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Elucidation of the Role of Leucine Aminopeptidase A in the Wound Response Pathway in Tomato

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DEDICATION

To all the graduate students who put in more hours than anyone knows and to the family and friends who support them

ABSTRACT OF THE DISSERTATION

Elucidation of the Role of Leucine Aminopeptidase A in the
Wound Response Pathway in Tomato

by

Melissa Ann Scranton

Doctor of Philosophy, Graduate Program in Plant Biology
University of California, Riverside, March 2013
Dr. Linda L. Walling, Chairperson

Historically, peptidases have been considered as housekeeping proteins involved in protein degradation and amino acid turnover. However, recent work has highlighted the fact that peptidases, including aminopeptidases, have critical regulatory roles in the cell including modulation of growth, development and stress responses. Recently, plant leucine aminopeptidases (LAPs) have been recognized for their roles in modulation of late wound-responses and insect defense. While no known mechanism of action has been identified, LAPs were presumed to act through their aminopeptidase function to affect the stability, activity, or localization of a peptide and protein involved in defense. In my dissertation studies, I have identified two new molecular functions for plant LAPs as molecular chaperones and Cys-Gly dipeptidases. In addition, microarray analyses has demonstrated the tomato LAP-A regulates early and late wound responses both positively and negatively. In particular, microarray analyses identified two new sets of genes modulated by LAP-A: late wound *dehydrins* and *Pathogenesis-Related 1*. This study also provides evidence LAP-A may act though the negative regulator salicylic acid or the positive regulator hydrogen peroxide to modulate wound signaling. The role of LAP-A in glutathione metabolism is also discussed. Together this study has provided evidence that LAP-A's role in wound signaling and insect defense is more complex than initially anticipated

and may be the result of LAP-A utilizing multiple functions (aminopeptidase, chaperone and/or Cys-Gly dipeptidase activities) in different environments and in response to different stresses.

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INTRODUCTION

Wound Signaling

In nature, plants must cope with multitude of stresses individually and simultaneously. These abiotic (rain, hail, wind) and biotic stresses (herbivory) breach cellular integrity causing membrane disruption, desiccation, lipid and protein oxidation, and protein aggregation (Bostock 2005). This damage can range from mild (responses to phloem-feeding whiteflies, psyllids and aphids) to extreme (responses to pruning, hail or herbivores that chew and tear plant tissues; Chao et al. 2000b). The most resistant and successful plants rapidly respond to its injured status and activate pathways to limit spread of cellular damage and pathogen ingression into wound sites, promote cellular healing, and interfere with herbivore success (Heil 2009; Howe and Jander 2008; Walling 2009).

Wound signaling begins when a plant perceives a combination of one or more damage-associated molecular patterns (DAMPs). These include cell wall fragments (ex. oligogalacturonides, oligosaccharides) and potentially increases in released extracellular sucrose and ATP (Heil 2009; Maffei et al. 2007). Wounding also initiates responses to membrane depolarization to activate defense signaling. Wounding causes increases in cytosolic Ca^{2+}, inactivation of the H^+-ATPase, K^+ and H^+ fluxes, and generation of reactive oxygen species (ROS) (Bergey et al. 1999; Felix and Boller 1995; Narváez-Vásquez et al. 1999; Schaller and Oecking 1999; Stennis et al. 1998). These signals in turn can lead to sustained membrane depolarization and to downstream signaling including mitogen-activated protein (MAP) kinase cascade and phospholipase activation and calmodulin action.

During chewing insect feeding, plants can also respond to molecular cues that are unique to herbivore feeding [herbivore-associated molecular patterns (HAMPs) (Mithofer and Boland 2008)]. HAMPs include fatty acid-amino acid conjugates (FACs) and plant proteins that have been partially digested by insect proteases, which are presumed to bind to plant receptor proteins to trigger defense. These HAMPs are sufficient to induce wound responses, significantly amplify

responses to mechanical damage, or can induce specific defense responses. However, to date no HAMP has been discovered to elicit defenses in tomato (Schmelz et al. 2009).

Together early wound signals (DAMPs and HAMPs) trigger the activation of the octadecanoid pathway, which is responsible for the generation of biologically active oxygenated lipids, including 12-oxo-phytodienoic acid (12-OPDA) and jasmonic acid (JA) and its biologically active isoleucine conjugate (JA-Ile) (Li et al. 2005; Sun et al. 2011). While JA-Ile is responsible for the majority of wound-induced gene expression, there are JA-independent wound signaling events in tomato, which are less well studied, and some may be dependent on the JA precursor 12-ODPA (Böttcher and Pollmann 2009; Howe 2004; Ryan 2000; Wasternack 2007). The octadecanoid pathway begins in the plastids of companion cells and sieve elements (Hause et al. 2003). Phospholipases (PLD, PLA_2) release linolenic acid from the plastid membrane, which is converted by the action of lipoxygenases (LOX), allene oxide synthase (AOS) and allene oxidase cyclase (AOC) to form 12-OPDA. 12-OPDA is transported to peroxisomes via an ATP-transporter (COMATOSE) where it metabolized by OPDA reductase and a series of β-oxidations to form JA (Theodoulou 2005).

