. The growth of *T. ni* was affected by COI1-dependent defenses for extended periods of larval development, whereas the growth of *L. decemlineata* was mainly affected at an early time point during the feeding trial. In contrast to this effect on larval development, JA-based defenses had a greater effect on the survivorship of *L. decemlineata* (50% mortality) than *T. ni* (34% mortality). These results provide evidence that JA-regulated defenses have differential effects on the mortality and growth of different insect herbivores. The decreased growth of *T. ni* on wild-type plants correlated with an enrichment of JIPs in *T. ni* frass. One interpretation of this observation is that *T. ni*, as a generalist herbivore, is less adapted than solanaceous specialists such as *M. sexta* and *L. decemlineata* for digesting JIPs that have anti-insect activity.

Previous studies have compared the growth of *L. decemlineata* larvae on *Solanum tuberosum* (potato) plants that were elicited with MeJA treatment or that were impaired in the JA pathway as a result of transgenic manipulation (Bolter and Jongsma, 1995; Royo et al., 1999). Consistent with these studies, we observed that the JA pathway has only a minor effect on the growth of *L. decemlineata* larvae. However, we found that the JA signaling pathway is responsible for high mortality of *L. decemlineata* larvae on tomato. The effect of JA-dependent defenses on weight gain after two days of feeding was not accentuated over time, implying that the surviving *L. decemlineata* larvae grew at the same rate on the wild-type and *jai1-1* plants. These observations suggest that JA-dependent defenses are mostly effective against *L. decemlineata* during early larval development and that the surviving larvae adapt quickly to the host defenses. Although *L. decemlineata* has only recently expanded its host range from the wild *Solanum rostratum* (Hitchner et al., 2008), it seems to adapt easily to other *Solanum* species (Gruden et al., 2004; Lyytinen et al., 2007). Mechanisms involved in the adaptability of *L. decemlineata* to different host plants may facilitate the insect's ability to rapidly develop resistance against pesticides (Roush et al., 1990).

Keys to the success of *L. decemlineata* on tomato

In comparison to the lepidopteran insects, frass from tomato-reared *L. decemlineata* contained relatively high levels of energy-related proteins, including RbcL, several subunits of ATP synthase and photosystem components. One interpretation of this observation is that incomplete digestion of plant material by *L. decemlineata* reflects a

strategy to minimize exposure to plant defense proteins. Limited protein digestion could result from a short transit time of plant material in the digestive tract. However, despite the voracity of the beetle, the transit time of food through *L. decemlineata* larvae (150 min) is reported to be similar to that of 5th instar *M. sexta* (120-160 min) (Martin et al., 1987; Krishnan et al., 2007). Another potential explanation for undigested protein in *L. decemlineata* frass is incomplete breakdown of chloroplasts or leaf tissue. Excretion of unprocessed TD2 in the frass of *L. decemlineata* could also be explained by protection of TD2 inside chloroplasts. However, the absence of intact RbcL in *L. decemlineata* frass argues against this idea. Additionally, some of the most abundant proteins identified in the leaf were Rubisco activase 1 and a chloroplastic phosphoglycerate kinase, which together account for 6.8% of the identified spectra from the leaf. That these proteins were not identified in the frass of *L. decemlineata* indicates that bulk chloroplastic protein is efficiently digested.

Our data support the idea that *L. decemlineata* is less efficient than Lepidoptera insects at digesting tomato leaf protein, which may reflect the different chemical properties of the coleopteran and lepidoptern digestive sytems. It has been suggested that the acidic gut of *L. decemlineata* may be protected from the action of PPO and other oxidative enzymes that are active at alkaline pH (Felton et al., 1992).

Degradation of Rubisco during herbivory

Because of its high abundance in plants leaves, Rubisco is a major source of amino acids for insect herbivores. Immunoblot analysis showed that breakdown of RbcL is initiated in

the herbivore-damaged leaf. Degradation of leaf Rubisco occurs in response to a wide range of environmental stresses, including exposure to low temperature, heavy metals, and ozone (Hajduch et al., 2001; Agrawal et al., 2002; Yan et al., 2006). Oxidative stress caused by these adverse conditions is thought to be an important factor in promoting Rubisco degradation (Desimone et al., 1996). An active mechanism for degrading Rubisco in response to wounding may indirectly allow the plant to recycle amino acids for use in the synthesis of defense-related proteins before the tissue is consumed by herbivores.

Chen and colleagues (2007) reported that RbcL is quickly degraded in the gut of *M. sexta*. It has also been shown that *L. decemlineata* and *M. sexta* gut fluids gratituously degrade Rubisco within minutes of exposure to digestive proteases (Martin et al., 1987; Brunelle et al., 1999). The use of more sensitive LC-MS/MS techniques allowed us to identify RbcL-derived peptides in each of the leaf and frass samples analyzed. Immunoblot analysis confirmed the presence of an RbcL degradation product in the *L. decemlineata* frass sample. The extent to which Rubisco and other bulk dietary protein is digested may be affected by interactions between insect proteases and plant PIs or other plant compounds that affect the solubility and digestibility of dietary protein (Johnson and Felton, 1996; McNabb et al., 1998). Interestingly, we identified an aspartic protease (CND41) in the lepidopteran frass samples that were highly depleted in RbcL. Because CND41 was previously shown to be involved in the degradation of RbcL during senescence (Kato et al., 2004), we speculate that this protease may also play a role in RbcL turnover during tomato-lepidopteran insect interactions.

TD2 and other JIPs

JIPs having a known or proposed defensive function against lepidopteran insects were identified in the frass of *L. decemlineata*, and included LAP-A, PPO-F, TD2, and TCI21. The acidic gut environment of *L. decemlineata*, together with its unique complement of digestive proteases, may render the defensive function of these host proteins inactive. TD2 is a good example of this. Lack of proteolytic processing in *L. decemlineata* is predicted to restrict the enzyme's ability to efficiently degrade Thr because of negative feedback inhibition by Ile, which presumably is present in the coleopteran gut. The lack of TD2 processing indicates that either the cleavage of the regulatory domain is inhibited in the *L. decemlineata* gut or that the protease responsible for cleaving TD2 is not present in the beetle gut. Identification of the protease responsible for processing TD2 will facilitate the testing of these hypotheses.

Detection of host defense proteins in insect frass does not necessarily imply a defensive role for the protein in the source insect because the activity of plant defensive proteins is often tailored to the gut physiological conditions of specific insects. For example, it has been suggested that the alkalophilic enzymes LAP-A, ARG2, and TD2 may act synergistically to deplete essential amino acids in the high pH environment of the lepidopteran gut (Chen et al., 2005; Felton, 2005). The relatively low activity of these enzymes under acidic conditions would likely limit their effectiveness in the acidic gut of *L. decemlineata* (Gu et al., 1999; Chen et al., 2004; Chen et al., 2007).

Only a subset of the proteins that were annotated as JIPs on the basis of the microarray data were identified in the frass. Because many JA-inducible genes encode

transcription factors and other low abundance proteins involved in signal transduction, this result is to be expected.

Trends of stable proteins

Tomato proteins that were enriched in the frass compared to the leaf proteome tended to belong to one of several defense-related categories, including perception and response to stimuli, protein degradation, and protease inhibitor activity. We also found that proteins (i.e., JIPs) encoded by JA-regulated genes were among the most abundant proteins in the frass samples. Taken together, these findings support the idea that defense-related plant proteins have been selected for increased stability. As is the case for PIs that impair insect digestive enzymes, high stability and resistance to proteolysis is expected to be a requisite feature of plant proteins that act in the insect gut.

The list of plant proteins identified in insect frass may provide insight into the biochemical determinants of protein stability. For example, disulfide bonds are known to confer stability and proteolytic resistance to PIs (Betz, 1993). Metals and cofactors can also contribute to protein stability (Woo et al., 2000; Mukhopadhyay and Lecomte, 2004; Bertini, 2007; Yogavel et al., 2008). Annotations based on information provided by UniProt, Expasy, and Percudani and Peracchi (2003) provide evidence that many tomato proteins excreted in the frass bind cofactors and metals. Pyridoxal phosphate-binding proteins were the most represented cofactor-containing proteins in the leaf sample. Because fewer pyridoxal phosphate-containing proteins were identified in the frass, binding of pyridoxal phosphate does not appear to be sufficient to confer stability (Figure

4.8). A similar trend was observed for Fe/S and metal (Ca^{2+}, K^{+}, Na^{+}, Mg^{2+}) containing proteins. Proteins containing heme and various heavy metals (Cu^{2+}, Fe^{2+}, Zn^{+}) were equally abundant in the leaf and frass, suggesting that these proteins may be more resistant to digestion by insect gut proteases.

Extracellular proteins tend to be more stable than cytosolic proteins (Xia et al., 2007). Because the apoplastic space is rich in hydrolytic enzymes, extracellular proteins are generally more resistant to proteolysis (Kusumawati et al., 2008). We observed a significant increase in the proportion of extracellular and cell wall-associated proteins in the frass compared to the leaf (Figure 4.9). A significant proportion (26%) of the plant proteins exclusively identified in *L. decemlineata* frass were annotated as pathogenesis-related (PR) proteins. PR proteins are typically targeted to either vacuoles or the apoplastic space (Fernandez et al., 1997), both of which have a pH comparable to that of the beetle gut (Rayle and Cleland, 1992; Lodish, 2000). The inherent stability of PR proteins in acidic and protease-rich environments may explain, at least in part, why they are so stable in the *L. decemlineata* digestive tract. The high stability of two PR proteins, PR-1 and germin, can be attributed to a compact quaternary structure that is stabilized by hydrogen bonds and hydrophobic interactions (Van Loon and Van Strien, 1999; Woo et al., 2000).

Oxidative stress in the gut

Plants often respond to biotic invasion with a rapid increase in enzyme-mediated production of reactive oxygen species (Levine et al., 1994). Increased levels of hydrogen peroxide and other reactive oxygen species have been negatively correlated with insect performance (Ramputh et al., 2002; Lou and Baldwin, 2006). Reactive oxygen species - generating enzymes are thought to decrease the palatability of plant tissues and impair insect digestive processes (Bi and Felton, 1995). Plants also produce secondary metabolites, including phenols and alkaloids that participate in reactive oxygen species production in the lepidoptera gut, thus reducing protein quality. It is possible that the activity of oxidative stress enzymes persist in the gut, exacerbating reactive oxygen species accumulation.

We identified peptides corresponding to tomato superoxide dismutase, catalase, ferredoxin, and peroxidases in the frass samples. Germin and germin-like proteins that generate H_2O_2, were among the most abundant proteins in the frass (Lane, 2000; Bernier and Berna, 2001). We also identified enzymes involved in the detoxification of oxidative stress: dehydroascorbate reductase (DHAR) and glutathione reductase (GR). It has been suggested that the resistance of oxidative stress enzymes to harsh environments is linked to their ability to generate ROS (Xia et al., 2007). It remains to be determined whether the tomato proteins identified here are active in the insect gut and, if so, whether they contribute to ROS generation or dissipation in the insect, or serve a role in reducing herbivore performance.

Plant proteases and protease inhibitors

Plant proteases appear to play several roles in anti-insect defense, including remobilization of amino acids within plant tissue, processing of plant proteins to release signaling peptides (Moura et al., 2001), and destruction of the peritrophic membrane that protects the midgut epithelium (Mohan et al., 2006). The latter group of proteins are cysteine proteases that exert toxic effects on lepidopteran insects (Pechan et al., 2002; Konno et al., 2004). Several tomato proteases that we identified in insect frass belong to the subtilisin-like family of serine proteases. In tomato, 15 subtilases have been classified into five subfamilies that exhibit distinct patterns of expression (Meichtry et al., 1999). The function of the large majority of these proteases in tomato and other plants remains unknown. We identified only one subtilisin-like protease in the leaf sample, whereas multiple isoforms (13 in *M. sexta*, 8 in *T. ni* , and 6 in *L. decemlineata*) were identified in the frass. The stability of these proteins during passage through the insect gut is consistent with their high thermostability conferred by the stabilizing effect of Ca^{2+} binding (Siezen et al., 1991). The fact that many subtilisin-like proteases from plants have an alkaline pH optimum (Hamilton et al., 2003) suggests that they may be catalytically active in the lepidopteran gut.

We identified the SLW1-like protein (also known as Drought Inducible Protein-1, DIP-1) an abundant protein in the frass of *L. decemlineata*. The tomato protein shares 66% and 69% amino acid identity with SLW1 from squash and DIP-1 from watermelon, respectively. The genes encoding the cucurbit proteins are induced by silverleaf whitefly feeding, MeJA and ethylene treatment, and drought stress (Kawasaki et al., 2000; van de Ven et al., 2000; Yokota et al., 2002). SLW1 and DIP-1 are cytosolic proteins belonging to the M20B family of proteases that have broad substrate specificity. Members of the

family have been implicated in deacetylation of peptides and amino acids and as carboxypeptidases (van de Ven et al., 2000). In watermelon it has been involved in the production of citrulline from glutamate under drought conditions (Kawasaki et al., 2000). The bacterial counterpart of these proteases has a neutral pH optimum (Javid-Majd and Blanchard, 2000). The role of the SLW1-like protein in plant-insect interactions, if any, remains to be determined.

Tomato leaves produce a wide range of PIs in response to wounding and insect herbivory. These defensive compounds are well characterized for their ability to inhibit digestive proteases in the insect gut (Ryan, 1990). Our proteomic analysis of insect frass identified PIs belonging to three mechanistic classes (serine-, aspartic-, and cysteine-type) proteases. Serine PIs were the most abundant class of PI identified in the frass of the two lepidopteran insects. This finding is consistent with the fact that most lepidopteran digestive proteases are serine-type proteases and are sensitive to serine PIs (Christeller et al., 1992). Genetic and biochemical evidence indicates that the serine PIs identified in our study serve a role in defense against lepidopteran insects and other biotic stresses (Zavala et al., 2004; Hermosa et al., 2006; Lison et al., 2006). Serine PIs also accounted for the major class of PIs identified in the frass of *L. decemlineata*. Cathepsin D-like proteases have been reported to be important for the initiation of protein digestion in *L. decemlineata*, whereas cysteine- and serine-proteases participate in later stages of protein hydrolysis (Brunelle et al., 1999). A cysteine protease inhibitor (multicystatin) was also detected in the lepidopteran frass, despite the fact that lepidopterans do not use cysteine proteases for digestion (Christeller et al., 1992) .

One rationale for cataloging plant dietary proteins in insect feces is to identify candidate proteins that impair insect nutrition. We successfully identified known tomato proteins that target insect digestive processes, including PIs, TD2, arginase, and cysteine proteases. Other excreted proteins, including SLW1-like, Wound Inducible Carboxypeptidase, and the YjgF family protein, were known to be JA-inducible but have unknown functions. Our findings raise the possibility that these proteins have a role in plant defense related to disruption of an insect digestive process. Transgenic approaches or feeding assays performed with heterologously expressed protein incorporated into artificial diet could be used to test this hypothesis.

Materials and Methods

Plant material and growth conditions

Wild-type and *jai1-1* tomato plants (*Solanum lycopersicum* cv. Castlemart) were grown on soil as described previously (Chen et al., 2005). *Leptinotarsa decemlineata* (Say) eggs were obtained from the Phillip Alampi Beneficial Insect Laboratory at the New Jersey Department of Agriculture. *Trichoplusia ni* (Hübner) eggs were obtained from Benzon Research. *Manduca sexta* (Linnaeus) eggs were obtained from the North Carolina State University Entomology Insectary. All eggs were hatched at 27°C (17 hours of light and 7 hours of dark).

Insect feeding bioassays

Fifteen newly hatched *L. decemlineata* larvae were placed on each of ten 5-week-old *jail-1* and wild-type tomato plants arranged in a randomized design, with each plant being the unit of replication. Movement of the larvae between the genotypes was prevented by bagging the pots inside a 8x16x18 in spun-polyester pot sleeve closed at the top (Hummert International, Earth City, MO, USA). Larvae were kept on plants at 17 h of light at 27°C and 7 hours of dark at 18°C. At each time point, three larvae were randomly selected from each pot and weighed. These readings were considered repeated measurements from the same experimental unit. After weighing, insects were returned to their plant of origin. At the end of the feeding trial, all larvae were collected and counted to determine the larval survivorship on each genotype. The conditions for the *T. ni* experiment were similar except that 10 larvae were used to infest 15 plants of each genotype.

Analysis of Variance was used to determine whether the mean weight and survivorship were significantly different between host genotypes. Larval weight data was log-transformed to obtain homogeneity of variance, and analyzed with two-way ANOVA with repeated measures. Both the effect of host genotype and time (after infestation) were evaluated. Untransformed data were used in figures. Survivorship, expressed as the percentage of the total number of larvae recovered at the end of the trial, was analyzed using one-way ANOVA. The statistical analysis was made with SAS® software, Version 9.1.3 of the SAS System for Windows (Copyright © 2002-2003).

Frass collection, protein extraction, and mass spectrometry

Four- to five-week-old wild-type tomato plants were used to feed *L. decemlineata* larvae. Twice a day, third and fourth instar insects were removed from the plants and transferred to Petri dishes for one hour. Feces were collected from the Petri dishes and the insects were returned to the plants. This method facilitated the collection of the watery feces of *L. decemlineata* and also minimized contamination of collected feces with plant material. Frass (approximately 200 mg) collected over a period of five days was pooled. Damaged leaves from the *L. decemlineata* feeding trial were collected and used for protein extraction.

In the case of *T. ni* and *M. sexta*, cut leaves from five-week-old tomato plants were used to feed the larvae. Cut leaves were replaced daily with fresh leaves. Frass pellets were collected from 4^{th} to 5^{th} instar larvae over a period of three to four days. Frass samples were stored at -20°C until subsequent use in protein extraction.

Proteins were extracted with a modified phenol-based extraction method described elsewhere (Chen et al., 2007) and quantified with a Bradford assay. For mass spectrometry analysis, one hundred mg of total protein were electrophoresed 1 cm into a 10% denaturing polyacrylamide gel. The gel was stained with Coomassie Brilliant Blue and destained overnight. Gel pieces containing the protein were excised and subjected to in-gel trypsin digestion. Briefly, the proteins were reduced with dithiothreitol, alkylated with iodoacetamide and digested with trypsin (Promega) (Jensen et al., 1999). Proteins were extracted with a modified phenol-based extraction method described elsewhere (Chen et al., 2007) and quantified with a Bradford assay.