JA is conjugated to Ile by JASMONATE RESISTANT 1 (JAR1) to form JA-Ile (Suza et al. 2010). JA-Ile then binds to the F-box protein CORONATINE-INSENSITIVE1 (COI1). COI1 is part of Skp1/Cullin/F-box complex (SCF^{COI1}) (Browse 2009; Li et al. 2004). The binding of JA-Ile to COI1 enhances COI1's binding to JASMONATE ZIM-DOMAIN (JAZ) transcriptional repressors. JAZ proteins are then targeted for ubiquitination and degradation by the 26S proteasome, freeing jasmonate transcription factors such as MYC2 to promote the transcription of JA-response genes. JAZ proteins repress expression by recruiting the Groucho (Gro)/Tup1-type co-repressor family of proteins (TOPLESS, TPL) either alone or through the aid of NOVEL INTERACTOR of JAZ (NINJA) (Pauwels et al. 2010). TPL in turn is proposed to act through the recruitment of histone-modifying enzymes to suppress transcription (Kazan and Manners 2012). There are 12 JAZ proteins in *Arabidopsis thaliana* (Arabidopsis) with unique expression, COI1-binding and motif patterns (Kazan and Manners 2012; Pauwels and Goossens 2011). The unique combination JAZ

proteins present and actively repressing transcription or being targeted for degradation is thought to be responsible for JA's unique roles in multiple pathways of development and stress responses.

The regulation of MYC2 by the JA-Ile-stimulated turnover of JAZ proteins has primarily been studied in Arabidopsis. However, components of this pathway are present in a wide array of plant species and examples of this type of regulation has been demonstrated in plants species as diverse as *Nicotiana tabacum*, Madagascar periwinkle (*Catharanthus roseus*), and *Artemisia annua* (De Geyter et al. 2012). In particular, COI1 and MYC homologues have been shown to be essential regulators of defense responses in tomato (*Solanum lycopersicum;* Boter et al. 2004; Li et al. 2006).

In tomato, JA biosynthesis primarily occurs in damaged leaves. Grafting studies with JA biosynthesis and perception mutants indicate that a peroxisome-generated oxylipin, likely JA, is phloem-transported and activates defenses in apical, unwounded leaves (systemic response) (Koo and Howe 2009; Wasternack et al. 2006). This contrasts to Arabidopsis where it was shown that a non-JA signal triggered systemic biosynthesis of JA and JA-Ile, which was required for systemic wound response (Koo et al. 2009). Thus, JA signaling is complex and distinct among these two model organisms (Sun et al. 2011).

Tomato is also unique from Arabidopsis in its temporal responses to wounding (Kim et al. 2011; Sun et al. 2011). In tomato, mechanical damage results in temporal (early and late) and spatial (locally and systemically) changes in gene expression (Ryan 2000). The early wound-response genes are up-regulated 0.5 to 2 hr after wounding and primarily involved in amplification of the octadecanoid pathway (LOX, AOS, AOC, polygalacturonases, prosystemin). The late wound-response genes are up-regulated 4 to 24 hr and primarily consist of genes involved in insect deterrence. These genes include polyphenol oxidase (PPO), serine proteinase inhibitors (PinI and PinII), arginase, and Thr deaminase, which are known to damage the insect midgut or have anti-nutritive roles (Chen et al. 2005; Felton et al. 1989; Green and Ryan 1972; Howe 2004). In some Solanaceous plants, leucine aminopeptidase A (LAP-A) also increases during the late-

wound response and is important for insect defense (Chao et al. 1999; Fowler et al. 2009; Hartl et al. 2008). However, LAP-A's mechanism of action remains unknown (discussed below).

Hormone Cross-Talk in Wound Signaling

While oxylipin-regulated defenses are often the dominant response to damage, this signaling pathway is integrated into a complex and dynamic defense signaling network that involves the plant hormones salicylic acid (SA), ethylene (ET), abscisic acid (ABA), gibberellic acid (GA), brassinosteroids (BR), auxin (indole acetic acid, IAA), cytokinins (CK), as well as H_2O_2 and redox changes (Erb and Glauser 2010; Erb et al. 2012; Pieterse et al. 2009; Robert-Seilaniantz et al. 2011; Robert-Seilaniantz et al. 2007). These responses are also influenced by herbivore elicitors and effectors to stimulate or suppress defenses (Felton and Tumlinson 2008; Heil 2009; Howe and Jander 2008; Schmelz et al. 2009; Walling 2009).