For mass spectrometry analysis, one hundred mg of total protein were run 1 cm into a 10% denaturing polyacrylamide gel. The gel was stained with Coomassie Brilliant Blue and destained overnight. The piece of gel containing the proteins was excised and subjected to in-gel trypsin digestion. Briefly, the proteins were reduced with dithiothreitol, alkylated with iodoacetamide and digested with trypsin (Promega) (Jensen et al., 1999). The extracted peptides were re-suspended in a solution of 2% Acetonitrile/0.1% Trifluoroacetic Acid to 20 µl. From this, 10 µl were automatically injected by a Waters nanoAcquity Sample Manager and loaded for 5 minutes onto a Waters Symmetry C18 peptide trap (5 µm, 180 µm x 20 mm) at 4µL/min in 2%ACN/0.1%Formic Acid. The bound peptides were then eluted onto a Waters BEH C18 nanoAcquity column (1.7 µm, 100 µm x 100 mm) and eluted over 240 minutes with a gradient of 2% B to 30% B in 200 min at a flow rate of 0.45µl/min. Separations were done using a Waters nanoAcquity UPLC (Buffer A = 99.9% Water/0.1% Formic Acid, Buffer B = 99.9% Acetonitrile/0.1% Formic Acid) and sprayed into a ThermoFisher LTQ-FTICR mass spectrometer using a Thermo nano-spray source. Survey scans were taken in the FT (25000 resolution at m/z 400) and the top ten ions in each survey scan were then subjected to automatic low energy collision induced dissociation (CID) in the LTQ. The resulting MS/MS spectra were converted to peak lists using BioWorks Browser v3.2 (ThermoFisher) using the default LTQ-FT parameters and searched against tomato Transcript Assembly (TA) database (Release 2) found at TIGR Plant Transcript Assemblies website (http://plantta.tigr.org/cgi-bin/plantta_release.pl), using the Mascot searching algorithm (Matrix Science, version 2.1). The Mascot output was then analyzed using Scaffold (Proteome Software Inc., Portland, OR) to probabilistically validate

protein identifications using the ProteinProphet computer algorithm (Nesvizhskii et al., 2003). Assignments validated above the Scaffold 95% confidence filter were considered true.

Identification of tomato proteins by LC-MS/MS

Proteins were identified using a translated database constructed from the tomato Transcript Assembly (TA) database (Release 2) at the TIGR Plant Transcript Assemblies website (http://plantta.tigr.org/cgi-bin/plantta_release.pl). The following criteria were used for protein identification: the protein probability was ≥95% as determined by the Protein Prophet algorithm and at least two individual peptides per protein were identified. For protein quantification using spectral count data (Table 4.1 and Figure 4.7), we imposed the more stringent criterion of accepting peptides only if their probability of identification was ≥95%.

Identified tomato proteins were classified into functional categories according to the FunCat classification scheme (Ruepp et al., 2004). To do this, BLASTX was used to identify the best hit in the TAIR8 protein set for the TIGR Tomato Transcript Assemblies. Categories associated with the Arabidopsis top hit were then assigned by inheritance to the TIGR Tomato TA and thus represent putative functional assignments. In the few cases were no match was found but the protein has a known function (i.e. polyphenol oxidase F), the functional category was manually assigned.

Microarray analysis

Wild-type and *jai1-1* plants were treated with vaporous MeJA for either 8 or 24 h as previously described (Li et al., 2004). Equal amounts of total RNA from the 8 and 24 h time points were pooled prior to RNA labeling with Cy5 or Cy3 dUTP as previously described (Zhao et al., 2003). Three biological replicate RNA samples were used for hybridization experiments with Cy5-labeled WT RNA and Cy3-labeled *jai1-1* RNA that were mixed in equal molar ratios prior to hybridization. Labeled RNA was hybridized to the TOM1 cDNA array that contains 12,899 ESTs corresponding to ~8500 genes, which represents ~25% of all tomato genes (Van der Hoeven et al., 2002).

We used the following procedure to merge the microarray and proteomics data. Clone IDs for each element on the array were updated to determine the current Unigene cluster membership (SGN-U). The sequence of each SGN-U ID was used to perform BLASTN searches against the tomato Transcript Assembly .(*Solanum lycopersicum* release 2) with an E-value cutoff of $1e^{-100}$. The list of TA accessions identified in all proteomic experiments was used to search the BLAST output to identify SGN-U sequences that match the identified proteins.

Acknowledgements

We would like to thank Doug Whitten for doing the LC-MS/MS analysis, and Kevin Carr for the bioinformatics work. Nora Bello assisted us with the statistical analysis.

Table 4.1. Common proteins identified in the three frass samples

Description	Accession	WT leaf damaged by *L. decemlineata*	*L. decemlineata* frass	*T. ni* frass	*M. sexta* frass
Energy generation					
ATP synthase CF1 beta subunit	TA19970_4081	* 71[a]	* 25	2	* 6
Chlorophyll a-b binding protein CP24 10A	TA17514_4081	* 11	* 3	* 2	* 1
Cytochrome f	TA28980_4081	* 13	* 9	* 9	* 24
Light harvesting chlorophyll a /b binding protein	BI924800	*	*	*	*
Light harvesting chlorophyll a /b binding protein	BI928497	*	* 1	* 6	* 1
Photosystem I P700 chlorophyll a apoprotein A2	TA34808_4081	-	* 3	* 3	* 4
Photosystem I reaction center subunit II	AW442599	* 16	* 4	* 6	* 4
Plastocyanin, chloroplast	AI774561	* 5	* 1	* 12	* 15
Ribulose bisphosphate carboxylase large chain	TA24863_4081	* 65	* 22	* 12	* 14
Ribulose bisphosphate carboxylase small chain	TA17625_4081	* 7	* 2	*	*
Type I (26 kD) CP29 polypeptide	BW692506	* 15	* 4	* 4	* 6
JA-inducible proteins					
Acetylornithine deacetylase, putative	TA19203_4081	* 19	* 23	* 22	* 10
Carbonic anhydrase	TA22924_4081	-	*	*	* 7
Cathepsin D Inhibitor	TA17990_4081	* 8	*	* 5	* 2
Leucine aminopeptidase 1, chloroplast	TA18810_4081	* 26	* 6	* 63	* 36
Perchloric acid soluble translation inhibitor	BW687354	* 5	* 5	* 10	* 26
Polyphenol oxidase F	TA17484_4081	* 13	* 3	* 3	* 4
Threonine dehydratase	TA17896_4081	* 21	* 22	* 45	* 99
Wound/stress protein	BG135795	-	* 2	* 1	* 5
Pathogenesis-related					
24K germin like protein	TA18731_4081	* 6	* 15	* 10	* 63
Acidic 26 kDa endochitinase	TA19846_4081	-	* 2	* 1	* 2
Germin-like protein	BI210469	-	* 1	* 3	* 1

Table 4.1 (cont 'd)

Description	Accession	WT leaf damaged by *L. decemlineata*	*L. decemlineata* frass	*T. ni* frass	*M. sexta* frass
Glucan endo-1,3-beta-D-glucosidase	TA19657_4081	-	* 2	* 4	* 9
Glucan endo-1,3-beta-glucosidase B	TA18261_4081	-	* 10	* 4	* 2
Peroxidase	TA29420_4081	- 1	* 18	* 15	* 17
Suberization-associated anionic peroxidase 1	TA19712_4081	-	* 1	* 2	* 6
Superoxide dismutase	TA20387_4081	*	*	* 1	* 7
Thioredoxin peroxidase	TA17869_4081	* 4	* 1	* 4	* 2
Proteases					
Cucumisin-like serine protease	BP901605	-	*	*	*
Nucleoid DNA-binding-like protein	BP896163	-	*	*	* 9
P69C protein	TA17373_4081	-	* 1	* 1	* 3
Subtilisin-like protease	LESUBTILI	-	* 6	* 11	* 40
Protease inhibitors					
Proteinase inhibitor clone TPP11	BI923963	*	*	* 7	* 22
Proteinase inhibitor 1 PPI3A4	TA18695_4081	* 4	* 4	* 4	* 3
Proteinase inhibitor I	AW442733	* 3	*	* 3	* 5
Unknown					
Elicitor-inducible protein EIG-J7	TA26153_4081	-	*	*	*
OSJNBa0043A12.27 protein	TA22010_4081	- 1	* 2	* 3	* 7
Similar to SOUL Protein	TA23932_4081	- 5	* 4	*	* 2
Similarity to receptor protein kinase	TA28106_4081	* 2	* 3	* 3	* 8
Other					
Auxin-binding protein ABP19a	BG128560	* 8	* 2	1	* 7
Lipase/hydrolase, putative	BI930964	-	* 1	* 2	* 11

* : protein was positively identified in the sample with a protein probability ≥ 95%

- : protein was not identified in the sample with a protein probability ≥ 95%

a : spectral counts identified with ≥ 95% protein and peptide probability

Table 4.2. Abundance of JA-regulated proteins

				WT leaf damaged		*L. dec* frass		*T. ni* frass		*M. sex* frass	
SGN UniGene ID (SGN-U)	Ratio WT/*jai1* *	TIGR TA	TA Definition	SC **	% SC ***	SC	% SC	SC	% SC	SC	% SC
SGN-U313509	146.2	BG735335	Proteinase inhibitor I								
SGN-U313384	108.7	BI923963	Trypsin inhibitor-like protein					7	1.45	22	2.33
SGN-U313384	108.7	BI927980	Trypsin inhibitor-like protein	9	0.54	7	1.79	3	0.62	4	0.42
SGN-U313029	108.5	BW687354	YjgF family protein	5	0.30	5	1.28	10	2.07	26	2.75
SGN-U312622	105.7	TA17990_4081	Cathepsin D Inhibitor	8	0.48			5	1.04	2	0.21
SGN-U312574	85.7	TA17896_4081	Threonine deaminase 2	21	1.27	22	5.64	45	9.34	99	10.47
SGN-U312599	82.0	TA17484_4081	Polyphenol oxidase F	13	0.79	3	0.77	3	0.62	4	0.42
SGN-U312588	72.6	TA18489_4081	Proteinase inhibitor II	11	0.67			7	1.45	7	0.74
SGN-U316036	43.7	TA20325_4081	Proteinase inhibitor								
SGN-U315987	38.1	AY656838	Arginase 2	12	0.73			4	0.83	21	2.22
SGN-U312825	38.0	AW442733	Proteinase inhibitor I	3	0.18			3	0.62	5	0.53
SGN-U312825	38.0	TA19511_4081	Wound-induced proteinase inhibitor 1			1	0.26	3	0.62	1	0.11
SGN-U312822	34.7	TA18695_4081	Proteinase inhibitor 1 PPI3A4	4	0.24	4	1.03	4	0.83	3	0.32
SGN-U312377	17.3	TA18810_4081	Leucine aminopeptidase 1	26	1.57	6	1.54	63	13.07	36	3.81
SGN-U313835	14.9	TA19203_4081	Silverleaf whitefly-induced 1-like	19	1.15	23	5.90	22	4.56	10	1.06
SGN-U315434	13.9	TA26617_4081	Wound-inducible carboxypeptidase	7	0.42	5	1.28				
SGN-U316257	12.1	BI928273	Acid phosphatase-like protein								
SGN-U319550	9.9	TA22924_4081	Carbonic anhydrase							7	0.74
SGN-U313233	7.6	TA18815_4081	Pectin methylesterase								
SGN-U313233	7.6	TA18816_4081	Pectin methylesterase	3	0.18	6	1.54	4	0.83		
SGN-U326694	7.6	TA30868_4081	Multicystatin								
SGN-U312849	7.3	TA17913_4081	2-oxoglutarate-dependent dioxygenase 2								
SGN-U312623	6.9	TA17999_4081	Tomato chymotrypsin inhibitor 21	15	0.91	7	1.79	8	1.66	20	2.11
SGN-U313693	6.3	TA19093_4081	ATP synthase delta chain	6	0.36						
SGN-U316288	5.7	TA21680_4081	Subtilisin-like protease					2	0.41	15	1.59

Table 4.2 (cont 'd)

				WT leaf damaged		*L. dec* frass		*T. ni* frass		*M. sex* frass	
SGN UniGene ID (SGN-U)	Ratio WT/*jai1*	TIGR TA	TA Definition	SC	% SC	SC	% SC	SC	% SC	SC	% SC
SGN-U312380	4.4	TA17694_4081	Ferredoxin-1	2	0.12			1	0.21	3	0.32
SGN-U313117	4.3	TA18648_4081	Viroid RNA-binding protein	1	0.06			4	0.83		
SGN-U314922	4.1	TA17359_4081	Chaperonin-60 beta subunit	17	1.03						
SGN-U339179	4.1	TA17499_4081	Chaperonin-60 beta subunit								
SGN-U313537	3.9	TA19033_4081	Dehydroascorbate reductase	6	0.36			4	0.83	4	0.42
SGN-U312978	3.7	CN384512	NME2 protein	6	0.36			5	1.04		
SGN-U316550	3.6	TA21078_4081	Allene oxide cyclase								
SGN-U313930	3.5	TA19440_4081	Pyridoxine biosynthesis protein isoform B	5	0.30						
SGN-U312802	-3.0	TA17852_4081	Glyceraldehyde-3-phosphate dehydrogenase B	18	1.09			2	0.41		
SGN-U312802	-3.1	TA17893_4081	Glyceraldehyde-3-phosphate dehydrogenase B	11	0.67			2	0.41		
SGN-U312344	-3.2	TA17571_4081	Plastidic aldolase	7	0.42					1	0.11
SGN-U312344	-3.2	TA18103_4081	Plastidic aldolase NPALDP1	5	0.30						
SGN-U312344	-3.2	TA18110_4081	Plastidic aldolase NPALDP1	24	1.45	1	0.26				
SGN-U312336	-3.3	TA17514_4081	Chlorophyll a-b binding protein CP24 10A	11	0.67	3	0.77	2	0.41	1	0.11
SGN-U313206	-3.7	TA17799_4081	Light harvesting chlorophyll a /b binding protein of PSII			3	0.77			2	0.21
SGN-U313206	-3.7	TA18096_4081	Light harvesting chlorophyll a /b binding protein of PSII	4	0.24	5	1.28				
SGN-U312888	-3.8	TA18261_4081	Glucan endo-13-beta-glucosidase B			10	2.56	4	0.83	2	0.21
SGN-U312660	-3.9	TA18386_4081	Thiazole biosynthetic protein								

Table 4.2 (cont 'd)

SGN UniGene ID (SGN-U)	Ratio WT/*jai1* *	TIGR TA	TA Definition	WT leaf damaged SC**	WT leaf damaged % SC***	*L. dec* frass SC	*L. dec* frass % SC	*T. ni* frass SC	*T. ni* frass % SC	*M. sex* frass SC	*M. sex* frass % SC
SGN-U314750	-4.2	BI924800	Light harvesting chlorophyll a /b binding protein of PSII								
SGN-U314750	-4.2	BI928497	Light harvesting chlorophyll a /b binding protein of PSII			1	0.26	6	1.24	1	0.11

*: Expression ratio in wild-type to *jai1* plants treated with MeJA.

**: Number of spectral counts corresponding to the protein.

***: Percentage of spectral counts from total spectral counts identified in the sample.

Figure 4.1. Jasmonate defenses reduce *Trichoplusia ni* fitness on tomato.

Ten newly hatched larvae were transferred to fifteen 5-week old wild-type (WT) or *jai1-1* plants.

(a) Weight gain of *T. ni* was measured at 3, 6 and 8 days after infestation (dai) of the indicated host genotype. Values are the mean weight ± standard error. $F_{2,180}=78.78$, $P<0.0001$ for the interaction between feeding time and host genotype. $F_{1,180}=842.08$, $P<0.0001$ for host genotype. $F_{1,180}=122.63$, $P<0.0001$ for larval weight at 3 days; $F_{1,180}=467.68$, $P<0.0001$ for 6 days; $F_{1,180}=709.58$, $P<0.0001$ for 8 days.

(b) Photograph of representative larvae recovered at the 8-day time point.

(c) Percent recovery of larvae at the end of the experiment (8 days) as a measure of survivorship. Values are the mean percentage of recovery ± standard error. $F_{1,28}=15.05$, $P=0.0006$.

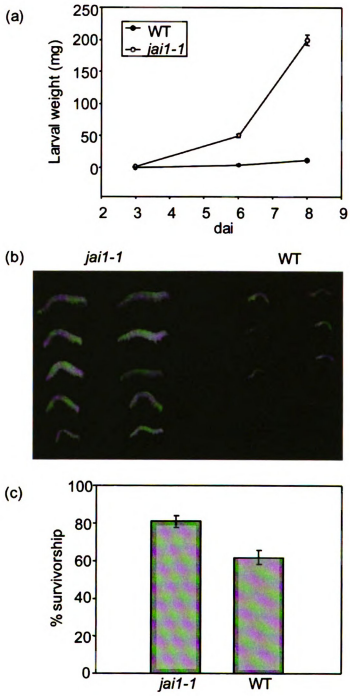

(a)
Larval weight (mg)
250
200
150
100
50
0
WT
jai1-1
2 3 4 5 6 7 8 9
dai
(b)
jai1-1
WT
(c)
% survivorship
100
80
60
40
20
0
jai1-1
WT

Figure 4.2. Jasmonate defenses reduce *Leptinotarsa decemlineata* fitness on tomato.

Fifteen newly hatched larvae were transferred to ten 5-week old wild-type (WT) or *jai1-1* plants.

(a) Weight gain of *L. decemlineata* was measured at 2, 4, 6 and 7 days after infestation (dai) of the indicated host genotype. Values are the mean weight ± standard error. $F_{1,160}=13.0$, $P=0.0004$ for host genotype. $F_{3,160}=0.98$, $P=0.4025$ for interaction between time and host genotype.

(b) Photograph of representative larvae recovered at the 7-day time point.

(c) Percent recovery of larvae at the end of the experiment (7 days) as a measure of survivorship. Values are the mean percentage of recovery ± standard error. $F_{1,18}=54.19$, $P<0.0001$.

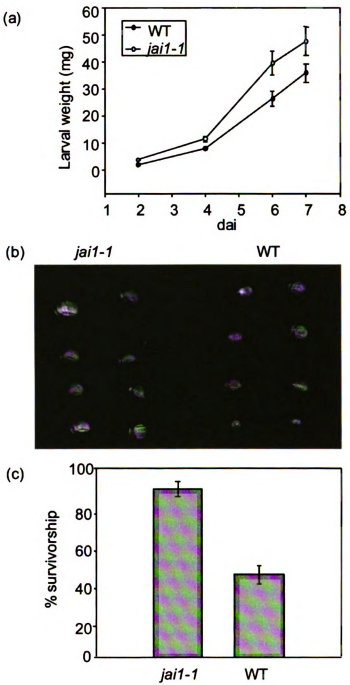

(a)
Larval weight (mg)
WT
jai1-1
dai
(b)
jai1-1
WT
(c)
% survivorship
jai1-1
WT

Figure 4.3. Distribution of proteins identified in the wild-type tomato leaf and frass of *L. decemlineata*.

(a) The Venn diagram shows the intersection of sets of tomato proteins identified in wild-type leaf damaged by *L. decemlineata* and the frass of tomato-reared *L. decemlineata* larvae. The diagrams were drawn to scale (proportional area) with 3Venn Applet.

(b) Distribution of proteins by functional category according to FunCat. The cumulative chart displays the percentage that each category contributes to the total. The identity of each functional category is shown in the legend (listed from top to bottom).

A, proteins identified in the leaf only; B, proteins identified in both leaf and frass; and C, proteins identified only in the *L. decemlineata* frass sample.

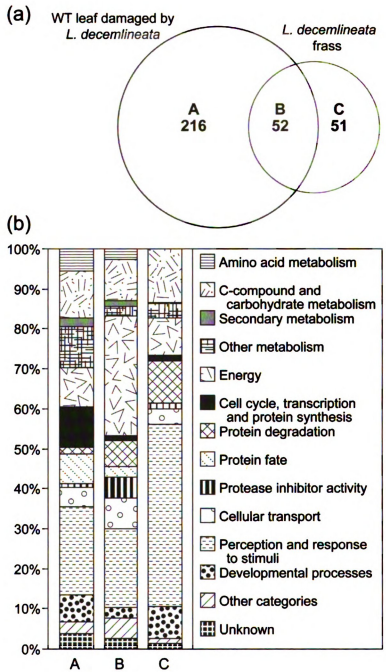

(a)
WT leaf damaged by
L. decemlineata
L. decemlineata
frass
A
216
B
52
C
51
(b)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
A
B
C
Amino acid metabolism
C-compound and carbohydrate metabolism
Secondary metabolism
Other metabolism
Energy
Cell cycle, transcription and protein synthesis
Protein degradation
Protein fate
Protease inhibitor activity
Cellular transport
Perception and response to stimuli
Developmental processes
Other categories
Unknown

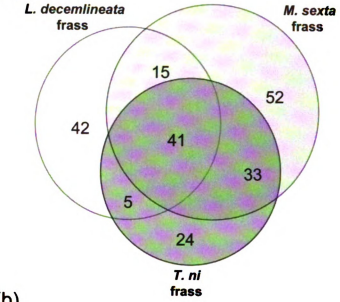
Figure 4.4. Distribution of the plant proteins identified in the three insect frass samples.

(a) Venn diagram showing the plant proteins identified in the frass. The diagrams were drawn to scale (proportional area) with 3Venn Applet .

(b) Distribution of identified proteins among functional categories according to FunCat. The cumulative chart displays the percentage that each category contributes to the total. The identity of each functional category is shown in the legend (listed from top to bottom).

L. dec: *Leptinotarsa decemlineata*; T. ni: *Trichoplusia ni*; and M. sex: *Manduca sexta*

(a)


L. decemlineata frass
M. sexta frass
T. ni frass
15
52
42
41
33
5
24

(b)

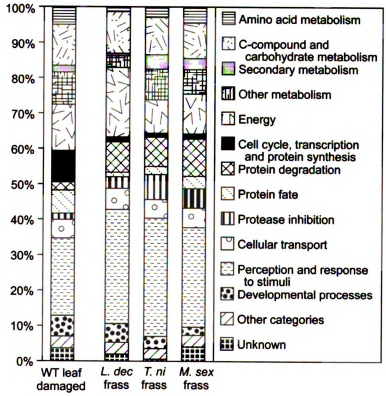
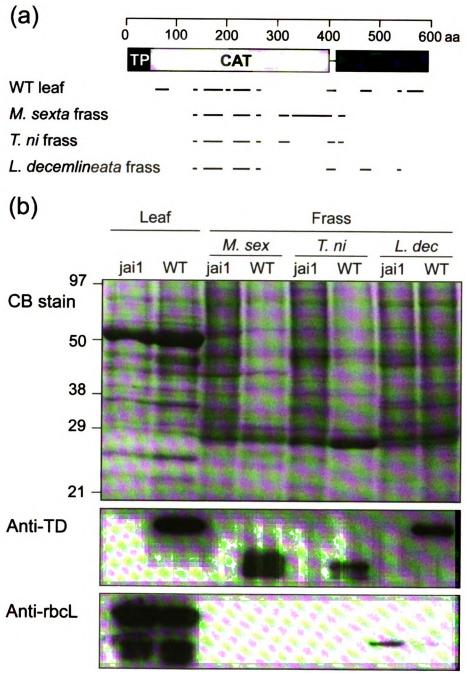

100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
WT leaf damaged
L. dec frass
T. ni frass
M. sex frass
Amino acid metabolism
C-compound and carbohydrate metabolism
Secondary metabolism
Other metabolism
Energy
Cell cycle, transcription and protein synthesis
Protein degradation
Protein fate
Protease inhibition
Cellular transport
Perception and response to stimuli
Developmental processes
Other categories
Unknown

Figure 4.5. Differential digestion of TD2 in the lepidopteran gut.

(a) Peptide coverage of TD2 in wild-type (WT) leaf and frass samples. The peptides identified by LC-MS/MS were mapped to the different regions of the protein: transit peptide (TP); catalytic domain (CAT); regulatory domain (REG) of TD2.

(b) Total protein was extracted from tomato leaves that were damaged by *L. decemlineata* (leaf), and feces of *M. sexta*, *T. ni,* and *L. decemlineata* reared on tomato (frass). Protein extracts (20 μg) were analyzed on a 10% SDS-polyacrylamide gel and stained with Coomassie blue. The same samples were used for Western blot analysis with an anti-TD2 and anti-RbcL antibodies


(a)
0
100
200
300
400
500
600 aa
TP
CAT
WT leaf
M. sexta frass
T. ni frass
L. decemlineata frass
(b)
Leaf
Frass
M. sex
T. ni
L. dec
jai1
WT
jai1
WT
jai1
WT
jai1
WT
97
CB stain
50
38
29
21
Anti-TD
Anti-rbcL

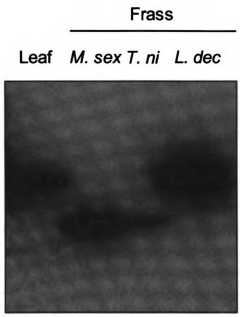


Figure 4.6. Processing of potato TD2.

Larvae from *M. sexta*, *T. ni* were grown on artificial diet until third to fourth instar. At that stage, larvae were transferred to potato plants (*Solanum tuberosum* cv White Red Craigg Royal) and frass was collected two days after infestation. *L. decemlineata* neonates were grown on potato until third instar. At that point they were transferred to new plants and frass collected two days later. Leaves from plants damaged by *L. decemlineata* were included as a control (Leaf). An antibody raised against tomato TD2 was used for immunoblotting.
L. dec: Leptinotarsa decemlineata; T. ni: Trichoplusia ni; and M. sex: Manduca sexta

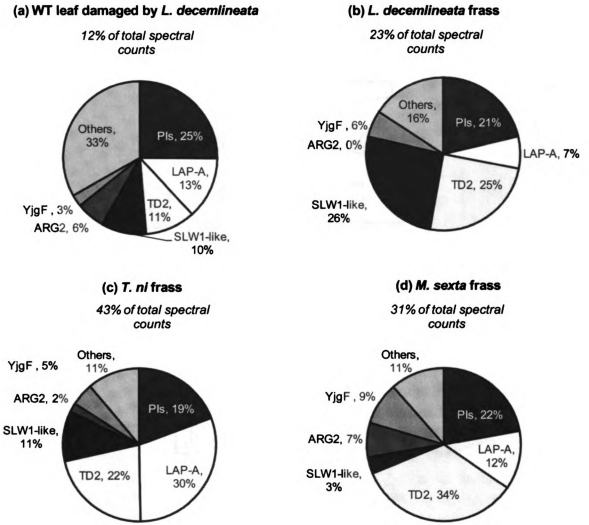


Figure 4.7. Identification of JA-inducible proteins.

The relative abundance of JA-inducible proteins (JIPs) identified in each of the four samples was estimated using the number of spectral counts.

PIs, protease inhibitors; LAP-A, Leucine aminopeptidase A, TD2: Threonine deaminase 2; SLW1-like, silverleaf whitefly-induced protein 1-like; ARG2, Arginase 2.

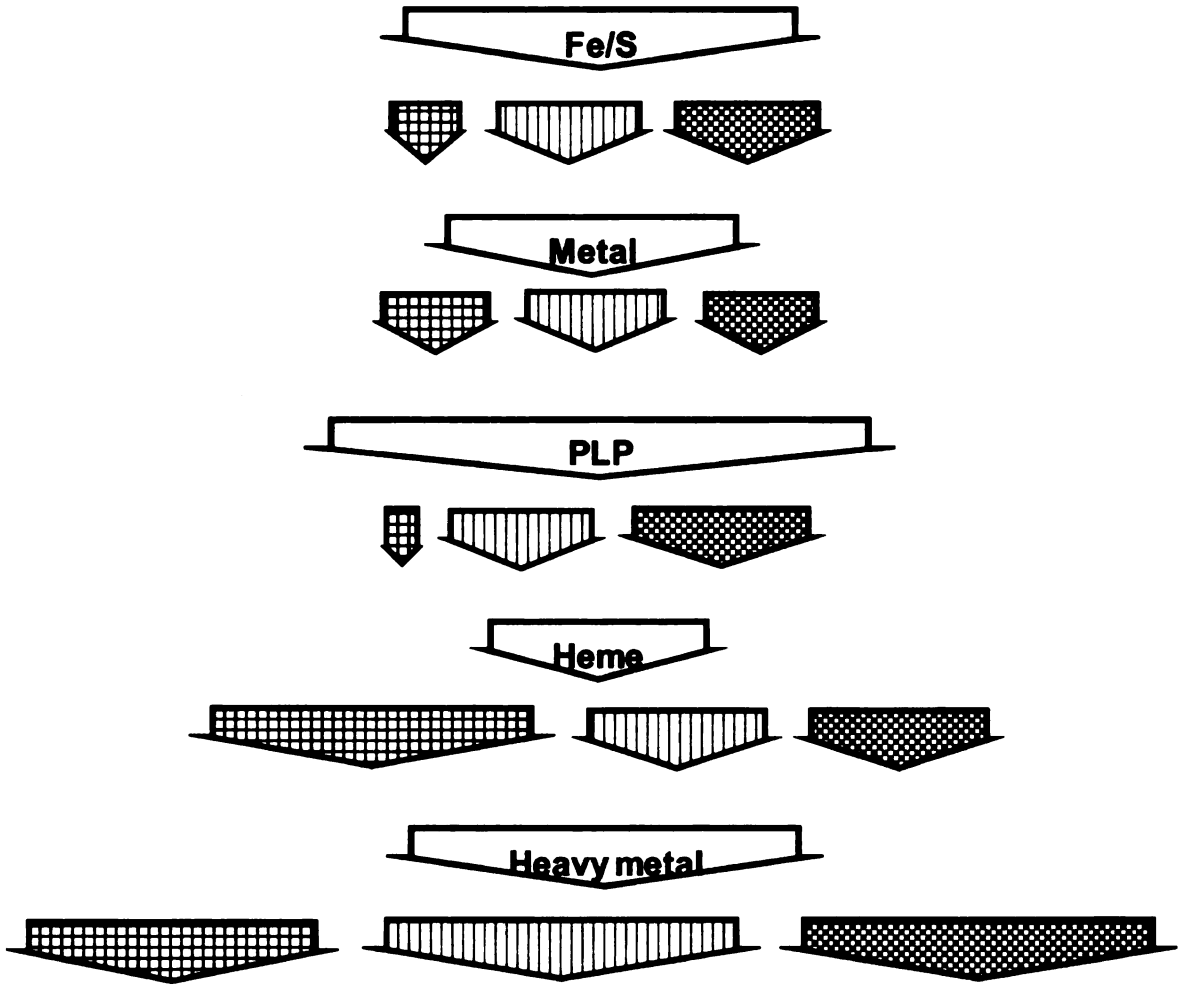


Figure 4.8. Distribution of proteins with cofactors.

The width of the arrow is proportional to the number of proteins containing the cofactor identified in the sample. Solid pattern: Wild-type leaf damaged by *L. decemlineata*; grid: *L. decemlineata* frass; stripped: *T. ni* frass; checkered: *M. sexta* frass. PLP: pyridoxal-phosphate.

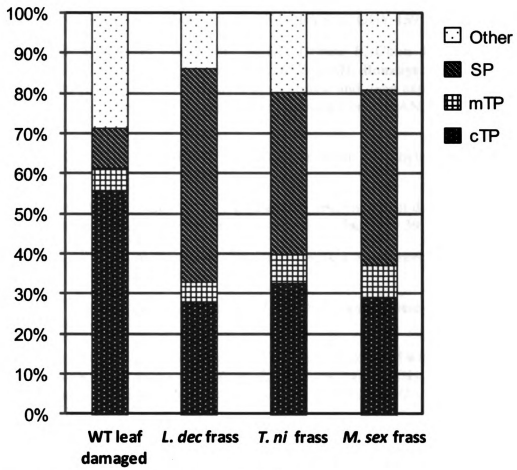


Figure 4.9. Subcellular localization of identified proteins.

For all of the TIGR Tomato TAs (*Solanum lycopericum* Release 2) the most similar Arabidopsis protein (TAIR v.8) was identified by using BLASTX (Tomato TA nucleotide translated vs. TAIR 8 proteins). The set of Arabidopsis proteins identified as best matches to the identified Tomato TAs were analyzed using the TargetP, ChloroP, SignalP programs which search for known target signal peptides at the N-terminus of a protein sequence.(SP: Secretory pathway, mTP: Mitochondrial transit peptide, cTP: Chloroplast transit peptide, *L. dec*: *Leptinotarsa decemlineata*; *T. ni*: *Trichoplusia ni*; and *M. sex*: *Manduca sexta*)

LITERATURE CITED

Agrawal GK, Rakwal R, Yonekura M, Kubo A, Saji H (2002) Rapid induction of defense/stress-related proteins in leaves of rice (Oryza sativa) seedlings exposed to ozone is preceded by newly phosphorylated proteins and changes in a 66-kDa ERK-type MAPK. JOURNAL OF PLANT PHYSIOLOGY **159:** 361-369

Alba R, Fei ZJ, Payton P, Liu Y, Moore SL, Debbie P, Cohn J, D'Ascenzo M, Gordon JS, Rose JKC, Martin G, Tanksley SD, Bouzayen M, Jahn MM, Giovannoni J (2004) ESTs, cDNA microarrays, and gene expression profiling: tools for dissecting plant physiology and development. PLANT JOURNAL **39:** 697-714

Bernays EA, Chapman RF (1994) Host-plant selection by phytophagous insects. Chapman & Hall, New York

Bernier F, Berna A (2001) Germins and germin-like proteins: Plant do-all proteins. But what do they do exactly? Plant Physiology and Biochemistry **39:** 545-554

Bertini I (2007) Biological inorganic chemistry : structure and reactivity. University Science Books, Sausalito, Calif.

Betz SF (1993) Disulfide Bonds and the Stability of Globular-Proteins. Protein Science **2:** 1551-1558

Bi JL, Felton GW (1995) Foliar Oxidative Stress and Insect Herbivory - Primary Compounds, Secondary Metabolites, and Reactive Oxygen Species as Components of Induced Resistance. JOURNAL OF CHEMICAL ECOLOGY **21:** 1511-1530

Bolter C, Jongsma MA (1997) The adaptation of insects to plant protease inhibitors. J Insect Physiol **43:** 885-895

Bolter CJ, Jongsma MA (1995) Colorado Potato Beetles (Leptinotarsa-Decemlineata) Adapt to Proteinase-Inhibitors Induced in Potato Leaves by Methyl Jasmonate. Journal of Insect Physiology **41:** 1071-1078

Browse J (2009) Jasmonate: preventing the maize tassel from getting in touch with his feminine side. Sci Signal **2:** pe9

Browse J, Howe GA (2008) New weapons and a rapid response against insect attack. Plant Physiol **146:** 832-838

Brunelle F, Nguyen-Quoc B, Cloutier C, Michaud D (1999) Protein hydrolysis by Colorado potato beetle, Leptinotarsa decemlineata, digestive proteases: The catalytic role of cathepsin D. Archives of Insect Biochemistry and Physiology **42:** 88-98

Chao WS, Gu YQ, Pautot VV, Bray EA, Walling LL (1999) Leucine aminopeptidase RNAs, proteins, and activities increase in response to water deficit, salinity, and the wound signals systemin, methyl jasmonate, and abscisic acid. Plant Physiol **120:** 979-992

Chen H, Gonzales-Vigil E, Wilkerson C, Howe G (2007) Stability of plant defense proteins in the gut of insect herbivores. Plant Physiol **143:** 1954-1967

Chen H, Gonzales-Vigil E, Wilkerson CG, Howe GA (2007) Stability of plant defense proteins in the gut of insect herbivores. Plant Physiol **143:** 1954-1967

Chen H, McCaig B, Melotto M, He S, Howe G (2004) Regulation of plant arginase by wounding, jasmonate, and the phytotoxin coronatine. J Biol Chem **279:** 45998-46007

Chen H, McCaig BC, Melotto M, He SY, Howe GA (2004) Regulation of plant arginase by wounding, jasmonate, and the phytotoxin coronatine. J Biol Chem **279:** 45998-46007

Chen H, Wilkerson C, Kuchar J, Phinney B, Howe G (2005) Jasmonate-inducible plant enzymes degrade essential amino acids in the herbivore midgut. Proc Natl Acad Sci U S A **102:** 19237-19242

Chen H, Wilkerson CG, Kuchar JA, Phinney BS, Howe GA (2005) Jasmonate-inducible plant enzymes degrade essential amino acids in the herbivore midgut. Proc Natl Acad Sci U S A **102:** 19237-19242

Christeller JT, Laing WA, Markwick NP, Burgess EPJ (1992) Midgut Protease Activities in 12 Phytophagous Lepidopteran Larvae - Dietary and Protease Inhibitor Interactions. Insect Biochemistry and Molecular Biology **22:** 735-746

Desimone M, Henke A, Wagner E (1996) Oxidative stress induces partial degradation of the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase in isolated chloroplasts of barley. PLANT PHYSIOLOGY **111:** 789-796

Devoto A, Ellis C, Magusin A, Chang HS, Chilcott C, Zhu T, Turner JG (2005) Expression profiling reveals COI1 to be a key regulator of genes involved in wound- and methyl jasmonate-induced secondary metabolism, defence, and hormone interactions. Plant Mol Biol **58:** 497-513

Dow JAT (1984) Extremely High Ph in Biological-Systems - a Model for Carbonate Transport. American Journal of Physiology **246:** R633-R636

Duffey SS, Stout MJ (1996) Antinutritive and toxic components of plant defense against insects. Archives of Insect Biochemistry and Physiology **32:** 3-37

Felton GW (1996) Nutritive quality of plant protein: Sources of variation and insect herbivore responses. Archives of Insect Biochemistry and Physiology **32:** 107-130

Felton GW (2005) Indigestion is a plant's best defense. Proc Natl Acad Sci U S A **102:** 18771-18772

Felton GW, Bi JL, Summers CB, Mueller AJ, Duffey SS (1994) POTENTIAL ROLE OF LIPOXYGENASES IN DEFENSE AGAINST INSECT HERBIVORY. Journal of Chemical Ecology **20:** 651-666

Felton GW, Donato KK, Broadway RM, Duffey SS (1992) Impact of Oxidized Plant Phenolics on the Nutritional Quality of Dietary-Protein to a Noctuid Herbivore, Spodoptera-Exigua. Journal of Insect Physiology **38:** 277-285

Felton GW, Workman J, Duffey SS (1992) Avoidance of Antinutritive Plant Defense - Role of Midgut Ph in Colorado Potato Beetle. JOURNAL OF CHEMICAL ECOLOGY **18:** 571-583

Fernandez C, Szyperski T, Bruyere T, Ramage P, Mosinger E, Wuthrich K (1997) NMR solution structure of the pathogenesis-related protein P14a. JOURNAL OF MOLECULAR BIOLOGY **266:** 576-593

Feys B, Benedetti C, Penfold C, Turner J (1994) Arabidopsis Mutants Selected for Resistance to the Phytotoxin Coronatine Are Male Sterile, Insensitive to Methyl Jasmonate, and Resistant to a Bacterial Pathogen. Plant Cell **6:** 751-759

Franca FH, Plaisted RL, Roush RT, Via S, Tingey WM (1994) Selection Response of the Colorado Potato Beetle for Adaptation to the Resistant Potato, Solanum-Berthaultii. ENTOMOLOGIA EXPERIMENTALIS ET APPLICATA **73:** 101-109

Green TR, Ryan CA (1972) Wound-Induced Proteinase Inhibitor in Plant Leaves: A Possible Defense Mechanism against Insects. Science **175:** 776-777