The primary phytohormones studied in JA signal modification are SA and ET. Broadly, SA is primarily involved in defense signaling against biotrophic pathogens, while JA and ET are essential for defenses against necotrophic pathogens and chewing insects (Glazebrook 2005). SA and JA mutual antagonism is the best studied relationship in phytohormone crosstalk (Robert-Seilaniantz et al. 2011; Thaler et al. 2012). The effect of this antagonism has been shown to be dependent on timing and concentration of each elicitor (Koornneef et al. 2008; Thaler et al. 2002b). However, SA and JA can also act additively or synergistically depending on the intensity and duration of each signal (Mur et al. 2006). For example, SA can enhance or suppress JA-responsive *THIONIN*, while JA can enhance or suppress SA-responsive *Pathogenesis Related protein 1* (*PR1*) depending on the concentration of modulating elicitor. The exact mechanism of cross-talk has yet to be elucidated though many potential key players have been identified such as glutaredoxin GRX480, MAP KINASE4 (MPK4), NPR1, and transcription factors such as MYC2, ET-response factor 1 (ERF1), NACs (petunia NAM and Arabidopsis ATAF1, ATAF2, and CUC2), and various WRKYs and JAZs (Koornneef and Pieterse 2008; Thaler et al. 2012; Zheng et al. 2012). Glutathione has also been implicated in JA-SA signal crosstalk (Koornneef et al. 2008).

In Arabidopsis, SA repression appears to regulate JA signaling downstream of JA biosynthesis (Leon-Reyes et al. 2010), while in tomato SA repression has been seen up- and down-stream of JA biosynthesis (Doares et al. 1995; Peña-Cortés et al. 1993). While the exact mechanism of cross-talk may not be conserved, mutual antagonism is seen at least throughout angiosperms (Thaler et al. 2012). This cross-talk can also be exploited by insect and microbial pathogens to suppress defenses through activation of the inappropriate defense pathway. The most famous example is of *Pseudomonas syringae*, which utilizes the JA-Ile mimic coronatine to induce JA responses to suppress SA defenses and promote virulence (Brooks et al. 2005). Conversely, whiteflies (*Bemisia tabaci B*) up-regulate SA signaling in order to suppress JA defenses and promote whitefly fitness (Zarate et al. 2007).

Overall, ET positively modulates JA responses in defense (Adie et al. 2007; Broekaert et al. 2006). While ET acts synergistically with JA in pathogen defense, ET more often has a negative relationship with JA signaling in response to wounding and insect feeding (Adie et al. 2007). Most studies examining ET modulation of JA signaling have been performed in Arabidopsis. These studies have shown that JA accumulation leads to de-repression of ET transcription factors (EIN3/EIL1), which in turn up-regulates ERF1 and ORA59 (OCTADECANOID-RESPONSIVE ARABIDOPSIS AP2/ERF 59), which redundantly regulate ET and JA (Lorenzo et al. 2003; Pre et al. 2008; Zhu et al. 2011). These genes control signaling in a MYC2-dependent manner and help to induce JA-regulated pathogen defenses but suppress JA-regulated wound-responsive genes (Dombrecht et al. 2007; Lorenzo et al. 2004; Lorenzo and Solano 2005). In addition, ET has also been implicated in modulating the NPR1-dependency of JA-SA antagonism in pathogen defense (Leon-Reyes et al. 2009). In contrast to Arabidopsis, in tomato, ET itself does not induce wound responses, but ET is essential for a robust JA or wound-induced *PinII* induction (O'Donnell 1996).

Another key phytohormone modulator of wound defenses is the drought-stress hormone ABA. Wounding and drought responses are tightly correlated since desiccation is known to occur in wounded and neighboring cells (Birkenmeier and Ryan 1998). ABA accumulates in response to wounding and systemin in tomato (Birkenmeier and Ryan 1998; Peña-Cortés et al. 1995; Peña-

Cortés et al. 1996). Moreover, ABA is essential for a robust wound response and defense against chewing insects (Peña-Cortés et al. 1996; Peña-Cortés et al. 1989; Pena-Cortes et al. 1991). In Arabidopsis, it has been shown that ABA and JA act synergistically through the transcription factor MYC2 to regulate wound responses and that mechanical wounding and dehydration stress regulate many of the same genes (Boter et al. 2004; Dombrecht et al. 2007; Reymond et al. 2000). The *At*MYC2 homologs JAMYC2 and JAMYC10 have also been shown to regulate JA-dependent wound-responsive genes in tomato (Boter et al. 2004). Together these studies have shown that in tomato desiccation and ABA play an essential synergistic role in generating a robust wound response.

GA also undergoes cross-talk with wound and JA signaling. In Arabidopsis, GA increases resistance to necrotrophic pathogens, while decreasing resistance to biotrophs by potentiating JA signaling and attenuating SA signaling (Navarro et al. 2008). Recently, an elegant model has been proposed in which GA modulates JA signaling through DELLA GA-signaling repressor in Arabidopsis (Gao et al. 2011; Hou et al. 2010). Hou et al. demonstrated that DELLAs competitively bind to JAZ proteins, which in turn frees JA-signaling transcription factors such as MYC2 to up-regulate JA-response genes. However, when GA is present, it promotes the degradation of DELLAs, allowing JAZ proteins to bind to MYC2 and other transcription factors and repress JA signaling. Conversely, JA has been shown to repress DELLA degradation and GA signaling (Yang et al. 2012). Consistent with this, in potato, GA was shown to prevent chitosan-induced *PinII* transcript accumulation (Peña-Cortés et al. 1989). However, one study in tomato has shown that GA can positively regulate JA- and wound-responsive class I chitinase (*Chi9*) and β-1,3-glucanase (*GluB*) (Wu and Bradford 2003). Therefore, it is not clear whether the Arabidopsis model will applicable in tomato.

Overall, BRs promote SA defenses while suppressing JA-related defenses. BR compromised tomato defense to insects by negatively regulating trichome density, terpene accumulation, and *PinII* transcript accumulation upstream of JA biosynthesis (Campos et al. 2009). In contrast, silencing of the BR-signaling gene *BRI1-associated kinase 1* (*BAK1*) in *N. attenuata* attenuated

wound signaling (Yang et al. 2011). This is confounded by the fact that the Arabidopsis BAK1 is known to associate with many LRR-receptor proteins involved in recognition and responses to many microbial associated molecular patterns (MAMPs), which are SA-dependent (Heese et al. 2007). Therefore, BAK1 may act independently of BR in wound or herbivory detection and signaling. In Arabidopsis, tobacco, and rice, BR also is involved in pattern-triggered immunity (PTI) and promotes resistance to a number of biotrophic pathogens through its promotion of SA accumulation and *PR1* expression (Divi et al. 2010; Nakashita et al. 2003). Therefore, BR's effect on wound signaling may be due to promotion of JA-SA crosstalk.

IAA's relationship with JA signaling is complex. In most instances, IAA promotes JA defenses against necrotrophs and suppresses SA defenses against biotrophs (Bari and Jones 2009). Several members of the IAA signaling pathway affect both IAA and JA signaling and response (Feng et al. 2003; Nagpal et al. 2005; Ren et al. 2005; Tiryaki and Staswick 2002). For example, SGT1 (SUPPRESSOR OF G-TWO ALLELE OF SKP1) is important for auxin and JA signaling in Arabidopsis, as well as wounding and insect defenses in *N. attenuata* (Gray et al. 2003b; Meldau et al. 2011). In tomato, one study has also shown that auxin can induce *PinII* expression in roots (Taylor et al. 1993). This last study is interesting given that IAA and JA appear to have an antagonistic relationship regulating root growth in tomato (Chen et al. 2011; Gutierrez et al. 2012). Moreover, IAA has been shown to repress the *PinII* promoter *in vitro* and *in vivo* in *N. tabacum* leaves (Kernan and Thornburg 1989). This is consistent with studies in different plant species that IAA and JA's relationship appears to be tissue, species and stressor specific (Bari and Jones 2009; Erb et al. 2012; Kazan and Manners 2008).

Little is known about the relationship of CK with wound and JA signaling. A series of studies in *N. attenuata* and one in poplar have demonstrated that CK promotes wound and JA signaling (Erb et al. 2012). On the other hand, CKs increased SA accumulation and *PR1* expression to promote resistance to biotrophic pathogen *P. syringae* in Arabidopsis (Choi et al. 2010). These differences may be due to differing signaling networks in different plant species that lead to different roles of CK in defense or perhaps differences in timing and dosage of elicitors and/or

stressor. CK's role in tomato defenses is unexplored. However, a tomato cytokinin-response factor (*SlCRF1/Pti6*) has been implicated in defense against biotrophic pathogens, suggesting that there may be some cross-talk (Gu et al. 2002; Shi et al. 2012; Zhou et al. 1997).

Nitric Oxide and Reactive Oxygen Species in Defense

Nitric oxide (NO) and reactive oxygen species (ROS) have been shown to be important signaling molecules and modulators of defense responses. NO is generated throughout the cell by either nitrate reductases or NO synthases and has been implicated in modulating many developmental and stress pathways (Wilson et al. 2008). During pathogen responses in Arabidopsis, NO works synergistically with hydrogen peroxide (H_2O_2) to promote disease resistance (Torres et al. 2006). However, in tomato, NO represses pathogen and wound-induced H_2O_2 accumulation and H_2O_2-dependent resistance to the necrotrophic fungal pathogen *Botrytis cinerea* and late wound-induced responses (Małolepsza and Rózalska 2005; Orozco-Cárdenas et al. 2001; Orozco-Cárdenas and Ryan 2002).

ROS production and metabolism is ubiquitous in the cell and throughout all organismal kingdoms. In plants, ROS in is generated naturally as a byproduct of aerobic metabolism in photosynthesis, respiration, and photorespiration (Møller and Sweetlove 2010). ROS are also produced by a suite of enzymes found throughout the cell including xanthine and glucose oxidases, as well as a range of peroxidases that utilize many different cellular components as substrates (Mittler 2002). However, ROS are highly reactive and can damage cellular components such as DNA, proteins, lipids, as well as small molecules such as hormones (Apel and Hirt 2004; Mori 2009). Therefore, plants also have a complex network of enzymes and small antioxidant compounds to maintain ROS homeostasis (Mittler et al. 2004). The best studied antioxidant network is the ascorbate-gluathione cycle (discussed below) (Foyer and Noctor 2011). Perturbation in primary metabolism due to abiotic or biotic stress can readily induce accumulation of ROS. At low levels, ROS act as signaling molecules in a wide variety of developmental and stress pathways (Dat et al. 2000; Foreman et al. 2003; Gapper and Dolan 2006; Tsukagoshi et al. 2010; Kerchev et al. 2012; Suzuki et al. 2012; Torres et al. 2006). However, elevated levels of

ROS often leads to programmed cell death, which is important not only in hypersensitive response in pathogen defense but also in development, senescence and plant-plant interactions (Bais et al. 2003; Bethke and Jones 2001; Gechev and Hille 2005).