Gruden K, Kuipers AGJ, Guncar G, Slapar N, Strukelj B, Jongsma MA (2004) Molecular basis of Colorado potato beetle adaptation to potato plant defence at the level of digestive cysteine proteinases. Insect Biochemistry and Molecular Biology **34:** 365-375

Gu Y, Holzer F, Walling L (1999) Overexpression, purification and biochemical characterization of the wound-induced leucine aminopeptidase of tomato. Eur J Biochem **263:** 726-735

Hajduch M, Rakwal R, Agrawal GK, Yonekura M, Pretova A (2001) High-resolution two-dimensional electrophoresis separation of proteins from metal-stressed rice (Oryza sativa L.) leaves: Drastic reductions/fragmentation of ribulose-1,5-bisphosphate carboxylase/oxygenase and induction of stress-related proteins. Electrophoresis **22:** 2824-2831

Halitschke R, Schittko U, Pohnert G, Boland W, Baldwin IT (2001) Molecular interactions between the specialist herbivore Manduca sexta (Lepidoptera, Sphingidae) and its natural host Nicotiana attenuata. III. Fatty acid-amino acid

conjugates in herbivore oral secretions are necessary and sufficient for herbivore-specific plant responses. PLANT PHYSIOLOGY **125:** 711-717

Hamilton JM, Simpson DJ, Hyman SC, Ndimba BK, Slabas AR (2003) Ara12 subtilisin-like protease from Arabidopsis thaliana: purification, substrate specificity and tissue localization. Biochem J **370:** 57-67

Hermosa MR, Turra D, Fogliano V, Monte E, Lorito M (2006) Identification and characterization of potato protease inhibitors able to inhibit pathogenicity and growth of Botrytis cinerea. PHYSIOLOGICAL AND MOLECULAR PLANT PATHOLOGY **68:** 138-148

Hermsmeier D, Schittko U, Baldwin I (2001) Molecular interactions between the specialist herbivore Manduca sexta (Lepidoptera, Sphingidae) and its natural host Nicotiana attenuata. I. Large-scale changes in the accumulation of growth- and defense-related plant mRNAs. Plant Physiol **125:** 683-700

Hildmann T, Ebneth M, Peña-Cortés H, Sánchez-Serrano J, Willmitzer L, Prat S (1992) General roles of abscisic and jasmonic acids in gene activation as a result of mechanical wounding. Plant Cell **4:** 1157-1170

Hildmann T, Ebneth M, Peña-Cortés H, Sánchez-Serrano JJ, Willmitzer L, Prat S (1992) General roles of abscisic and jasmonic acids in gene activation as a result of mechanical wounding. Plant Cell **4:** 1157-1170

Hitchner EM, Kuhar TP, Dickens JC, Youngman RR, Schultz PB, Pfeiffer DG (2008) Host plant choice experiments of Colorado potato beetle (Coleoptera : Chrysomelidae) in Virginia. Journal of Economic Entomology **101:** 859-865

Howe GA, Jander G (2008) Plant immunity to insect herbivores. Annu Rev Plant Biol **59:** 41-66

Ishiga Y, Uppalapati SR, Ishiga T, Elavarthi S, Martin B, Bender CL (2009) The phytotoxin coronatine induces light-dependent reactive oxygen species in tomato seedlings. New Phytologist **181:** 147-160

Javid-Majd F, Blanchard JS (2000) Mechanistic analysis of the argE-encoded N-acetylornithine deacetylase. Biochemistry **39:** 1285-1293

Jensen O, Wilm M, Shevchenko A, Mann M (1999) Sample preparation methods for mass spectrometric peptide mapping directly from 2-DE gels. Methods Mol Biol **112:** 513-530

Johnson KS, Felton GW (1996) Physiological and dietary influences on midgut redox conditions in generalist lepidopteran larvae. Journal of Insect Physiology **42:** 191-198

Johnson KS, Felton GW (1996) Potential influence of midgut pH and redox potential on protein utilization in insect herbivores. Archives of Insect Biochemistry and Physiology **32:** 85-105

Kato Y, Murakami S, Yamamoto Y, Chatani H, Kondo Y, Nakano T, Yokota A, Sato F (2004) The DNA-binding protease, CND41, and the degradation of ribulose-1,5-bisphosphate carboxylase/oxygenase in senescent leaves of tobacco. Planta **220:** 97-104

Katsir L, Chung HS, Koo AJK, Howe GA (2008) Jasmonate signaling: a conserved mechanism of hormone sensing. CURRENT OPINION IN PLANT BIOLOGY **11:** 428-435

Kawasaki S, Miyake C, Kohchi T, Fujii S, Uchida M, Yokota A (2000) Responses of wild watermelon to drought stress: Accumulation of an ArgE homologue and citrulline in leaves during water deficits. Plant and Cell Physiology **41:** 864-873

Konno K, Hirayama C, Nakamura M, Tateishi K, Tamura Y, Hattori M, Kohno K (2004) Papain protects papaya trees from herbivorous insects: role of cysteine proteases in latex. PLANT JOURNAL **37:** 370-378

Krishnan N, Kodrik D, Turanli F, Sehnal F (2007) Stage-specific distribution of oxidative radicals and antioxidant enzymes in the midgut of Leptinotarsa decemlineata. Journal of Insect Physiology **53:** 67-74

Kusumawati L, Imin N, Djordjevic M (2008) Characterization of the secretome of suspension cultures of Medicago species reveals proteins important for defense and development. JOURNAL OF PROTEOME RESEARCH **7:** 4508-4520

Lane BG (2000) Oxalate oxidases and differentiating surface structure in wheat: germins. Biochemical Journal **349:** 309-321

Lee KP, Raubenheimer D, Simpson SJ (2004) The effects of nutritional imbalance on compensatory feeding for cellulose-mediated dietary dilution in a generalist caterpillar. Physiological Entomology **29:** 108-117

Levine A, Tenhaken R, Dixon R, Lamb C (1994) H_2O_2 from the Oxidative Burst Orchestrates the Plant Hypersensitive Disease Resistance Response. Cell **79:** 583-593

Li L, Zhao Y, McCaig B, Wingerd B, Wang J, Whalon M, Pichersky E, Howe G (2004) The tomato homolog of CORONATINE-INSENSITIVE1 is required for the maternal control of seed maturation, jasmonate-signaled defense responses, and glandular trichome development. Plant Cell **16:** 126-143

Lison P, Rodrigo I, Conejero V (2006) A novel function for the cathepsin D inhibitor in tomato. PLANT PHYSIOLOGY **142:** 1329-1339

Lodish HF (2000) Molecular cell biology, Ed 4th. W.H. Freeman, New York

Lou Y, Baldwin IT (2006) Silencing of a germin-like gene in Nicotiana attenuata improves performance of native herbivores. Plant Physiol **140:** 1126-1136

Lundberg P, Palo RT (1993) Resource Use, Plant Defenses, and Optimal Digestion in Ruminants. OIKOS **68:** 224-228

Lyytinen A, Lindstrom L, Mappes J, Julkunen-Tiitto R, Fasulati SR, Tiilikkala K (2007) Variability in host plant chemistry: behavioural responses and life-history parameters of the Colorado potato beetle (Leptinotarsa decemlineata). Chemoecology **17:** 51-56

Martin JS, Martin MM, Bernays EA (1987) FAILURE OF TANNIC-ACID TO INHIBIT DIGESTION OR REDUCE DIGESTIBILITY OF PLANT PROTEIN IN GUT FLUIDS OF INSECT HERBIVORES - IMPLICATIONS FOR THEORIES OF PLANT DEFENSE. JOURNAL OF CHEMICAL ECOLOGY **13:** 605-621

Mattson W (1980) Herbivory in Relation to Plant Nitrogen Content. Annual Review of Ecology and Systematics **11:** 119-161

McNabb WC, Peters JS, Foo LY, Waghorn GC, Jackson FS (1998) Effect of condensed tannins prepared from several forages on the in vitro precipitation of ribulose-1,5-bisphosphate carboxylase (Rubisco) protein and its digestion by trypsin (EC 2.4.21.4) and chymotrypsin (EC 2.4.21.1). Journal of the Science of Food and Agriculture **77:** 201-212

Meichtry J, Amrhein N, Schaller A (1999) Characterization of the subtilase gene family in tomato (Lycopersicon esculentum Mill.). Plant Mol Biol **39:** 749-760

Mohan S, Ma PWK, Pechan T, Bassford ER, Williams WP, Luthe DS (2006) Degradation of the S. frugiperda peritrophic matrix by an inducible maize cysteine protease. Journal of Insect Physiology **52:** 21-28

Moura DS, Bergey DR, Ryan CA (2001) Characterization and localization of a wound-inducible type I serine-carboxypeptidase from leaves of tomato plants (Lycopersicon esculentum Mill.). Planta **212:** 222-230

Mukhopadhyay K, Lecomte JTJ (2004) A relationship between heme binding and protein stability in cytochrome b(5). Biochemistry **43:** 12227-12236

Murdock LL, Shade RE (2002) Lectins and protease inhibitors as plant defenses against insects. Journal of Agricultural and Food Chemistry **50:** 6605-6611

Nesvizhskii AI, Keller A, Kolker E, Aebersold R (2003) A statistical model for identifying proteins by tandem mass spectrometry. Anal Chem **75:** 4646-4658

Paschold A, Halitschke R, Baldwin IT (2007) Co(i)-ordinating defenses: NaCOI1 mediates herbivore- induced resistance in Nicotiana attenuata and reveals the role of herbivore movement in avoiding defenses. Plant J **51:** 79-91

Pautot V, Holzer FM, Reisch B, Walling LL (1993) Leucine aminopeptidase: an inducible component of the defense response in Lycopersicon esculentum (tomato). Proc Natl Acad Sci U S A **90:** 9906-9910

Pechan T, Cohen A, Williams WP, Luthe DS (2002) Insect feeding mobilizes a unique plant defense protease that disrupts the peritrophic matrix of caterpillars. Proceedings of the National Academy of Sciences of the United States of America **99:** 13319-13323

Percudani R, Peracchi A (2003) A genomic overview of pyridoxal-phosphate-dependent enzymes. Embo Reports **4:** 850-854

Ramputh AI, Arnason JT, Cass L, Simmonds JA (2002) Reduced herbivory of the European corn borer (Ostrinia nubilalis) on corn transformed with germin, a wheat oxalate oxidase gene. PLANT SCIENCE **162:** 431-440

Rautengarten C, Steinhauser D, Büssis D, Stintzi A, Schaller A, Kopka J, Altmann T (2005) Inferring hypotheses on functional relationships of genes: Analysis of the Arabidopsis thaliana subtilase gene family. PLoS Comput Biol **1:** e40

Rayle DL, Cleland RE (1992) The Acid Growth Theory of Auxin-Induced Cell Elongation Is Alive and Well. PLANT PHYSIOLOGY **99:** 1271-1274

Reymond P, Bodenhausen N, Van Poecke RM, Krishnamurthy V, Dicke M, Farmer EE (2004) A conserved transcript pattern in response to a specialist and a generalist herbivore. Plant Cell **16:** 3132-3147

Reymond P, Weber H, Damond M, Farmer E (2000) Differential gene expression in response to mechanical wounding and insect feeding in Arabidopsis. Plant Cell **12:** 707-720

Roush RT, Hoy CW, Ferro DN, Tingey WM (1990) Insecticide Resistance in the Colorado Potato Beetle (Coleoptera, Chrysomelidae) - Influence of Crop-Rotation and Insecticide Use. Journal of Economic Entomology **83:** 315-319

Royo J, León J, Vancanneyt G, Albar JP, Rosahl S, Ortego F, Castañera P, Sánchez-Serrano JJ (1999) Antisense-mediated depletion of a potato lipoxygenase reduces wound induction of proteinase inhibitors and increases weight gain of insect pests. Proc Natl Acad Sci U S A **96:** 1146-1151

Ruepp A, Zollner A, Maier D, Albermann K, Hani J, Mokrejs M, Tetko I, Guldener U, Mannhaupt G, Munsterkotter M, Mewes H (2004) The FunCat, a functional annotation scheme for systematic classification of proteins from whole genomes. NUCLEIC ACIDS RESEARCH **32:** 5539-5545

Sasaki-Sekimoto Y, Taki N, Obayashi T, Aono M, Matsumoto F, Sakurai N, Suzuki H, Hirai M, Noji M, Saito K, Masuda T, Takamiya K, Shibata D, Ohta H (2005) Coordinated activation of metabolic pathways for antioxidants and defence compounds by jasmonates and their roles in stress tolerance in Arabidopsis. Plant J **44:** 653-668

Schilmiller A, Howe G (2005) Systemic signaling in the wound response. Curr Opin Plant Biol **8:** 369-377

Siezen RJ, de Vos WM, Leunissen JA, Dijkstra BW (1991) Homology modelling and protein engineering strategy of subtilases, the family of subtilisin-like serine proteinases. Protein Eng **4:** 719-737

Simpson SJ, Simpson CL (1989) The mechanisms of nutritional compensation by phytophagous insects. *In* EA Bernays, ed, Insect-Plant Interactions, Vol II. CRC Press, Inc, Boca Raton, pp 111-160

Steppuhn A, Baldwin IT (2007) Resistance management in a native plant: nicotine prevents herbivores from compensating for plant protease inhibitors. Ecol Lett **10:** 499-511

Strassner J, Schaller F, Frick U, Howe G, Weiler E, Amrhein N, Macheroux P, Schaller A (2002) Characterization and cDNA-microarray expression analysis of 12-oxophytodienoate reductases reveals differential roles for octadecanoid biosynthesis in the local versus the systemic wound response. Plant J **32:** 585-601

Terra WR (1987) Evolution of holometabola insect digestive systems: physiological and biochemical aspects. *In* Mem. Inst. Oswaldo Cruz, Vol 82, Rio de Janeiro, Brasil, pp 25-28

Terra WR (1990) Evolution of Digestive Systems of Insects. ANNUAL REVIEW OF ENTOMOLOGY **35:** 181-200

Tornero P, Conejero V, Vera P (1996) Primary structure and expression of a pathogen-induced protease (PR-P69) in tomato plants: Similarity of functional domains to subtilisin-like endoproteases. Proc Natl Acad Sci U S A **93:** 6332-6337

Tumlinson J, Lait C (2005) Biosynthesis of fatty acid amide elicitors of plant volatiles by insect herbivores. Arch Insect Biochem Physiol **58:** 54-68

Uppalapati SR, Ayoubi P, Weng H, Palmer DA, Mitchell RE, Jones W, Bender CL (2005) The phytotoxin coronatine and methyl jasmonate impact multiple phytohormone pathways in tomato. PLANT JOURNAL **42:** 201-217

Uppalapati SR, Ishiga Y, Wangdi T, Urbanczyk-Wochniak E, Lshiga T, Mysore KS, Bender CL (2008) Pathogenicity of Pseudomonas syringae pv. tomato on tomato seedlings: Phenotypic and gene expression analyses of the virulence function of coronatine. MOLECULAR PLANT-MICROBE INTERACTIONS **21:** 383-395

van de Ven WT, LeVesque CS, Perring TM, Walling LL (2000) Local and systemic changes in squash gene expression in response to silverleaf whitefly feeding. Plant Cell **12:** 1409-1423

Van der Hoeven R, Ronning C, Giovannoni J, Martin G, Tanksley S (2002) Deductions about the number, organization, and evolution of genes in the tomato genome based on analysis of a large expressed sequence tag collection and selective genomic sequencing. PLANT CELL **14:** 1441-1456

van Loon LC, Rep M, Pieterse CMJ (2006) Significance of inducible defense-related proteins in infected plants. ANNUAL REVIEW OF PHYTOPATHOLOGY **44:** 135-162

Van Loon LC, Van Strien EA (1999) The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. PHYSIOLOGICAL AND MOLECULAR PLANT PATHOLOGY **55:** 85-97

Vera P, Conejero V (1988) Pathogenesis-Related Proteins of Tomato : P-69 as an Alkaline Endoproteinase. Plant Physiol **87:** 58-63

Vogel H, Kroymann J, Mitchell-Olds T (2007) Different transcript patterns in response to specialist and generalist herbivores in the wild Arabidopsis relative Boechera divaricarpa. PLoS ONE **2:** e1081

Wang J, Constabel CP (2004) Polyphenol oxidase overexpression in transgenic Populus enhances resistance to herbivory by forest tent caterpillar (Malacosoma disstria). Planta **220:** 87-96

Woo EJ, Dunwell JM, Goodenough PW, Marvier AC, Pickersgill RW (2000) Germin is a manganese containing homohexamer with oxalate oxidase and superoxide dismutase activities. Nature Structural Biology **7:** 1036-1040

Xia K, Manning M, Hesham H, Lin Q, Bystroff C, Colon W (2007) Identifying the subproteome of kinetically stable proteins via diagonal 2D SDS/PAGE. Proceedings of the National Academy of Sciences of the United States of America **104:** 17329-17334

Yan SP, Zhang QY, Tang ZC, Su WA, Sun WN (2006) Comparative proteomic analysis provides new insights into chilling stress responses in rice. Molecular & Cellular Proteomics **5:** 484-496

Yang YL, Joern A (1994) Compensatory Feeding in Response to Variable Food Quality by Melanoplus-Differentialis. Physiological Entomology **19:** 75-82

Yang YL, Joern A (1994) Influence of Diet Quality, Developmental Stage, and Temperature on Food Residence Time in the Grasshopper Melanoplus-Differentialis. Physiological Zoology **67:** 598-616

Yogavel M, Mishra PC, Gill J, Bhardwaj PK, Dutt S, Kumar S, Ahuja PS, Sharma A (2008) Structure of a superoxide dismutase and implications for copper-ion chelation. Acta Crystallographica Section D-Biological Crystallography **64:** 892-901

Yokota A, Kawasaki S, Iwano M, Nakamura C, Miyake C, Akashi K (2002) Citrulline and DRIP-1 protein (ArgE homologue) in drought tolerance of wild watermelon. Annals of Botany **89:** 825-832

Yoshida Y, Sano R, Wada T, Takabayashi J, Okada K (2009) Jasmonic acid control of GLABRA3 links inducible defense and trichome patterning in Arabidopsis. Development **136:** 1039-1048

Zavala JA, Patankar AG, Gase K, Hui D, Baldwin IT (2004) Manipulation of endogenous trypsin proteinase inhibitor production in Nicotiana attenuata demonstrates their function as antiherbivore defenses. Plant Physiol **134:** 1181-1190

Zhang B, VerBerkmoes NC, Langston MA, Uberbacher E, Hettich RL, Samatova NF (2006) Detecting differential and correlated protein expression in label-free shotgun proteomics. J Proteome Res **5:** 2909-2918

Chapter V

Conclusions and Future Perspectives

At the beginning of this thesis research, studies of tomato TD had focused on a single gene (*TD2*) that was reported to encode the most abundant protein in tomato flowers (Samach et al., 1991). Subsequent studies showed that the potato ortholog of this gene is strongly induced by wounding and JA treatment (Hildmann et al., 1992; Dammann et al., 1997). The extraordinary level of expression of this gene in floral organs and wound-stressed leaves was not consistent with a role in primary metabolism (i.e., Ile biosynthesis). To investigate the possibility that tomato and potato have multiple isoforms of TD, a search of the tomato EST database was performed, which led to the identification of a second *TD* gene in these species. This uncharacterized gene was called *TD1*, to differentiate it from the jasmonate-inducible gene *TD2*.

The expression pattern and biochemical properties of tomato TDs are consistent with functional divergence of the two isoforms. Results described in this thesis indicate that *TD1* likely serves a primary metabolic role in Ile synthesis because the gene is constitutively expressed in all tissues and is orthologous to *TD*s from plants such as rice and Arabidopsis that contain a single *TD* gene. In contrast, *TD2* expression is strictly regulated in an inducible manner by the jasmonate pathway (Chen et al., 2007). Moreover, TD2 is highly abundant in the frass of *M. sexta*, *T. ni*, and *L. decemlineata* larvae reared on tomato. These findings led to the hypothesis that TD2 performs a post-ingestive defensive role in depleting the essential amino acid Thr from the insect's diet.

The increased weight of *S. exigua* larvae growing on transgenic tomato plants silenced in TD2 expression provides direct evidence for this hypothesis. Duplication and subsequent neofunctionalization of genes involved in primary metabolism may be a recurrent strategy in the evolution of plant defense proteins that impair the ability of arthropod herbivores to acquire nutrients from plant tissue. Similar examples are available in the biosynthesis of secondary metabolites (Bohlmann et al., 1996; Ober, 2005). For example, in *Ruta graveolens a* tryptophan –sensitive anthranilate synthase enzyme is responsible for amino acid synthesis, whereas a tryptophan-resistant anthranilate synthase has been recruited for the biosynthesis of anthranilate-derived alkaloids (Bohlmann et al., 1996).

Our results also show that TD2's role in insect resistance is associated with certain biochemical properties that optimize the enzyme's capacity to function in the extreme environment of the insect gut. The Thr-degrading activity of TD2 in the insect gut is enhanced by proteolytic cleavage of the enzyme's regulatory domain following ingestion of tomato leaves by insect larvae. TD2 is presumably maintained in a latent state inside the chloroplast, and is subsequently activated by an insect chymotrypsin-like protease in the midgut of lepidopteran herbivores. Removal of the regulatory domain results in an enzyme that is insensitive to feedback inhibition by Ile and, as a consequence, is highly efficient in degrading Thr in the Ile-rich environment of the lepidopteran gut.

Another key feature of TD2 is its thermostability and resistance to insect digestive proteases. Further studies are needed to determine the structural features that underlie these properties. Answers to this question promise to provide basic insight into why plant proteins accumulate in the digestive tract, and may also facilitate the rationale design of

hyperstable proteins that are useful as pesticides or other commercial applications. The TD1 and TD2 isoforms provide an attractive comparative model system in which to study this question. As an initial step toward this goal, the structure of TD1 and TD2 was modeled using the X-ray structure of *E. coli* TD (Gallagher et al., 1998). These models suggest that the overall structure of TD1 and TD2 are very similar (Figure 5.1), despite the sequence divergence of the two isoforms. Superimposition of the structures, however, revealed an α-helix in the regulatory domain of TD1 that is not present in TD2. This observation suggests that the regulatory domain of TD2 may not be subject to the functional constraints of the regulatory domain of TD1 and other biosynthetic-type TDs. More detailed information is needed to establish the structural basis of the differential stability of TD1 and TD2. Efforts to determine the X-ray crystal structure of the two isoforms are underway in collaboration with Chris Bianchetti and George Phillips at the University of Wisconsin.

A significant part of this thesis is devoted to the analysis of the frass proteome of different insects reared on tomato. These results show that shotgun proteomic analysis of the insect feces provides a rapid and robust approach to identify hyperstable proteins that may have a role in plant defense. The approach was validated through the identification of well characterized proteins that impair insect digestion, including a variety of protease inhibitors, cysteine protease, a germin-like protein, and the amino acid-degrading enzymes arginase and TD2. Other JA-inducible proteins with unknown roles were also identified. Among these were a member of the highly conserved YjgF family of proteins and a silverleaf whitefly-1 like protein (van de Ven et al., 2000; Leitner-Dagan et al., 2006). Plant proteins that are both stable in the insect gut and regulated by the JA

pathway are good candidates for additional testing as anti-insect compounds using transgenic approaches.

The comparison of the tomato leaf proteome before and after passage through *L. decemlineata* indicated that most leaf proteins are digested in the insect. Nevertheless, enrichment of a subset of leaf proteins, including defense-related and/or JA-inducible proteins, in the insect gut was observed. This finding suggests that plant anti-insect proteins have been selected during evolution for increased stability and resistance to proteolysis. The fact that many gut-accumulating tomato proteins are encoded by JA-responsive genes indicates that herbivore-induced transcriptional reprogramming is an important part of the plant's defensive strategy to limit nutrient acquisition. This idea is supported by the observation that the generalist insect *T. ni*, whose growth is severely affected by JA-based defenses, excreted a higher proportion of JA-inducible proteins compared to the Solanaceae specialists.

Proteomic analysis indicated that both nutritional (e.g., RbcL) and defense-related (TD2) host proteins are digested less efficiently by *L. decemlineata* in comparison to the lepidopteran insects. Reduced digestion of dietary protein by *L. decemlineata* may represent a counteradaptive strategy to minimize exposure to host defense proteins. Pathogenesis-related (PR) proteins also appeared to be more stable in the gut of *L. decemlineata* compared to the lepidopterans. Although it is widely accepted that PR proteins serve important roles in plant resistance to pathogens, the biological function of many PR proteins remains unknown (van Loon et al., 2006). The potential function of PR proteins as a post-ingestive defense against insects deserves further investigation.

For the vast majority of stable tomato proteins identified in this thesis research, additional work is needed to determine whether they have a role in anti-insect defense. This goal could be accomplished by altering the expression of the corresponding gene in transgenic plants, or by incorporating the purified protein in insect artificial diet. Because of its amenability to reverse genetic analysis, genetic approaches may be facilitated with the use of *Arabidopsis thaliana* as a host plant. Proteomic analysis frass from *T. ni* and *S. exigua* fed on Arabidopsis showed that the most abundant Arabidopsis protein excreted by both insects is a cystine lyase encoded by locus At4g23600 (E Gonzales-Vigil and G Howe, unpublished results). RNA interference technology was subsequently used to silence the expression of this gene. Insect feeding assays performed with At4g23600-silenced lines showed a small but consistent effect of the gene in promoting Arabidopsis resistance to *S. exigua* (E Gonzales-Vigil and G Howe, unpublished results). These preliminary results provide additional evidence that shotgun proteomic analysis of insect frass is a valid approach for identifying plant genes that mediate resistance to insect herbivores.

Restricted occurrence in a subset of species within the *Solanaceae* is an interesting feature of *TD2* as a JA-regulated gene (Chen et al., 2007). Thus, despite the conservation of the JA-signaling pathway in the plant kingdom, some JA-inducible defenses (e.g., TD2) are plant-lineage specific, whereas others (e.g., PIs) are widespread. This observation indicates that the discovery of novel plant anti-insect proteins may be facilitated by extending proteomic-based surveys to other plant species.

In summary, this thesis research has generated new insight into the role of plant proteins in defense against insect herbivores. Based on the results obtained, further

experiments will be designed to understand the mechanisms underlying the role of these proteins in plant defense. The ultimate goal of this work is to develop transgenic crops with increased resistance to arthropod herbivores.

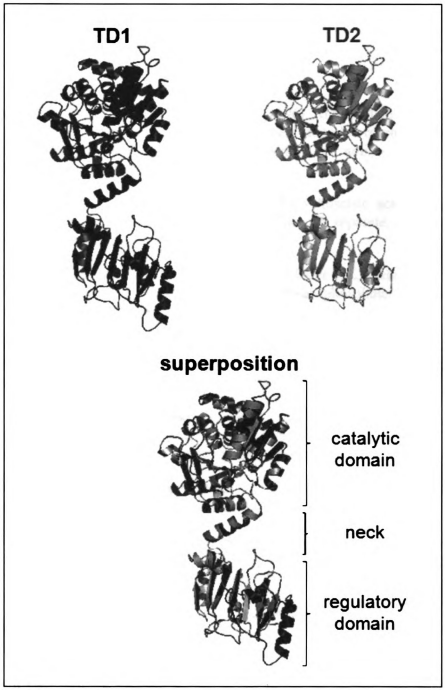


Figure 5.1. Homology models of tomato TDs.

The X-ray structure of *E. coli* TD (Gallagher et al., 1998) was used as a template to construct the homology models with SWISS-MODEL. Images were rendered with PyMol.

LITERATURE CITED

Bohlmann J, Lins T, Martin W, Eilert U (1996) Anthranilate synthase from Ruta graveolens. Duplicated AS alpha genes encode tryptophan-sensitive and tryptophan-insensitive isoenzymes specific to amino acid and alkaloid biosynthesis. Plant Physiol **111:** 507-514

Dammann C, Rojo E, Sánchez-Serrano JJ (1997) Abscisic acid and jasmonic acid activate wound-inducible genes in potato through separate, organ-specific signal transduction pathways. Plant J **11:** 773-782

Gallagher DT, Gilliland GL, Xiao G, Zondlo J, Fisher KE, Chinchilla D, Eisenstein E (1998) Structure and control of pyridoxal phosphate dependent allosteric threonine deaminase. Structure **6:** 465-475

Hildmann T, Ebneth M, Peña-Cortés H, Sánchez-Serrano JJ, Willmitzer L, Prat S (1992) General roles of abscisic and jasmonic acids in gene activation as a result of mechanical wounding. Plant Cell **4:** 1157-1170

Leitner-Dagan Y, Ovadis M, Zuker A, Shklarman E, Ohad I, Tzfira T, Vainstein A (2006) CHRD, a plant member of the evolutionarily conserved YjgF family, influences photosynthesis and chromoplastogenesis. Planta **225:** 89-102

Ober D (2005) Seeing double: gene duplication and diversification in plant secondary metabolism. Trends in Plant Science **10:** 444-449

Samach A, Hareven D, Gutfinger T, Ken-Dror S, Lifschitz E (1991) Biosynthetic threonine deaminase gene of tomato: isolation, structure, and upregulation in floral organs. Proc Natl Acad Sci U S A **88:** 2678-2682

van de Ven WT, LeVesque CS, Perring TM, Walling LL (2000) Local and systemic changes in squash gene expression in response to silverleaf whitefly feeding. Plant Cell **12:** 1409-1423

van Loon LC, Rep M, Pieterse CMJ (2006) Significance of inducible defense-related proteins in infected plants. ANNUAL REVIEW OF PHYTOPATHOLOGY **44:** 135-162

Appendix 1. Protein report

Description	Accession	WT leaf damaged by *L. dec*				*L. decemlineata* frass				*T. ni* frass				*M. sexta* frass				Sum TS
		%†	P	US	TS	%	P	US	TS	%	P	US	TS	%	P	US	TS	
10 kDa chaperonin	BW689841									43				100	2	2	3	3
14-3-3 protein	BW689602	100																0
14-3-3 protein 9	TA18907_4081	99																0
24K germin like protein precursor	TA18731_4081	100	2	3	6	100	3	5	15	100	4	5	10	100	5	9	63	94
30S ribosomal protein S1, chloroplast precursor	TA20234_4081	100	2	2	2													2
30S ribosomal protein S9, chloroplast precursor	TA21483_4081	100	3	3	4													4
34 kDa outer mitochondrial membrane protein porin	TA19515_4081	100								91								0
40S ribosomal protein S16	CN385797	99																0
40S ribosomal protein S20	AI780034	98																0
40S ribosomal protein S5	TA18754_4081	100	3	3	5													5
4D11_17	TA22513_4081									99	1	1	1	100	4	5	8	9
50S ribosomal protein L11, chloroplast precursor	TA21526_4081	99																0
50S ribosomal protein L12, chloroplast precursor	AW038647	98																0

† %: Protein probability; P: number of identified peptides; US: number of unique spectra; TS: number of total spectra

Appendix 1. (cont 'd)

50S ribosomal protein L15, chloroplast precursor	TA20020_4081	99														0
50S ribosomal protein L18, chloroplast precursor	TA24516_4081	99														0
50S ribosomal protein L21, chloroplast precursor	TA18095_4081	98														0
50S ribosomal protein L24, chloroplast precursor	TA19700_4081	100														0
50S ribosomal protein L3, chloroplast precursor	TA20651_4081	100	2	2	3											3
50S ribosomal protein L5, chloroplast	TA18398_4081	100	4	4	5											5
50S ribosomal protein L9, chloroplast precursor	TA25046_4081	96														0
6,7-dimethyl-8-ribityllumazine synthase	TA26137_4081									77				100	2	2	3	3
60 kDa chaperonin	TA34085_4081									100	5	5	5					5
60S ribosomal protein L13	BE353475	100	2	2	3													3
60S ribosomal protein L22-2	AW222378	100	2	2	3													3
7-domain trypsin inhibitor	TA18489_4081	100	7	8	11	81				100	5	5	7	100	7	7	7	25
Acetylornithine deacetylase, putative	TA19203_4081	100	10	10	19	100	12	14	23	100	13	16	22	100	6	7	10	74
Acid phosphatase-like protein	BI928273	99																0
Acidic 26 kDa endochitinase precursor	TA19846_4081					91	1	1	2	100	1	1	1	100	2	2	2	5

Appendix 1. (cont 'd)

Actin	BT013524	100	3	3	4										4
Actin	TA20198_4081					99									0
Adenosylhomocysteinase	TA18170_4081	100	2	2	2										2
Adenylosuccinate synthase	DV105280	100													0
ADP ribosylation factor 002	AW219305	99													0
ADP-glucose pyrophosphorylase large subunit	TA26818_4081	100	2	2	3										3
AlaT1	TA19225_4081	100	9	10	17										17
Aldehyde dehydrogenase 1 precursor	TA19166_4081	100	4	4	4										4
Aldehyde oxidase	TA22020_4081											100	3	3	3
Allene oxide cyclase precursor	TA21078_4081									99		100			0
Alpha-mannosidase	TA32559_4081					91				100					0
Aminomethyltransferase, mitochondrial precursor	TA17349_4081	100	7	7	13										13
Anionic peroxidase swpb3	TA19669_4081					100	5	5	8						8
Arginase 2	AY656838	100	7	7	12					100	3	3	4	100	11	12	21	37
Aspartate aminotransferase	AW221110									100					0
Aspartate aminotransferase-like	TA21048_4081	100													0
At1g62740	TA18881_4081	100	2	2	2										2

Appendix 1. (cont 'd)

At1g67280/F1N21_10	TA17808_4081	99														0
AT3g15190/F4B12_10	TA21042_4081	100	2	2	3											3
At5g19860	TA29989_4081													100	4	4	7	7
ATP synthase alpha chain, mitochondrial	TA20572_4081	100	3	4	5											5
ATP synthase B' chain, chloroplast precursor	BG127384	100	2	2	4											4
ATP synthase CF1 alpha subunit	TA21015_4081	100	13	13	32	100	9	9	15	92	1	1	1	100	2	2	3	51
ATP synthase CF1 beta subunit	TA19970_4081	100	21	26	71	100	14	14	25	92	1	1	2	100	5	5	6	104
ATP synthase delta chain, chloroplast precursor	TA19093_4081	100	4	4	6											6
ATP synthase gamma chain, chloroplast precursor	TA18781_4081	100	7	7	8	100	2	2	3							11
ATP-dependent Clp protease ATP-binding subunit clpA homolog CD4B, chloroplast precursor	TA18602_4081	100	4	5	5					88						5
Auxin-binding protein ABP19a precursor	BG128560	73	1	2	8	91	1	2	2	92	1	1	1	100	3	4	7	18
Basic PR-1 protein precursor	AI895584					98												0
Beta-cyanoalanine synthase like protein	TA22181_4081													100	2	2	2	2

Appendix 1. (cont 'd)

Beta-glucosidase	TA19521_4081									100	1	1	1	100	2	2	3	4
Beta-mannosidase enzyme	TA17782_4081					100												0
CAPIP2	CN385695					99								100				0
Carbonic anhydrase	TA17850_4081	100	13	15	33	91	1	1	1	100	7	10	20	100	3	3	5	59
Carbonic anhydrase	TA22924_4081					94				98				100	4	5	7	7
Carotenoid cleavage dioxygenase 1B	TA20176_4081									92	1	1	1	100	4	4	5	6
Catalase isozyme 1	TA18296_4081	100	7	7	10	100	2	2	2									12
Catalase isozyme 2	TA17392_4081	100	6	6	11					83								11
Cathepsin D Inhibitor precursor	TA17990_4081	100	3	3	8					100	3	3	5	100	2	2	2	15
Cathepsin D Inhibitor precursor	TA17999_4081	100	7	7	15	100	5	5	7	100	3	3	8	100	9	10	20	50
Cell division cycle protein 48 homolog	TA19623_4081	100	3	3	3													3
Cell division protein ftsH homolog 1, chloroplast precursor	TA18303_4081	100	2	2	3													3
ceramidase	TA22476_4081	73	1	1	1					100	2	2	2	89	1	1	1	4
Chaperonin 21 precursor	TA18597_4081	100	3	3	6													6

Appendix 1. (cont 'd)

Chaperonin 21 precursor	TA19000_4081	100	4	4	6											6
Chaperonin-60 beta subunit precursor	TA17359_4081	100	10	10	17											17
Chaperonin-60 beta subunit precursor	TA17499_4081	100														0
Chaperonin-60 beta subunit precursor	TA20937_4081	100	2	2	3					92	1	1	1			4
Chitinase, class V	AW032116					100										0
Chlorophyll a/b-binding protein CP29 precursor	TA18428_4081	100	3	3	3	100	3	3	4	92	1	1	1			8
Chlorophyll a-b binding protein 8, chloroplast precursor	TA17640_4081	100	2	2	9	91	1	1	2							11
Chlorophyll a-b binding protein CP24 10A, chloroplast precursor	TA17514_4081	100	4	4	11	100	2	2	3	98	1	1	2	89	1	1	1	17
Chloroplast ferredoxin-NADP+ oxidoreductase precursor	TA18636_4081	100	9	9	18	99	1	1	1	92	1	1	1			20
Chloroplast inorganic pyrophosphatase	TA19016_4081	100	5	5	9											9
Chloroplast photosystem II 22 kDa component	TA18154_4081	100	2	2	3	91	1	1	1							4
Chloroplast polyprotein of elongation factor Ts	TA24092_4081	98														0
Chloroplast protease precursor	TA19255_4081	100	7	7	9											9

Appendix 1. (cont 'd)

Chloroplast ribosomal protein L10	TA22312_4081	100	2	2	4											4
Chromosome 10 SCAF12030, whole genome shotgun sequence	TA18952_4081	73	1	1	2					100	2	2	2			4
Chromosome 9 SCAF14729, whole genome shotgun sequence	BT012711	100	2	2	2											2
CND41, chloroplast nucleoid DNA binding protein precursor	TA19516_4081									100	2	2	3	100	3	4	7	10
Coatomer alpha subunit	AI894417													100	2	2	3	3
Coproporphyrinogen III oxidase, chloroplast precursor	TA20713_4081	100	1	1	2	98	1	1	1	100	3	3	3					6
Cucumisin-like serine protease; subtilisin-like protease	BP901605									58				99				0
Cucumisin-like serine protease; subtilisin-like protease	TA25211_4081					100	2	2	2	92	1	1	2	100	4	5	5	9
Cucumisin-like serine protease; subtilisin-like protease	TA31417_4081	73	1	1	2					100	2	2	3	98	1	1	2	7
Cyc07-like protein	BP875908	99																0
CYP1	AF172856	34				100	3	3	4					81				4
Cysteine proteinase 3 precursor	BW685706					99								89				0