The major ROS in plants are superoxide ($O_2^{\cdot -}$), singlet oxygen ($^1O_2$), the hydroxyl radical superoxide ($HO^{\cdot}$), and H_2O_2 (Møller and Sweetlove 2010). These ROS have been postulated to be signaling molecules themselves. However, most of these molecules are too reactive to travel any significant distance in the cell, let alone pass through any membranes. The exception is H_2O_2 which is relatively stable and has been shown to pass through membranes alone or with the aide of aquaporins (Bienert et al. 2007; Miller et al. 2010; Mubarakshina et al. 2010; Petrov and Van Breusegem 2012). In bacteria, H_2O_2 has been shown to directly regulate gene expression by oxidizing transcription factors (Imlay 2008; Lee and Helmann 2006; Lee et al. 2009). However, in eukaryotic cells, ROS is produced in different organelles and in response to many stimuli (Møller and Sweetlove 2010). Despite this, different types of ROS from different locations in the cell have been shown to induce unique gene expression signatures suggesting that there is specificity in ROS signaling (Gadjev et al. 2006). Therefore, several models have been proposed to explain ROS specificity (Mittler et al. 2011; Møller and Sweetlove 2010): (i) the intensity and dynamics of ROS accumulation could generate a unique ROS wave signature similar to that seen in Ca^{2+} signaling; (ii) each cellular compartment has their own ROS receptors and affect nuclear gene expression through its own signaling cascade; (iii) organelle-specific ROS-damaged molecules, such as oxidized lipids or peptides, can act as secondary messengers; and (iv) ROS simply modulates responses to other secondary signals present in stress conditions through an unknown pathway.

In plants, the primary sources of wound- and pathogen-induced ROS are plasma membrane-localized respiratory burst oxidase homologues (Rboh) and pH-dependent peroxidases (Suzuki et al. 2011; Torres et al. 2006). The best studied are the *Rboh* genes, which utilize NADPH to reduce O_2 to the superoxide anion (Marino et al. 2012). The highly reactive $O_2^{\cdot -}$ is then readily converted by superoxide dismutase (SOD) to H_2O_2, which is more stable and is able to travel into

the cytosol either passively or through aquaporins (Miller et al. 2010; Mittler et al. 2011). In tomato, it has been shown that H_2O_2, most likely originating from *Rboh*, is required for a robust expression of late-wound response genes, but H_2O_2 does not modulate early wound responses (Orozco-Cárdenas and Ryan 1999; Orozco-Cárdenas et al. 2001; Sagi et al. 2004). In addition, in Arabidopsis, Rboh not only generates local ROS in response to wounding and biotic stress, but also is required for ROS waves that can travel to systemic tissue through an auto-propagation of ROS production in cells along the path (Miller et al. 2009). Stress-induced ROS at the plasma membrane can also have anti-microbial effects either directly or through its role in strengthening the cell wall (Torres et al. 2006). While apoplastic ROS has been the primary focus of stress responses, ROS and redox changes occur throughout the cell in response to stress. In particular, a role for chloroplastic ROS in defense signaling has been highlighted recently (discussed below) (Baier and Dietz 2005; Galvez-Valdivieso and Mullineaux 2010; Padmanabhan and Dinesh-Kumar 2010).

In addition to enhancing wound signaling, ROS has also been shown to have direct anti-nutritional activity towards insects. The best characterized examples are polyphenol oxidases and other peroxidases that catalyze the oxidation of phenolics to quinones (Felton et al. 1989). Quinones in turn bind to nucleophilic side chains of proteins and amino acids. These modified proteins are resistant to degradation and therefore deprive the insect of essential amino acids. This is consistent with another study in potato showing that increasing foliar levels of the antioxidant ascorbic acid (AsA), which scavenges damaging ROS species, lead to increased *Myzus persicae* fitness (Goggin et al. 2010). However, this is in contrast to two studies in Arabidopsis and *Medicago sativa* in which the fitness of the caterpillar *Spodoptera littoralis* and aphids (*Therioaphis trifolii maculate* and *Acyrthosiphon kondo*), respectively, were negatively correlated with levels the antioxidant glutathione (GSH) and AsA (Miles and Oertli 1993; Schlaeppi et al. 2008). The negative relationship of GSH and insect fitness is confounded by the fact that GSH is the precursor for anti-insect glucosinolates (Schlaeppi et al. 2008). However, in the AsA-deficient *vtc1-1* Arabidopsis mutant, glucosinolate concentrations were not significantly

different. Miles and Oertli (1993) postulate that these discrepancies may due to the need to maintain the appropriate level of phenol semiquinones, since accumulation of too many quinones may result in self-polymerization and loss of radical capacity to damage essential proteins (Miles and Oertli 1993).