Appendix 1. (cont 'd)

Cysteine proteinase 3 precursor	TA19249_4081	100	4	4	6	67				100	2	2	6	100	2	2	4	16
Cytochrome b6-f complex iron-sulfur subunit, chloroplast precursor	AW096705	99				53												0
Cytochrome f	TA28980_4081	100	6	8	13	100	5	6	9	100	5	6	9	100	9	12	24	55
Cytosolic ascorbate peroxidase 1	DQ096286	100	3	4	7													7
Cytosolic cysteine synthase	TA20429_4081													100	8	9	14	14
Dehydroascorbate reductase	TA19033_4081	100	4	5	6					100	4	4	4	100	3	3	4	14
D-glycerate 3-kinase, chloroplast precursor	TA20706_4081	100																0
Dihydrolipoamide dehydrogenase	TA20033_4081	100	4	4	5	50												5
DnaK protein, putative	TA18122_4081	100	11	12	19									99	1	1	1	20
Elicitor-inducible protein EIG-J7	TA26153_4081					99				92				99				0
Elongation factor TS, putative	TA23917_4081	100																0
Elongation factor TuA, chloroplast precursor	TA19432_4081	100	10	11	19													19
Elongation factor TuA, chloroplast precursor	TA19807_4081	100	3	3	3													3
Enolase	TA18379_4081	100	4	4	5													5
Eukaryotic translation initiation factor 5A-4	AI482632	99																0

Appendix 1. (cont 'd)

Expressed protein	AW929577					100	3	3	5	91				38				5
F21B7.21	TA21261_4081	100																0
F3F9.11	CN384743	100																0
F8K7.10 protein	TA25080_4081									47				100				0
Ferredoxin-1, chloroplast precursor	TA17694_4081	73	1	1	2					100	1	1	1	100	2	2	3	6
FKBP-type peptidyl-prolyl cis-trans isomerase 3, chloroplast precursor	AI777652	100																0
Formate--tetrahydrofolate ligase	TA20725_4081	100	2	2	2													2
Fructose 1,6, bisphosphate aldolase	TA19769_4081	100	4	4	5													5
Fructose-1,6-bisphosphatase	TA20612_4081	100																0
GDP-mannose 3,5-epimerase 1	TA18352_4081	100	2	2	2													2
Geranylgeranyl reductase	TA18887_4081	100	3	3	5					100	2	2	3					8
Germin-like protein	BI210469					100	1	1	1	100	2	2	3	99	1	1	1	5
Glucan endo-1,3-beta-D-glucosidase precursor	TA19657_4081					100	1	1	2	100	3	3	4	100	6	7	9	15
Glucan endo-1,3-beta-glucosidase B precursor (EC 3.2.1.39) ((1-3)- beta-glucan endohydrolase B) ((1-3)-beta-glucanase B)	TA18261_4081					100	6	6	10	100	3	3	4	89	1	1	2	16

Appendix 1. (cont 'd)

Glucose-1-phosphate adenylyltransferase small subunit, chloroplast precursor	TA17584_4081	100	2	2	4									4
Glucose-6-phosphate isomerase	TA20171_4081										100	3	3	5	5
Glutamate dehydrogenase	SLU48695										100	7	7	11	11
Glutamate-1-semialdehyde 2,1-aminomutase, chloroplast precursor	TA17703_4081	100	6	6	10		92	1	1	1					11
Glutamine synthetase	TA17912_4081	100	8	8	16										16
Glutathione reductase	TA19151_4081						98				89				0
Glutathione reductase, chloroplast precursor	TA21802_4081						100	8	8	13	100	8	8	10	23
Glyceraldehyde-3-phosphate dehydrogenase	TA18082_4081	100	3	3	6		64								6
Glyceraldehyde-3-phosphate dehydrogenase A, chloroplast precursor	TA17655_4081	100	15	18	30	88					100	1	1	1	31
Glyceraldehyde-3-phosphate dehydrogenase A, chloroplast precursor	TA17989_4081	73				88					100				0
Glyceraldehyde-3-phosphate dehydrogenase B, chloroplast precursor	TA17852_4081	100	10	11	18	88	100	1	1	2					20

Appendix 1. (cont 'd)

Glyceraldehyde-3-phosphate dehydrogenase B, chloroplast precursor	TA17893_4081	100	7	7	11	88				100	1	1	2				13
Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	TA17557_4081	100	5	5	8	100											8
Glycine decarboxylase complex H-protein	AW094248	100	2	2	4												4
Glycine dehydrogenase	TA17388_4081	100	3	3	5												5
Glycine dehydrogenase	TA17490_4081	100	5	5	11												11
Glyoxisomal malate dehydrogenase	BT012828	100	5	6	11	91	1	1	1	92	1	1	1	100	2	2	3	16
Harpin binding protein 1	TA23741_4081	100	2	2	2									100	8	9	13	15
Heat shock protein 70	TA21468_4081	100																0
Heat shock protein 70 precursor	TA18055_4081	100	5	5	8													8
Hsp90-2-like	TA18281_4081	100	2	2	2													2
Hydroxyphenylpyruvate reductase	TA19698_4081	100																0
Hydroxypyruvate reductase	TA22182_4081	100	4	4	6													6

Appendix 1. (cont 'd)

Hypothetical protein	TA19580_4081	100	3	3	5										5
Hypothetical protein	TA26195_4081												100	3	3	4	4
Hypothetical protein	TA17827_4081_f	98								58			89				0
Hypothetical protein	TA17913_4081	99															0
Hypothetical protein	TA17422_4081	99				100	2	2	2								2
Hypothetical protein	BI208816	73	1	1	2					100	3	3	3	100	3	4	6	11
Hypothetical protein	BM411868	100	3	3	4													4
Hypothetical protein	BM413312													100	4	4	5	5
Hypothetical protein	TA18422_4081	100	5	5	6									89	1	1	1	7
Hypothetical protein	TA19126_4081					91				99								0
Hypothetical protein	TA19447_4081	100	2	2	2													2
Hypothetical protein	AJ784661									92				98				0
Hypothetical protein At1g15140; F9L1.8	TA20410_4081	98																0
Hypothetical protein At1g62750	TA27999_4081	98																0
Hypothetical protein At3g26090; MPE11.24	TA27077_4081	100	2	2	3					100	2	2	3	100	7	9	13	19
Hypothetical protein AT4g39730	TA21820_4081													100	2	3	5	5

Appendix 1. (cont 'd)

Hypothetical protein F6I1.12	TA19459_4081	100	3	3	4											4
Hypothetical protein OSJNBa0035J16.9	TA19842_4081	100														0
Hypothetical protein P0577B11.138	TA20912_4081	99														0
Hypothetical protein precursor	BI923963									100	4	4	7	100	5	9	22	29
Hypothetical protein precursor	BI927980	100	5	5	9	100	4	4	7	92	1	1	3	100	1	1	4	23
Hypothetical protein precursor	TA19736_4081	100																0
Hypothetical protein T1E4.10	TA20023_4081	100	3	3	4	91	1	1	1					100	10	11	17	22
Hypothetical protein T6H20.190	TA19777_4081	100	8	8	14													14
Kunitz-type protease inhibitor-like protein	TA28946_4081					90				100	3	3	4	100	5	5	6	10
L1 protein	TA19075_4081	100	2	2	4													4
Leucine aminopeptidase 1, chloroplast precursor	TA18810_4081	100	14	15	26	100	5	5	6	100	19	25	63	100	18	25	36	131
LEXYL2 protein	TA19490_4081	99	1	1	1	100	4	4	4									5
Light harvesting chlorophyll a /b binding protein of PSII	BI924800	100				91								99				0
Light harvesting chlorophyll a /b binding protein of PSII	BI928497	100				100	1	1	1	100	2	2	6	99	1	1	1	8
Light harvesting chlorophyll a /b binding protein of PSII	TA17799_4081					100	2	2	3					89	1	1	2	5
Light harvesting chlorophyll a /b binding protein of PSII	TA17900_4081	100	4	5	10	100	4	5	11	100	4	6	12	100	4	5	10	43

Appendix 1. (cont 'd)

Light harvesting chlorophyll a /b binding protein of PSII	TA18096_4081	100	3	3	4	100	2	2	5							9
Light-induced protein, chloroplast precursor	TA18485_4081	99														0
Light-regulated chloroplast-localized protein	TA19697_4081	100	3	3	4											4
Lipase/hydrolase, putative	BI930964					100	1	1	1	100	2	2	2	100	5	6	11	14
Lipase/lipooxygenase, PLAT/LH2	TA25872_4081					100	1	1	2					100	2	2	3	5
Malate dehydrogenase	AJ320050	99																0
Malate dehydrogenase-like protein	TA18799_4081	100	5	5	7													7
Membrane-associated 30 kDa protein, chloroplast precursor	TA21801_4081	100	2	2	2													2
Methionine synthase	TA17930_4081	100	3	3	5									99	1	1	2	7
Mitochondrial ATPase beta subunit	TA18872_4081	100	4	4	7													7
Mitochondrial malate dehydrogenase	TA17846_4081	100	7	7	15	91	1	1	1	92	1	1	2	100	9	11	16	34
MRNA binding protein precursor	TA19245_4081	100	10	10	16													16
MRNA, complete cds, clone: RAFL22-48-I16	TA20680_4081	100																0
Multicystatin	TA27774_4081					37				100	2	2	2					2
Multicystatin	TA27775_4081					37								100				0

Appendix 1. (cont 'd)

Multicystatin	TA30868_4081												100				0
NAD-malate dehydrogenase precursor	TA22316_4081					91	1	1	2				100	2	2	3	5
NADP-dependent glyceraldehyde-3-phosphate dehydrogenase	TA19637_4081	100	3	3	3					100	3	3	4				7
NADPH:protochlorophyllide oxidoreductase	TA17491_4081	100	2	2	2												2
Neutral leucine aminopeptidase preprotein precursor	TA17427_4081	100	3	3	6									100	2	2	3	9
NIFS-like protein CpNifsp precursor	TA27495_4081													100	3	3	5	5
Nitrite reductase	TA19114_4081	100	3	3	4					100	1	1	2					6
NME2 protein	CN384512	100	4	4	6					100	3	3	5					11
No description	AJ832025_e	100	2	2	3													3
No description	TA20325_4081													99				0
Non-specific lipid transfer protein	AA824719					100	2	2	3					100	2	2	2	5
Nonspecific lipid-transfer protein 1 precursor	TA17462_4081					56								99				0
Nonspecific lipid-transfer protein 2 precursor	AW931821													100				0
Nucleoid DNA-binding-like protein	BP896163													100	2	4	9	9
Nucleoid DNA-binding-like protein	TA23713_4081	73	1	1	1	100	2	2	4	100	2	2	2	100	6	6	11	18

Appendix 1. (cont 'd)

Nucleoside diphosphate kinase	TA22803_4081	100	1	1	2	43				100	2	2	4				6
OSJNBa0020P07.3 protein	TA18515_4081	100	2	2	2												2
OSJNBa0043A12.27 protein	TA22010_4081	73	1	1	1	100	1	1	2	100	1	1	3	100	3	3	7	13
OSJNBa0084K20.14 protein	AW443345	100															0
Osmotin-like protein precursor	TA22462_4081													100	2	2	3	3
Oxygen-evolving enhancer protein 1, chloroplast precursor	TA17382_4081	100	3	3	5													5
Oxygen-evolving enhancer protein 1, chloroplast precursor	TA17771_4081	100	13	13	22													22
Oxygen-evolving enhancer protein 2, chloroplast precursor	TA18411_4081	100	7	10	17									64				17
P0 ribosomal protein-like	TA19201_4081	100	2	2	4													4
P69C protein	TA17373_4081	17				100	1	1	1	100	1	1	1	100	2	3	3	5
P69E protein	AW035790	17				71								100	2	2	3	3
P69F protein	TA30777_4081									84				100	3	4	6	6
P69F protein	TA35122_4081	17												100	2	2	3	3
Pathogenesis-related leaf protein 6 precursor	AI780393	73	1	1	1	100	2	2	5									6
Pectin methylesterase isoform alpha	TA23356_4081					99												0
Pectin methylesterase precursor	TA18815_4081													100				0

Appendix 1. (cont 'd)

Pectin methylesterase precursor	TA18816_4081	100	2	2	3	100	4	4	6	100	2	2	4				13
Pectinesterase U1 precursor	TA19076_4081					100	5	5	6								6
Peptide methionine sulfoxide reductase (EC 1.8.4.6) (Protein-methionine-S-oxide reductase) (Peptide Met(O) reductase)	TA19140_4081	100															0
Peptidyl-prolyl cis-trans isomerase CYP20-2, chloroplast precursor	TA18700_4081	100	2	2	3												3
Perchloric acid soluble translation inhibitor protein homolog	BW687354	100	4	4	5	100	4	4	5	100	6	6	10	100	7	11	26	46
Perchloric acid soluble translation inhibitor protein homolog	TA21647_4081									100	1	1	2	100	3	3	5	7
Peroxidase	TA29420_4081	73	1	1	1	100	6	6	18	100	7	9	15	100	6	9	17	51
Peroxidase	TA20935_4081	73	1	1	1	100	3	3	5									6
Peroxidase	TA29008_4081					100	1	1	1					100	2	2	3	4
Peroxidase precursor	TA18849_4081					100	3	3	4									4
Peroxiredoxin	TA22053_4081													100	2	2	3	3
Peroxiredoxin Q	TA21096_4081	100	5	6	9													9
Peroxisomal (S)-2-hydroxy-acid oxidase	TA18332_4081	100	8	8	15					100	1	1	1					16

Appendix 1. (cont 'd)

Phosphoglycerate kinase	TA17522_4081	100	16	17	34								89	1	1	1	35
Phosphoglycerate mutase	TA18385_4081	97															0
Phosphoribulokinase	TA18944_4081	100	7	7	14												14
Photosystem I P700 chlorophyll a apoprotein A2	TA34808_4081					100	2	2	3	100	2	2	3	100	2	2	4	10
Photosystem I reaction center subunit II, chloroplast precursor	AW442599	100	3	3	16	100	2	2	4	100	1	1	6	100	2	2	4	30
Photosystem I reaction center subunit IV B, chloroplast precursor (PSI-E B)	AW094263	100	4	4	7	100	2	2	2	92	1	1	1					10
Photosystem I reaction center subunit N, chloroplast precursor	AI775566	100	5	5	8													8
Photosystem II 10 kDa polypeptide, chloroplast precursor	AW040527	98																0
Photosystem II CP47 chlorophyll apoprotein	TA27197_4081					100	2	2	3									3
Photosystem II CP47 chlorophyll apoprotein	TA33932_4081					100												0
Photosystem II oxygen-evolving complex protein 3 precursor	TA17865_4081	100	3	4	12													12
Photosystem II phosphoprotein	BI922036	99																0

Appendix 1. (cont 'd)

Photosystem1 subunit A	TA32260_4081					97										0
Pi1 protein	LES277563					100										0
PII-like protein	BW685412													100	2	2	3	3
Plastidic aldolase	TA17571_4081	100	3	3	7									89	1	1	1	8
Plastidic aldolase NPALDP1	TA18103_4081	100	3	3	5													5
Plastidic aldolase NPALDP1	TA18110_4081	100	9	9	24	99	1	1	1									25
Plastidic cysteine synthase 2	TA22218_4081	100	3	3	5					85				100	3	3	3	8
Plastocyanin, chloroplast precursor	AI774561	100	3	3	5	100	1	1	1	100	3	5	12	100	3	5	15	33
Polygalacturonase inhibitor protein precursor	TA19908_4081					85				100	1	1	1	100	4	4	6	7
Polygalacturonase inhibitor-like protein	TA19262_4081	100	2	2	4	100	2	2	3	92	1	1	1					8
Polyphenol oxidase F, chloroplast precursor	TA17484_4081	100	8	9	13	100	2	2	3	100	3	3	3	100	3	3	4	23
Porphobilinogen deaminase, chloroplast precursor	TA20382_4081	100	3	3	4													4
PR5-like protein	AY257487					100	3	3	5									5
PREDICTED: similar to tubulin, beta 2	BT013893	100	5	5	6													6

Appendix 1. (cont 'd)

Probable aquaporin PIP-type pTOM75	TA17457_4081					100	2	2	2								2
Protein disulfide isomerase	TA18577_4081	100	4	4	6												6
Protein NP24 precursor	BI923079	23				100	2	2	3								3
Proteinase inhibitor 1 PPI3A4	TA18695_4081	100	2	2	4	100	3	3	4	100	3	3	4	100	3	3	3	15
Proteinase inhibitor I	AW442733	100	3	3	3	49				100	2	3	3	100	3	4	5	11
Proteinase inhibitor I	BG735335	99								100				100				0
PSI-H precursor	TA17698_4081	100																0
Putative alcohol dehydrogenase	BG124529	100																0
Putative aminotransferase AGD2	TA23087_4081	100	2	2	2													2
Putative chloroplast GrpE protein	TA22330_4081	98																0
Putative chloroplast GrpE protein	TA22331_4081	100	2	2	2													2
Putative chloroplast thiazole biosynthetic protein	TA18386_4081	100																0
Putative elongation factor	TA18790_4081	100	3	3	5													5
Putative GDSL-motif lipase/acylhydrolase	TA19171_4081					100	1	1	1					100	3	3	5	6
Putative L-ascorbate peroxidase, chloroplast precursor	TA21178_4081	100	2	3	3													3

Appendix 1. (cont 'd)

Putative ML domain protein	TA17458_4081	73	1	1	2	100	2	2	2	92	1	1	1				5
Putative peroxiredoxin	AI777234	100	3	3	4									100	1	1	2	6
Putative photosystem II protein reaction center W	TA23321_4081	100	2	2	2													2
Putative photosystem II stability/assembly factor HCF136, chloroplast	TA20754_4081	100	3	3	3													3
Putative proline-rich protein	TA21941_4081													100				0
Putative proline-rich protein APG isolog	TA17488_4081													100	6	7	11	11
Putative protein At4g01050	BG643273													100	2	2	3	3
Putative PSII 32kDa protein from chromosome 10 chloroplast insertion (Photosystem q(B) protein)	TA20986_4081	100	3	3	4	100	2	2	3					100	2	2	2	9
Putative pyridoxine biosynthesis protein isoform B	TA19440_4081	100	3	3	5													5
Putative ribose-5-phosphate isomerase	TA19773_4081	100	2	2	2	83												2
Putative rubisco subunit binding-protein alpha subunit	TA17716_4081	100	15	15	24													24
Putative serine protease inhibitor	TA26306_4081													100	5	5	6	6
Putative strictosidine synthase	TA23837_4081									68				100	2	2	3	3
Putative subtilisin-like proteinase	AW622160													100	2	2	4	4

Appendix 1. (cont 'd)

Putative thioredoxin m2	TA17883_4081	98								45							0
Putative thylakoid lumen rotamase	TA21377_4081	100	5	5	9												9
Putative transcription factor BTF3	AI778696	99															0
Quinone oxidoreductase-like protein	TA19411_4081	100	2	2	3												3
Ras-related GTP-binding protein	AW625996	98															0
Ribosomal protein L2	TA22694_4081	100	2	2	4												4
Ribosomal protein L25-like protein	BW691821	97															0
Ribosomal protein S5	TA19041_4081	99															0
Ribosome recycling factor, chloroplast precursor	TA23340_4081	100	2	2	4												4
Ribulose bisphosphate carboxylase large chain precursor	TA24863_4081	100	18	22	65	100	10	11	22	100	7	7	12	100	10	10	14	113
Ribulose bisphosphate carboxylase small chains, chloroplast precursor (EC 4.1.1.39) (RuBisCO small subunits)	AI774174	100	6	8	33	100	3	3	6									39
Ribulose bisphosphate carboxylase small chains, chloroplast precursor (EC 4.1.1.39) (RuBisCO small subunits)	AI780578	100	2	2	4													4

Appendix 1. (cont 'd)

Ribulose bisphosphate carboxylase small chains, chloroplast precursor (EC 4.1.1.39) (RuBisCO small subunits)	AW040461	100	2	4	17					100	1	1	2	89	1	1	1	20
Ribulose bisphosphate carboxylase small chains, chloroplast precursor (EC 4.1.1.39) (RuBisCO small subunits)	TA17625_4081	68				77				99	1	2	2	100	2	2	2	4
Ribulose bisphosphate carboxylase small chains, chloroplast precursor (EC 4.1.1.39) (RuBisCO small subunits)	TA17631_4081	100	3	4	7	99	1	1	2									9
Ribulose bisphosphate carboxylase/oxygenase activase 1, chloroplast precursor	TA17806_4081	100	15	19	55					92	1	1	1	100	2	2	2	58
Ribulose bisphosphate carboxylase/oxygenase activase, chloroplast precursor	TA17805_4081	100	3	3	5													5
Ribulose bisphosphate carboxylase/oxygenase activase, chloroplast precursor	TA17811_4081	100	3	3	9					92	1	1	1					10
Ribulose-phosphate 3-epimerase, chloroplast precursor	TA18809_4081	100	2	2	4													4
Rieske iron-sulfur protein-like	TA17377_4081													100	3	3	4	4
Ripening regulated protein DDTFR10-like	TA19398_4081	99																0

Appendix 1. (cont 'd)

S-adenosyl-L-methionine synthetase	TA18438_4081	100	4	4	7	77										7
SBT4B protein	AI895158					98								33				0
Sedoheptulose-1,7-bisphosphatase	TA19884_4081	100	7	7	12									98	1	1	1	13
Serine hydroxymethyltransferase	TA17718_4081	100	6	6	9													9
Serine hydroxymethyltransferase, mitochondrial precursor	TA19628_4081	100	5	5	9													9
Serine hydroxymethyltransferase, mitochondrial precursor	TA19629_4081	100	2	2	3													3
Serine-glyoxylate aminotransferase	TA17378_4081	100	5	5	9					100	2	2	3	100	3	3	4	16
SIEP1L protein precursor	TA18838_4081					98												0
Similar to SOUL Protein	TA23932_4081	100	3	3	5	100	2	2	4					100	1	1	2	11
Similar to SOUL Protein	TA29626_4081					100	1	1	1	100	1	1	2	100	6	7	9	12
Similarity to diaminopimelate decarboxylase	TA20755_4081	100	2	2	2													2
Similarity to receptor protein kinase	TA28106_4081	73	1	1	2	100	2	2	3	100	2	2	3	100	5	5	8	16
Single-stranded DNA binding protein precursor	TA19278_4081	100	2	2	2													2
Snakin2 precursor	TA17721_4081	73												99				0

Appendix 1. (cont 'd)

Sorbitol related enzyme	BG125422	17								99							0
Stress responsive cyclophilin	TA17988_4081	100															0
Stromal 70 kDa heat shock-related protein, chloroplast precursor	TA34404_4081	100	3	3	7												7
Suberization-associated anionic peroxidase 1 precursor	TA19712_4081					98	1	1	1	92	1	1	2	100	3	3	6	9
Subtilisin-like endoprotease	TA17581_4081	17												100	3	4	5	5
Subtilisin-like protease	LESUBTILI					100	4	4	6	100	8	8	11	100	22	23	40	57
Subtilisin-like protease	TA19997_4081					100	6	6	7					100	2	2	3	10
Subtilisin-like protease	TA21680_4081	20								100	2	2	2	100	12	12	15	17
Subtilisin-like protease precursor	TA23010_4081									100	4	4	4	100	7	7	9	13
Sulfate adenylyltransferase	TA21529_4081	99																0

Appendix 1. (cont 'd)

Sulfur	TA18962_4081	100	5	5	6											6
Superoxide dismutase	CN384616									100						0
Superoxide dismutase	AI778582	100	2	3	4					100	4	4	6	100	2	2	3	13
Superoxide dismutase	TA20387_4081	19				62				99	1	1	1	100	4	4	7	8
Superoxide dismutase	TA20147_4081									100	2	2	4	89	1	1	2	6
T31J12.6 protein	TA18673_4081	100	7	7	9													9
T5A14.12 protein	TA27483_4081	100	2	2	3													3
T6D22.2	TA17949_4081	100	6	6	8	91	1	1	1					89	1	1	1	10
Temperature-induced lipocalin	TA20409_4081									99				100	3	4	6	6
Temperature-induced lipocalin'	TA20210_4081									100	3	3	5	100	5	5	6	11
TGB12K interacting protein 3	TA18565_4081	100	2	2	3													3
Thaumatin-like protein	TA19251_4081					100	5	5	7									7
Thioredoxin F-type 2, chloroplast precursor	TA22363_4081	99																0
Thioredoxin M-type 4, chloroplast precursor	TA22065_4081	100																0
Thioredoxin peroxidase	TA17869_4081	100	3	3	4	100	1	1	1	100	3	3	4	100	2	2	2	11

Appendix 1. (cont 'd)

Threonine dehydratase biosynthetic, chloroplast precursor	TA17896_4081	100	13	14	21	100	11	11	22	100	12	17	45	100	16	25	99	187
Threonine synthase, chloroplast precursor	TA20716_4081	97																0
Thylakoid lumenal 16.5 kDa protein, chloroplast precursor	TA26629_4081	100	2	2	3													3
Thylakoid membrane phosphoprotein 14 kDa, chloroplast	TA19485_4081	95																0
Transketolase, chloroplast precursor	TA19239_4081	100	11	11	18					92	1	1	1	89	1	1	2	21
Translational elongation factor Tu	BI924701					99				92								0
Translationally controlled tumor protein-like protein	AW649133	100	2	2	3													3
Triose phosphate isomerase cytosolic isoform	TA18676_4081	100	4	4	8													8
Triosephosphate isomerase, chloroplast precursor	TA18257_4081	100	3	3	5													5
Type I (26 kD) CP29 polypeptide	BW692506	100	8	10	15	100	2	2	4	100	3	3	4	100	4	4	6	29
Ubiquitin C variant	BG713824	100												99				0
UTP:alpha-D-glucose-1-phosphate uridylyltransferase	TA18547_4081	100	4	4	8													8
UVB-resistance protein UVR8	TA35304_4081													100	2	2	3	3

Appendix 1. (cont 'd)

Vacuolar ATPase subunit B	TA18706_4081	100	3	3	6											6
Vacuolar H+-ATPase A1 subunit isoform	TA18543_4081	100	5	5	7											7
Viroid RNA-binding protein	TA18648_4081	100	1	1	1					100	2	2	4			5
Wound/stress protein precursor	BG135795					100	1	1	2	99	1	1	1	100	3	3	5	8
Wound/stress protein precursor	TA22659_4081					55				83				100	3	4	5	5
Wound-induced proteinase inhibitor 1 precursor	AW040514	100	2	2	4					100	1	1	2	89	1	1	1	7
Wound-induced proteinase inhibitor 1 precursor	TA19511_4081	26				99	1	1	1	100	3	3	3	89	1	1	1	5
Wound-inducible carboxypeptidase	TA26617_4081	100	2	2	4	100	2	2	2									6
YUP8H12.20 protein	TA19551_4081	100	2	2	3					67								3

Appendix 2. List of cDNA microarray clones that were differentially regulated ≥3-fold

SGN ID	Ratio WT/*jai1*	SD	Cluster	NR Definition line	NR E-value	TIGR TC#
TUS-32-E4	146.2	37.23	U144127	Ethylene-responsive proteinase inhibitor	2.00E-09	116378
TUS-21-H4	108.5	22.24	U143744	Translation-inhibitor protein	4.00E-50	116149
TUS-20-G14	105.7	16.44	U143342	Aspartic protease inhibitor 1	4.00E-72	123948
TUS-21-L17	85.73	31.33	U143321	Threonine dehydratase biosynthetic	0	115826
TUS-20-P8	82.04	26.73	U143365	Polyphenol oxidase F	0	124027
TUS-21-L4	77.33	55.26	U143859	Hypothetical protein precursor	1.00E-116	124174
TUS-44-M3	72.65	64.87	U143329	Wound-induced proteinase inhibitor II	8.00E-73	116040
TUS-45-F11	63.3	84.5	U144891	Expressed protein	4.00E-80	117076
TUS-43-H22	59.87	78.06	U143332	Proteinase inhibitor type II CEVI57	1.00E-106	126336
TUS-30-E5	58.73	22.1	U151205	No hits found		127041
TUS-22-P8	57.09	17.43	U149869	Expressed protein	2.00E-08	126495
TUS-19-D7	54.33	40.01	U144888	Pto-responsive gene 1 protein	1.00E-111	116861
TUS-18-J14	48.11	20.18	U145939	Expressed protein	1.00E-70	107323
TUS-17-N7	46.17	36.77	U143361	Polyphenol oxidase D	0	117469
TUS-19-D24	43.73	11.66	U144943	Putative proteinase inhibitor	3.00E-25	124725
TUS-23-E7	43.06	20.13	U147584	Unknown protein	1.00E-18	125767
TUS-34-H14	42.62	14.91	U149988	No hits found		121170
TUS-47-A8	39.21	48.81	U143905	Proteinase inhibitor type II	1.00E-129	124098
TUS-32-M5	38.14	16.9	U145219	Arginase	1.00E-128	124737
TUS-38-P12	37.97	14.66	U143556	Wound-induced proteinase inhibitor I	2.00E-34	116238
TUS-45-K21	36.08	14.38	U149449	Expressed protein	3.00E-04	122623
TUS-35-K21	35.54	11.79	U143387	1-aminocyclopropane-1-carboxylate oxidase	0	115848
TUS-38-D8	34.65	35.4	U143552	Wound-induced proteinase inhibitor I	1.00E-38	124387
TUS-37-L14	32.28	18.8	U146238	Expressed protein	8.00E-05	777711
TUS-47-K19	30.1	11.94	U144855	Extensin homolog	4.00E-70	116812
TUS-23-C13	27.37	5.03	U147292	Cytochrome P450	6.00E-83	125738
TUS-14-P15	27.07	9.27	U145539	Oxidoreductase 2OG-Fe(II) oxygenase	7.00E-70	125637
TUS-19-K22	25.84	6.28	U151986	Proton extrusion protein-related	4.00E-19	119955

Appendix 2. (cont'd)

TUS-28-N24	24.66	13.14	U143303	Lipoxygenase A	0	124943
TUS-47-D3	24.02	8.13	U144856	Transferase family protein	6.00E-39	116721
TUS-14-J20	22.23	5.1	U154698	Digalactosyldiacylglycerol synthase	1.00E-10	131200
TUS-20-B21	22.1	4.87	U147059	Putative 7-transmembrane G-protein	0	117732
TUS-22-K8	21.16	7.44	U144325	PnFL-2	1.00E-32	124337
TUS-14-I18	21.15	3.07	U145537	Oxidoreductase 2OG-Fe(II) oxygenase	8.00E-51	125636
TUS-44-I20	20.08	9.16	U145329	Unknown protein	3.00E-29	118394
TUS-20-D13	19.43	3.58	U143562	Hypothetical protein	1.00E-25	116087
TUS-19-C12	17.28	0.47	U143488	Aminopeptidase 1	0	124100
TUS-35-D21	16.61	5.38	U146391	Putative cytochrome P450	1.00E-157	117297
TUS-29-D22	16.15	2.5	U143809	Cinnamic acid 4-hydroxylase	0	124119
TUS-44-G5	15.54	1.9	U143781	WIZZ	4.00E-28	124157
TUS-44-E15	15.01	12.16	U146859	Myb-related protein Ph2	6.00E-59	118787
TUS-21-N19	14.94	4.92	U144188	DIP-1	0	116347
TUS-34-G5	14.87	11.72	U149140	RabGAP/TBC domain-containing protein	1.00E-103	121734
TUS-19-F14	14.67	2.68	U144573	4-coumarate--CoA ligase 1	0	116469
TUS-19-B6	14.44	1.26	U143549	Wound-induced proteinase inhibitor I	5.00E-44	116240
TUS-23-C12	14.41	6.48	U145924	Lipase	2.00E-68	117225
TUS-37-D16	14.23	10.67	U153310	Hypothetical 16.5K protein - common toba	6.00E-62	123215
TUS-30-E3	13.96	6.19	U152367	Leucine-rich repeat family protein	2.00E-23	128116
TUS-22-A3	13.86	2.07	U148185	Wound-inducible carboxypeptidase	1.00E-127	117874
TUS-17-A4	13.73	4.83	U148626	Serine/threonine protein kinase	9.00E-18	AW034894
TUS-44-G11	13.64	6.59	U146418	AP2 domain-containing transcription factor	1.00E-24	127078
TUS-43-C7	12.79	2.48	U144503	Putative WRKY transcription factor 30	9.00E-77	124512
TUS-27-K23	12.32	5.1	U145314	Calnexin 1 (CNX1)	0	116805
TUS-40-E3	12.13	7.37	U145330	Putative acid phosphatase	5.00E-68	116991
TUS-17-J4	12.13	2.61	U145740	Hydroxycinnamoyl transferase	0	125103
TUS-36-P22	11.83	4.03	U146652	Aspartyl protease family protein	7.00E-47	119540
TUS-19-C15	11.5	3.73	U143496	Arginine decarboxylase 1	0	124056
TUS-42-P11	11.5	3.76	U143896	YABBY2-like transcription factor YAB2	1.00E-62	124135

Appendix 2. (cont'd)

TUS-30-D18	11.37	2.24	U155823	Ammonium transporter 1	6.00E-99	118444
TUS-17-J8	10.73	0.57	U146433	Probable triacylglycerol lipase	1.00E-69	117304
TUS-19-G20	10.6	3.02	U149106	Expressed protein	9.00E-73	127882
TUS-21-J8	10.48	1.14	U149225	Expressed protein	5.00E-14	119017
SGN ID	Ratio	SD	Cluster	NR Definition line	NR E-value	TIGR TC#
TUS-34-I1	10.34	8.28	U144245	AKIN gamma	1.00E-146	124343
TUS-45-H14	10.22	15.39	U144562	Ascorbate peroxidase	1.00E-146	116499
TUS-38-J6	9.86	0.81	U150956	Beta-carbonic anhydrase	1.00E-108	127960
TUS-28-I20	9.75	9.08	U145346	Caffeoyl-CoA 3-O-methyltransferase	2.00E-79	124848
TUS-21-M23	9.65	5.01	U143779	WIZZ	1.00E-101	124156
TUS-21-A11	9.51	7.38	U145284	Mitochondrial substrate carrier family protein	1.00E-113	116866
TUS-36-O11	9.22	3.59	U148668	Aspartyl protease family protein	1.00E-111	119715
TUS-28-A13	9.13	1.51	U154619	protein F2D10.4	1.00E-126	BE459243
TUS-16-H16	9.03	7.22	U146661	Glycerol kinase	0	117672
TUS-30-I7	9.02	3.5	U147920	Unknown protein	9.00E-58	127678
TUS-43-M4	8.99	2.81	U147854	Pathogenesis-related genes transcription	1.00E-67	118897
TUS-19-L23	8.96	1.67	U143907	Lactoylglutathione lyase family protein	4.00E-39	124186
TUS-23-G5	8.91	0.77	U147275	No hits found		128310
TUS-21-D20	8.86	0.84	U144877	F1K23.17	7.00E-77	116728
TUS-17-P18	8.85	3.03	U146488	Oxidoreductase 2OG-Fe(II) oxygenase	3.00E-60	121271
TUS-21-J19	8.76	3.8	U143908	Lactoylglutathione lyase family protein	3.00E-44	124186
TUS-14-H6	8.75	0.66	U146788	Glycerophosphoryl diester phosphodiester	1.00E-144	125817
TUS-30-E15	8.42	3.75	U147722	ER lumen protein retaining receptor	1.00E-100	121118
TUS-40-O24	8.38	3.62	U155880	Phenylalanine ammonia-lyase	2.00E-42	130532
TUS-15-C18	8.36	5.83	U146079	Lipase class 3 family protein	1.00E-125	125257
TUS-36-D8	8.32	0.36	U148374	Probable bHLH transcription factor	1.00E-49	122558
TUS-14-O18	8.31	1.92	U145978	Expressed protein	1.00E-118	117214
TUS-45-E3	8.24	2.45	U153437	F-box family protein (FKF1) / adagio 3	1.00E-166	121791
TUS-25-P12	8.09	3.56	U147667	Tyrosine specific protein phosphatase	2.00E-66	118311
TUS-20-G3	7.71	6.05	U143560	Hypothetical protein	9.00E-24	116087

Appendix 2. (cont'd)

TUS-31-D18	7.69	2.85	U155584	Nuclear movement family protein	6.00E-80	127027
TUS-30-F9	7.62	1.24	U143853	Pectinesterase	3.00E-70	124144
TUS-36-C20	7.58	1.04	U153466	Multicystatin (MC)	7.00E-71	120994
TUS-20-N10	7.41	0.73	U144270	Probable cytochrome P-450 - curled-leave	1.00E-171	116472
TUS-29-G4	7.38	1.96	U143143	S-adenosylmethionine decarboxylase	4.00E-44	BG123372
TUS-21-H9	7.25	5.17	U143658	Oxidase like protein	0	123927
TUS-30-L17	7.04	2.91	U144408	Tobacco nucleolin	1.00E-106	117102
TUS-18-D2	7.01	2.72	U144670	MADS-box protein 15	4.00E-45	116193
TUS-36-H1	6.94	2.04	U150465	Unknown	1.00E-24	119403
TUS-30-K17	6.91	1.55	U145891	Heavy-metal-associated domain-containing	4.00E-26	123804
TUS-43-K4	6.84	4.16	U144010	Zinc finger protein	3.00E-93	116267
TUS-21-K12	6.81	1.18	U143858	Phospho-2-dehydro-3-deoxyheptonate aldolase	0	124172
TUS-41-D18	6.79	2.04	U146283	COP9 signalosome subunit 6 / CSN subunit	1.00E-130	118205
TUS-25-D8	6.74	3.55	U144444	Ripening regulated protein DDTFR18	0	116476
TUS-22-K20	6.7	0.9	U145228	12-oxophytodienoate reductase 3	0	124656
TUS-46-P10	6.7	3.06	U150532	Expressed protein	1.00E-92	128854
TUS-41-H12	6.66	7.47	U149892	DEM2	1.00E-178	119637
TUS-31-F20	6.54	2.04	U149861	Vacuolar sorting receptor protein	1.00E-150	124131
TUS-20-G23	6.52	2.28	U147748	Phosphoglycerate/bisphosphoglycerate mutase	7.00E-46	118835
TUS-31-M5	6.49	1.84	U144425	Shaggy protein kinase 4	0	116439
TUS-43-N21	6.44	1.87	U152461	Putative ethylene receptor	9.00E-59	124584
TUS-22-N3	6.4	1.55	U152615	Regulator of chromosome condensation	7.00E-88	128714
TUS-29-N22	6.37	3.25	U152590	Putative RAV-like B3 domain DNA binding	4.00E-53	120659
TUS-29-P14	6.36	1.03	U151203	OJ1092_A07.13	1.00E-26	775371
TUS-21-O1	6.27	2.49	U143736	Cathepsin B-like cysteine proteinase	1.00E-175	124059
TUS-47-F4	6.27	2.28	U145654	ATP synthase delta chain	1.00E-101	117379
TUS-19-L13	6.25	0.56	U146975	Calcium-binding protein	2.00E-30	125762
TUS-28-J2	6.22	2.27	U143969	Avr9/Cf-9 rapidly elicited protein 75	5.00E-28	123396
TUS-27-M23	6.12	1.74	U143764	Calreticulin precursor	0	124117
TUS-45-C18	6.11	1.85	U143563	Hypothetical protein	4.00E-25	116088