The relationship of ROS and plant-insect interactions is further complicated by the fact that some aphid and caterpillar species secrete their own glucose oxidase (GOX) (Celorio-Mancera et al. 2011; Eichenseer et al. 2010; Harmel et al. 2008). In the presence of glucose, GOX produces H_2O_2, which in most species including Arabidopsis (Weech et al. 2008), *M. truculata* (Bede et al. 2006), *N. attenuata* (Diezel et al. 2009), and *N. tabacum* (Musser et al. 2005; Musser et al. 2002), suppresses host defenses to insects, potentially either through the up-regulation of SA and pathogen responses or perhaps through ET crosstalk. However, in tomato, the caterpillar *Helicoverpa zea* saliva and GOX induces JA-dependent insect defenses such as the expression of *PinII* and *osmotin*, as well as stimulated additional trichome growth in response to injury (Tian et al. 2012). It was noteworthy that *H. zea* saliva did not affect tomato's early branch of wound responses which include signals that amplify JA biosynthesis. These data are consistent with previous studies that showed that H_2O_2 only enhanced late-wound gene expression in tomato (Orozco-Cárdenas et al. 2001). Taken together these studies demonstrate that the role of ROS in plant-insect interactions in complex, dependent on species-specific host-insect interactions as well as the location, metabolism, and specific species of ROS involved.

Growth Verses Defense

While constitutive defenses appear to be evolutionarily favorable, defense components can be autotoxic or can interfere with defense responses to other abiotic or biotic stresses (Baldwin and Callahan 1993; Frost et al. 2008; Kessler and Baldwin 2002). In addition, activating and maintaining defense is energetically costly (Bolton 2009). This has led to the hypothesis that plants must balance growth and development with defense responses (Herms 1992; Mole 1994). Consistent with this hypothesis, an antagonistic relationship between stress responses and photosynthesis has been established (Bilgin et al. 2010; Kempema et al. 2007; Kerchev et al.

2012). Although some studies have shown that maintaining photosynthesis is correlated with resistance to herbivory (Kerchev et al. 2012), the global down-regulation of photosynthesis gene expression appears to be a response that is subject to evolutionary selection, since it occurs in many plant-attacker interactions (Bilgin et al. 2010; Kerchev et al. 2012; Little et al. 2007; Schroder et al. 2005; Velikova et al. 2010). In addition, many studies have shown the trade-off between growth and defenses at the whole plant level (Bolton 2009). However, other studies have shown no trade-off or have seen a benefit in promoting primary metabolism in defense (Bolton 2009). Therefore, whether or not a plant can sustain constitutive defenses while maintaining growth may depend on the plant species and environmental factors/stressors present.

The Roles of Chloroplasts and Chloroplastic ROS in Defense

While chloroplasts have primarily been studied for their role in photosynthesis and primary metabolism, recent studies have focused on the regulatory role of plastids in pathogen, wound, and insect defense (Bonaventure and Baldwin 2010; Padmanabhan and Dinesh-Kumar 2010). In particular, plastids are also the site of many defense-response molecules including those involved in primary defense signaling (SA, JA, and other bioactive oxylipins), modulators of defense (ABA, GA, CK, NO), and volatile signals and defense (C_6 and terpenoid volatiles) (Dudareva et al. 2006; Torres et al. 2006; Uppalapati et al. 2007; Vranova et al. 2012; Wasternack 2007).Therefore, early signals, such as ROS that are generated primarily at the plasma membrane (discussed above), must be able to reach the plastid to stimulate the synthesis these key molecules. This may be done indirectly through affecting nuclear gene expression of plastid-targeted hormone biosynthesis genes or more directly through a recently described signaling pathway in which SA biosynthesis in PTI is regulated by the activation of a thylakoid calcium-sensing protein (CAS) by Ca^{2+} signals from the cytosol and stroma (Arimura et al. 2011; Bonaventure and Baldwin 2010; Nomura et al. 2012).

Due to the nature of its bivalent oxygen chemistry, heavy requirement for reductive power, and constant risk of excess electron pressure at PSII, the plastid is one of the major sources of

ROS and potentially oxidized metabolites (Foyer et al. 1994). While apoplastic H_2O_2 has been implicated as the primary signal essential for pathogen and wound signaling and defense (Miller et al. 2009; Orozco-Cárdenas et al. 2001; Sagi et al. 2004), plastid-generated ROS has been implicated in retrograde signaling not only in growth and development, but also in abiotic and biotic stress responses (Baier and Dietz 2005; Galvez-Valdivieso and Mullineaux 2010; Maruta et al. 2012; Padmanabhan and Dinesh-Kumar 2010).