Appendix 2. (cont'd)

TUS-29-G6	6.06	4.52	U143274	1-aminocyclopropane-1-carboxylate oxidase	1.00E-169	123932
TUS-43-A17	6.04	1.35	U144063	Putative metallophosphatase	0	124230
TUS-43-K21	6	1.96	U152747	OSJNBa0058K23.11	3.00E-51	130115
TUS-47-H22	5.94	1.73	U143272	Unnamed protein product	1.00E-102	125167
TUS-32-K10	5.78	0.41	U153221	No hits found		39783
TUS-46-J20	5.74	1.2	U145424	Unknown protein	5.00E-30	127048
TUS-47-J6	5.74	0.64	U145579	Similar to hsr203J	0	117270
TUS-29-N11	5.74	0.34	U147544	Putative sugar transporter	0	117919
TUS-17-F17	5.7	1.68	U146843	Subtilisin-like proteinase	1.00E-170	121451
TUS-17-G1	5.67	4.94	U148411	Expressed protein	1.00E-09	126151
TUS-35-P10	5.63	1.27	U148155	Putative ABA-induced protein	6.00E-50	126310
TUS-37-P14	5.62	3.61	U143713	Polygalacturonase 2A precursor	0	124082
TUS-20-N3	5.54	3.04	U145756	Protein transport protein sec61	1.00E-156	116976
TUS-27-F9	5.52	0.27	U144268	Iron-deficiency-responsive protein	4.00E-97	124440
TUS-32-C19	5.51	1.21	U144147	S-adenosylmethionine synthetase 2	0	116331
TUS-15-M21	5.51	6.52	U154812	Prf	3.00E-27	122461
TUS-40-I14	5.5	6.68	U146660	S-adenosyl-L-methionine:salicylic acid c	1.00E-103	125218
TUS-34-J13	5.49	1	U148430	Exostosin family protein	1.00E-166	119286
TUS-42-O4	5.43	6.01	U145231	Lipoxygenase	0	124825
TUS-44-B17	5.42	0.94	U158882	Avr9/Cf-9 rapidly elicited protein 264	1.00E-112	AI771942
TUS-25-J16	5.4	0.16	U144733	Acetyl-CoA C-acyltransferase	5.00E-91	125676
TUS-19-G12	5.38	1.18	U151677	Lipase class 3 family protein	3.00E-45	486175
TUS-45-D18	5.36	1.44	U144851	Histidine decarboxylase	6.00E-74	124718
TUS-44-N21	5.36	1.52	U150427	protein kinase family protein	5.00E-65	127411
TUS-40-A1	5.35	1.87	U152275	Hypothetical protein F2G14_50	3.00E-19	120893
TUS-31-D5	5.31	1.43	U148805	emp24/gp25L/p24 family protein	5.00E-59	118580
TUS-46-N21	5.31	1.67	U155394	Pathogen-responsive alpha-dioxygenase	2.00E-63	119265
TUS-16-D8	5.31	4.07	U156084	Phospholipid/glycerol acyltransferase	6.00E-36	896655
TUS-21-I11	5.3	0.62	U143686	Arginine decarboxylase	0	124097
TUS-23-B13	5.26	0.49	U146873	Steroid sulfotransferase	3.00E-90	117818

Appendix 2. (cont'd)

TUS-24-F20	5.2	4.16	U150670	Expressed protein	0	120945
TUS-46-I20	5.17	2.47	U143947	Putative E2 ubiquitin-conjugating enzyme	1.00E-74	116271
TUS-36-E4	5.14	1.14	U147658	Photoperiod responsive protein	9.00E-75	125906
TUS-28-M23	5.06	0.76	U146536	Transcription factor-related	8.00E-85	117279
TUS-36-B10	5.05	1.53	U148801	AP2 domain-containing transcription fact	3.00E-23	120802
TUS-17-C2	5.03	2.29	U144857	Selenium binding protein	0	124585
TUS-46-I11	5	2.15	U144875	Zinc finger (C3HC4-type RING finger)	3.00E-25	124777
TUS-22-G4	4.89	1.83	U150975	Expressed protein	2.00E-95	120037
TUS-24-K2	4.88	1.49	U148347	FAD-binding domain-containing protein	1.00E-105	120302
TUS-16-D13	4.85	2.91	U151052	50S ribosomal protein-related	8.00E-50	131554
TUS-37-F8	4.84	1.1	U153465	MYC transcription factor	8.00E-63	127925
TUS-42-E9	4.83	1.08	U143318	Histidine decarboxylase	1.00E-146	123916
TUS-20-C11	4.7	1.51	U146870	No hits found		125327
TUS-24-G9	4.68	0.56	U144949	Expressed protein	5.00E-15	116782
TUS-21-P4	4.66	0.25	U149291	transporter-like protein	1.00E-42	128432
TUS-16-H13	4.64	3.89	U148611	Calcium-binding EF hand family protein	3.00E-85	118960
TUS-14-C9	4.56	1.14	U145278	Lignin forming anionic peroxidase	1.00E-151	124298
TUS-20-I16	4.56	0.66	U146052	AAA-type ATPase family protein	2.00E-68	125191
TUS-14-E16	4.54	0.74	U144140	Putative spermine synthase	0	124221
TUS-22-K5	4.52	0.13	U143822	Embryo-abundant protein EMB	7.00E-73	116194
TUS-23-N18	4.49	0.72	U145573	Sorting nexin 1	1.00E-172	124902
TUS-46-C18	4.48	0.41	U145512	OSJNBa0086B14.2	4.00E-85	117067
TUS-46-G15	4.47	0.95	U146817	Homeobox-leucine zipper protein 13	5.00E-32	488544
TUS-47-E10	4.45	0.83	U168671	F-box family protein	5.00E-24	130340
TUS-45-N21	4.42	0.77	U143344	Chloroplast ferredoxin I	2.00E-60	124073
TUS-35-C14	4.3	2.68	U143762	Viroid RNA-binding protein	0	116099
TUS-21-K11	4.27	0.61	U149460	Putative phosphatidylcholine acyltransferase	6.00E-72	127351
TUS-19-F10	4.25	0.78	U148120	Miraculin-like protein	1.00E-30	125619
TUS-14-N18	4.24	0.61	U147700	Hypothetical protein	7.00E-10	126893
TUS-19-I22	4.2	1.3	U150880	Putative aspartate transaminase	1.00E-98	126428

Appendix 2. (cont'd)

TUS-46-F24	4.15	1.91	U150296	2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase	3.00E-86	127327
TUS-28-F9	4.14	1.18	U146498	Unknown	2.00E-96	117327
TUS-42-K17	4.12	0.68	U144471	Hypothetical protein R1	0	116544
TUS-31-G14	4.11	0.11	U144342	Probable protein disulfide-isomerase	0	124361
TUS-23-N17	4.09	0.61	U147634	Calcium-binding protein CAST	1.00E-93	117785
TUS-46-D19	4.05	1.62	U143261	Probable chaperonin 60 beta chain	0	115713
TUS-42-D11	4.04	0.47	U143697	CIG1	0	116127
TUS-47-H5	4.04	0.93	U145008	Ethylene-binding protein	1.00E-137	116750
TUS-22-H10	3.94	0.81	U144769	Expressed protein	6.00E-15	117053
TUS-47-H20	3.92	0.55	U145186	Systemin precursor gi|7489075|pir||T0714	3.00E-79	116818
TUS-21-F18	3.91	0.57	U143667	NAC domain protein NAC2	2.00E-83	116288
TUS-29-J3	3.89	1.53	U144004	Dehydroascorbate reductase	1.00E-106	116433
TUS-38-A19	3.87	0.76	U150615	Xyloglucan endotransglycosylase XET2	1.00E-98	130186
TUS-36-N24	3.87	0.48	U150924	DNAJ heat shock N-terminal domain-containing	4.00E-09	123010
TUS-18-L11	3.84	1.66	U144626	Zinc finger (CCCH-type) family protein	1.00E-143	124300
TUS-22-H23	3.81	0.69	U147087	Beta-fructofuranosidase	0	125260
TUS-15-A3	3.77	0.59	U145306	Calcium-dependent protein kinase 2	7.00E-14	AW029657
TUS-24-H21	3.76	1.05	U146634	2-oxoglutarate-dependent dioxygenase	3.00E-78	117485
TUS-20-B20	3.74	0.42	U150616	Cytochrome P450	2.00E-49	120324
TUS-42-K18	3.72	0.54	U143665	Triose phosphate/phosphate translocator	0	124128
TUS-24-N12	3.7	0.64	U149512	IAA16 protein	4.00E-58	119702
TUS-15-K1	3.66	0.42	U143909	Homeotic protein VAHOX1	1.00E-169	124192
TUS-20-B4	3.66	0.89	U144278	Nucleoside diphosphate kinase (NDK)	4.00E-76	124309
TUS-29-A15	3.66	0.95	U147018	Glutaminyl-peptide cyclotransferase	4.00E-91	125935
TUS-42-D17	3.64	0.97	U145523	Calmodulin-like protein	2.00E-29	124877
TUS-21-D9	3.63	0.28	U143841	Putative peroxidase	1.00E-151	116210
TUS-47-D18	3.57	1.11	U143286	Probable glutathione S-transferase	8.00E-79	124150
TUS-38-L17	3.57	1.44	U147422	Allene oxide cyclase	1.00E-105	117620
TUS-19-J13	3.54	1.34	U145901	Mitochondrial import receptor subunit TO	1.00E-78	125120

Appendix 2. (cont'd)

TUS-37-C10	3.53	0.12	U144781	Probable pyridoxin biosynthesis protein	1.00E-151	116989
TUS-34-E10	3.53	0.83	U150807	Expressed protein	6.00E-87	129121
TUS-18-C4	3.52	0.39	U146256	Expressed protein	2.00E-81	125255
TUS-48-C12	3.5	1.79	U160777	Putative gamma TIP	3.00E-88	116090
TUS-28-K23	3.43	0.55	U144858	Pyruvate dehydrogenase E1 beta subunit	1.00E-172	116814
TUS-43-A4	3.42	0.81	U145485	WRKY family transcription factor	1.00E-98	122662
TUS-42-G3	3.41	0.35	U144337	Citrate synthase	0	116816
TUS-23-F12	3.41	0.42	U145393	At1g17620	1.00E-40	116796
TUS-36-C11	3.41	1.55	U147792	RNA polymerase beta chain	0	119956
TUS-32-K2	3.36	1.23	U146545	NAD(P)H:quinone oxidoreductase	6.00E-91	127564
TUS-40-E1	3.36	0.7	U146690	Expressed protein (APS2)	5.00E-13	117543
TUS-17-F14	3.33	1.16	U144421	Putative chorismate mutase/prephenate de	1.00E-167	116428
TUS-28-F23	3.3	0.93	U143555	Metal-transporting ATPase-like protein	1.00E-134	117934
TUS-38-C17	3.29	0.54	U143504	Protein phosphatase 2A	1.00E-180	124599
TUS-18-M10	3.27	1.02	U147697	Basic helix-loop-helix (bHLH) family	4.00E-30	118036
TUS-28-D3	3.24	0.1	U152686	Phosphogluconate dehydrogenase	1.00E-121	123248
TUS-38-G18	3.22	0.87	U145175	Cellulose synthase family protein	1.00E-119	124881
TUS-22-D13	3.22	1.02	U151267	MATE efflux family protein	5.00E-92	127381
TUS-41-M14	3.2	1.31	U154981	Ethylene response factor 1	1.00E-117	122646
TUS-32-B12	3.19	1.32	U144112	Thioredoxin H-type 1	6.00E-52	116392
TUS-14-N17	3.18	0.3	U148495	LOB domain protein / lateral organ bound	5.00E-46	127274
TUS-45-N19	3.17	0.795	U143301	S-ADENOSYLMETHIONINE synthetase	0.00E+00	123896
TUS-48-F9	3.16	1.22	U147161	Probable UDPglucose 4-epimerase	1.00E-116	125202
TUS-23-C19	3.15	1.23	U149448	STS14 protein precursor	1.00E-77	127152
TUS-22-J22	3.14	1.37	U145384	Protein phosphatase 2C family protein	1.00E-140	142665
TUS-29-B8	3.13	0.96	U144939	Rcd1-like cell differentiation protein	1.00E-136	124652
TUS-21-C5	3.12	0.5	U143576	Cystathionine gamma synthase	0	123963
TUS-38-L6	3.12	0.43	U146009	Hypothetical protein	1.00E-131	125149
TUS-45-A17	3.12	1.24	U143290	Ubiquinol-cytochrome-c reductase	1.00E-127	147902
TUS-35-J6	3.11	0.08	U153200	receptor-like protein kinase	1.00E-24	120424

Appendix 2. (cont'd)

TUS-43-B21	3.07	1.39	U145379	Mitogen-activated protein kinase kinase	1.00E-99	144096
TUS-31-N22	3.06	1.36	U146095	Expressed protein	1.00E-100	117415
TUS-25-A19	3.05	0.44	U154673	NAD-dependent epimerase/dehydratase fami	1.00E-144	931860
TUS-38-G11	-3.06	0.36	U146692	Vacuolar ATP synthase subunit G 2	7.00E-48	125282
TUS-28-F20	-3.07	0.49	U144447	Band 7 family protein	1.00E-141	116470
TUS-30-D10	-3.13	0.96	U144536	Delta12 fatty acid desaturase	1.00E-160	116791
TUS-44-P4	-3.15	0.12	U143773	Glyceraldehyde 3-phosphate dehydrogenase	0	124167
TUS-40-G10	-3.17	0.64	U155488	Potassium channel	5.00E-59	122956
TUS-48-F16	-3.19	0.86	U155555	Plastidic aldolase	0	115712
TUS-40-K13	-3.32	1.31	U154023	Hypothetical protein	4.00E-15	128579
TUS-29-L14	-3.33	1.33	U143309	Secretory peroxidase	1.00E-170	115999
TUS-15-A17	-3.35	0.63	U143432	Late-embryogenesis protein homolog	2.00E-38	123969
TUS-41-D14	-3.43	0.73				
TUS-25-F24	-3.44	1.03	U151661	Homeotic protein - common sunflower	3.00E-58	131020
TUS-42-K14	-3.45	0.76	U155736	Bacterial-induced peroxidase	2.00E-79	AI773515
TUS-41-G16	-3.46	2.27	U147563	Putative polyamine oxidase	1.00E-134	119169
TUS-47-G11	-3.49	1.18	U144976	Hypothetical protein SEND32	1.00E-10	116757
TUS-43-D9	-3.52	4.6	U143283	Putative glutathione S-transferase T2	1.00E-118	124149
TUS-32-N7	-3.58	1.13	U152575	Expressed protein	2.00E-43	782809
TUS-47-F24	-3.63	1.29	U148335	Putative high-affinity nitrate transport	1.00E-17	118703
TUS-47-B8	-3.73	1.55	U143979	Putative leucine zipper protein	0	124228
TUS-43-M21	-3.73	0.8	U145262	CBL-interacting protein kinase 25	5.00E-97	116971
TUS-40-G22	-3.73	0.88	U154238	Self-incompatibility protein S3	1.00E-19	734743
TUS-18-K16	-3.74	1.38	U146073	No hits found		117135
TUS-31-D11	-3.75	1.26	U147756	Fatty acid desaturase family protein	1.00E-150	127187
TUS-36-C14	-3.84	1	U143856	Metallocarboxypeptidase inhibitor	1.00E-38	124072
TUS-41-G7	-3.88	2.36	U145441	Vacuolar processing enzyme-1b	0	116357
TUS-27-F20	-3.9	0.83	U143454	Chloroplast thiazole biosynthetic protein	1.00E-172	123957
TUS-28-E15	-4	1.43	U153930	probable B-box zinc finger protein T26J1	5.00E-40	128680
TUS-32-J15	-4.03	0.51	U153622	RSI-1 protein precursor	9.00E-18	130502

Appendix 2. (cont'd)

TUS-35-G20	-4.05	1.17	U146034	DNA methyltransferase	1.00E-153	125703
TUS-48-O13	-4.05	1.68	U160559	Chlorophyll a/b-binding protein	1.00E-109	124015
TUS-36-G20	-4.08	2.03	U154075	Unknown	1.00E-45	131448
TUS-46-D24	-4.24	2.83	U143467	Chlorophyll a-b binding protein 13	1.00E-145	124015
TUS-26-K11	-4.43	0.78	U145335	Expressed protein	1.00E-143	124627
TUS-46-F9	-4.46	0.61	U144517	Geranylgeranyl reductase	0	124431
TUS-31-L19	-4.54	3.45	U152821	Copper-binding protein-related	3.00E-08	123686
TUS-41-D10	-4.55	3.83				U32444
TUS-30-O3	-4.61	2.14	U159425	Protein kinase family protein	3.00E-05	778956
TUS-29-H14	-4.62	1.31	U155603	Chlorophyll a-b binding protein 1B	1.00E-145	123794
TUS-17-G9	-4.88	1.76	U145334	Serine acetyltransferase	1.00E-164	116875
TUS-31-P19	-5.02	2.3	U148445	Expressed protein	1.00E-161	126849
TUS-30-L6	-5.25	1.53	U152950	Putative zinc finger protein	6.00E-27	131314
TUS-17-H1	-5.36	4.42	U145536	LeAux=Arabidopsis auxin-regulated	1.00E-81	124816
TUS-48-M17	-5.63	4.19	U163803	Chlorophyll a/b binding protein	1.00E-81	126295
TUS-30-K13	-6.18	2.82	U151928	Protein kinase family protein	2.00E-58	121403
TUS-32-G16	-7.19	2.63	U143868	P-rich protein EIG-I30	2.00E-34	116130
TUS-20-A1	-8.08	8.39	U146372	NAC domain protein	1.00E-162	118131
TUS-38-H10	-10.83	15.09	U146025			486692
TUS-32-P19	-10.98	10.08	U143335	Chlorophyll a-b binding protein 5	6.00E-33	116423
TUS-16-L10	-11.12	16.33	U146602	Amidase-like protein	0	117676
TUS-16-I16	-14.29	11.16	U157109	CSN8	2.00E-69	130749
TUS-41-A6	-17.73	27.9	U144826	Pathogenesis-related protein STH-2	4.00E-83	124667
TUS-47-P19	-17.89	34.67	U143280	Probable glutathione S-transferase	1.00E-105	124148
TUS-35-L9	-25.27	40.45	U143404	Phytoene synthase 1	0	115970
TUS-34-D24	-72.95	122.3	U147628	Putative ubiquitin-specific protease otu	8.00E-22	127142