Relationships between chloroplasts and pathogen defense have been established. For instance, multiple pathogen effectors from *P. syringae* are targeted to the plastid and at least four have been shown to cause virulence (Fu et al. 2007; Guttman et al. 2002; Jelenska et al. 2010; Jelenska et al. 2007; Rodriguez-Herva et al. 2012). In addition, *Tobacco mosaic virus* (TMV) alters the location of the chloroplast N receptor-interacting protein 1 (NRIP1; (Caplan et al. 2008). This leads to NRIP's recognition by the plant cell and induction of defense responses. Moreover, there is a strong correlation between chloroplastic ROS and pathogen defense. In Arabidopsis, chloroplastic ROS is up-regulated after pathogen infection (Caplan et al. 2009; Liu et al. 2007b) and, in both Arabidopsis and tobacco, plastid ROS is required for a robust hypersensitive response (Liu et al. 2007b; Zurbriggen et al. 2009). In addition, studies of Arabidopsis lesion-mimic mutants demonstrate that intact chlorophyll biosynthesis and chloroplast regulation are required for proper hypersensitive responses (Padmanabhan and Dinesh-Kumar 2010). For example, the Arabidopsis *flu* mutant specifically generates $^1O_2$ in the chloroplast, which, in turn, induces expression of many nuclear-encoded SA- and JA-response genes, including *PR1* and several WRKYs (Danon et al. 2005). This expression pattern is different from that produced by superoxide/H_2O_2, which indicates there is specificity in ROS signaling (Gadjev et al. 2006). It should be noted that *PR* genes (including *PR1*) are also induced in response to cytosolic ROS accumulation (Baier and Dietz 2005); therefore, pathogen responses are regulated by the amount of ROS rather than the type or location of the ROS source.

Finally, chloroplast ROS has been implicated in defense due to the relationship between light quality and intensity and defense. Responses to microbial pathogens and acclimation to high-light

intensity strongly overlap (Mullineaux et al. 2000; Mullineaux and Baker 2010; Straus et al. 2010). Moreover, high-light treatment is correlated with enhanced disease resistance (Griebel and Zeier 2008; Karpinski et al. 2003; Muhlenbock et al. 2008). Finally, light is required for robust pathogen defense, including SA biosynthesis and *PR-1* gene expression (Chandra-Shekara et al. 2006; Genoud et al. 2002; Griebel and Zeier 2008; Zeier et al. 2004). This is presumably due to the fact that high light can lead to excess excitation energy in the plastid and, in turn, lead to excess chloroplast-localized ROS, which is important for biotic stress responses (discussed above) (Karpinski et al. 2003). Other potential roles for light in defense signaling include light's influence on primary metabolism and available resources for invading pathogens, as well as the role of photoreceptor signaling in defense cross-talk (Kangasjärvi et al. 2012). The latter is supported by the facts that changes in light quality, such as in shade, can also affect defense responses and that defense genes are regulated by light-dependent circadian rhythms (Kazan and Manners 2011; Roden and Ingle 2009; Shin et al. 2012; Wang et al. 2011).

Roles for plastids and light signaling in wounding and insect defense outside of JA and volatile biosynthesis have received little attention. While apoplastic ROS is essential for the wound response (Orozco-Cárdenas et al. 2001; Sagi et al. 2004), relatively little is known about chloroplastic ROS in wounding. Studies of the *flu* mutants of Arabidopsis implicate chloroplast $^1O_2$ in lipid oxidation leading to JA synthesis and signaling (Przybyla et al. 2008). In addition, in Arabidopsis light is required for a robust wound response (Morker and Roberts 2011), and changes in light quality (due to shade) repress JA responses and lead to increased insect feeding (Izaguirre et al. 2006; Moreno et al. 2009). Finally, JA responses are regulated by circadian rhythms, which also impact the timing of insect defense molecule accumulation (Goodspeed et al. 2012; Kazan and Manners 2011; Shin et al. 2012). Taken together, plastids and, in particular, light signaling play an important role in wound and insect defense, but their exact role and mechanism of action remain unknown.

Retrograde signaling from the chloroplast to the nucleus is well-established for developmental "biogenic" regulation, "operational" regulation in response to environmental

perturbations in mature plastids, and for “degradational” control of senescence (Fernández and Strand 2008; Pfannschmidt 2010; Pogson et al. 2008; Woodson and Chory 2008). However, despite over 30 years of research, no clear signaling pathway or mode of action has been determined for any of these critical regulatory pathways. Some potential players involved in retrograde signaling have been described and include: plastidial gene expression (PGE); intermediates of pigment biosynthesis; ROS or ROS/redox-regulated proteins or metabolites; and fluxes in metabolic pools. More recently, Ca^{2+} signaling has also been implicated in the plastid-mediated stress response (Nomura et al. 2012). However, due to the interconnected nature of these processes, teasing apart and identifying the role of any individual component has been difficult. For example, the most studied putative retrograde signals are the *genomes uncouple (gun)* mutants, which encode members of the tetrapyrrole biosynthesis pathway. *gun* mutants were initially identified by their ability to express Photosynthetic-Associated Nuclear Genes (PhANGs) in the presence of plastid function inhibitors, such as norflurazon (NF) and lincomycin (Linc) (Susek et al. 1993). These studies suggested that *gun* genes were negative regulators of PhANG expression during plastid stress. However, NF alters ROS-signaling and ABA biosynthesis, and Linc alters PGE and tetrapyrrole synthesis (Chamovitz et al. 1991; Gray et al. 2003a; Pfannschmidt 2010; Schon et al. 1986). Therefore, determining the functional relevance of the *gun* mutants under ambient and natural stress conditions has been difficult.

Glutathione Metabolism and its Roles in ROS Metabolism

Glutathione (GSH) is an essential metabolite in all kingdoms involved in ROS metabolism and redox homeostasis (Noctor et al. 2012). Pathways for GSH biosynthesis and catabolism have been long established in animal systems and understanding of plant GSH metabolism has been growing in recent years. GSH is involved in plant development, cell cycle regulation, xenobiotic and heavy metal detoxification, sulfur assimilation, and the resistance to a wide range of abiotic and biotic stresses. GSH is a tripeptide composed of Glu, Cys, and Gly in which the Glu attaches to the Cys at the γ–carboxyl group of Glu (differentiating it from a typical peptide bond) (Noctor et al. 2011). While GSH is the only known thiol tripeptide in Arabidopsis, other GSH homologues

have been found in other plant species including legumes and cereals. GSH is the predominant storage and long distance transport form of reduced sulfur in plants (Leustek and Saito 1999), typically accumulating to millimolar concentrations in healthy cells (Mhamdi et al. 2010; Noctor et al. 2012; Noctor et al. 2011). The primary GSH form in healthy cells is the reduced GSH, which is typically 20 times more abundant that the oxidized GSSG. However, when stressed, total levels of GSH increase and the GSH:GSSG ratio lowers. GSH levels also fluctuate diurnally, increasing in response to light (Koornneef et al. 2008; Noctor and Foyer 1998).

Similar to animals, GSH is synthesized in two ATP-dependent steps. The γ-EC synthase (γ-ECS; GSH1) forms the γ-Glu-Cys bond and GSH synthase (GSH-S; GSH2) joins the Gly to the γ-Glu-Cys dipeptide (Meister 1988; Mullineaux and Rausch 2005; Noctor et al. 2002; Rennenberg 1982). γ–ECS is the rate limiting enzyme and is primarily regulated post-transcriptionally by redox regulation of a regulatory disulfide bond within the enzyme or feedback inhibition by GSH (Hell and Bergmann 1990; Hothorn et al. 2006; Noctor et al. 2002). However, with the exception of exogenous H_2O_2, *GSH1* and *GSH2* transcripts also increase in response to JA and other stresses (Queval et al. 2009; Sung et al. 2009; Xiang and Oliver 1998). In Arabidopsis, GSH1 is encoded by one gene that is targeted to the plastid. In contrast, GSH2 is encoded by one gene that gives rise to two transcripts encoding protein with or without a plastid transit sequence (Wachter et al. 2005). In Arabidopsis, the cytosolic form of GSH2 is predominant. In addition to increases in γ–ECS activity, increases in Cys biosynthesis are associated with increases in GSH levels. In addition, Cys biosynthesis genes, including all three adenosine 5'-phosphosulphate reductases (APR) and the plastid-localized serine acetyltransferase (SAT), are up-regulated in Arabidopsis in response to stress (Noctor et al. 2012; Queval et al. 2009).

While GSH biosynthesis is localized to the plastid and cytosol, GSH is present at substantial levels in every organelle in the cell, with notably high concentrations in the mitochondria (Queval et al. 2011; Zechmann et al. 2008). A subset of transporters that aide in this distribution of GSH have been identified and include: the Arabidopsis plasma membrane oligopeptide transporter OPT6, which plays a role in long-distance transport (Cagnac et al. 2004); the Arabidopsis

chloroplast transporters CLT1-3, which can aide in bidirectional transport in the plastid (Maughan et al. 2010); and tonoplast multidrug resistance-associated protein (MRPs), which aid in uptake of GSSG in Arabidopsis and barley (Lu et al. 1998; Tommasini et al. 1993).

GSH has multiple functions within plant cell. The best characterized role of GSH is in ROS metabolism either alone or in combination with other antioxidants and/or enzymes (Noctor et al. 2012; Noctor et al. 2011). It is important to note that GSH reductive capabilities are dependent not only on the total amount of GSH, but also on the redox state of GSH (GSH:GGSG ratio). The classic example of GSH-mediated ROS metabolism is the ascorbate (Asc)-GSH pathway (Foyer and Noctor 2011). Briefly, Asc scavenges ROS via Asc peroxidase (APX) to generate monodehydroascorbate (MDHA). MDHA can either be regenerated to Asc by MDHA reductase (MDHAR) or is rapidly converted to dehydroascorbate (DHA) and requires DHA reductase (DHAR) to regenerate Asc. DHAR utilizes GSH to reduce DHA, forming GSSG which in turn is reduced back to GSH by GSH reductase (GR). This pathway has proven to be central to redox homeostasis in the cell and has been implicated in a wide array of developmental and stress pathways.

GSH can also be conjugated to proteins and small molecules by the diverse GSH transferase (GST) family or glutaredoxins (GRX) (Dixon and Edwards 2010; Rouhier 2010). Some GSTs have well-characterized functions such as DHAR in ROS metabolism and PCS in phytochelatin synthesis; however, most GSTs remain uncharacterized (Dixon and Edwards 2010). GRXs were originally studied for their role in GSH-dependent reduction of protein disulfide bridges; however, GRXs can also S-glutathionylated or de-glutathionylate Cys residues (Rouhier 2010). GRXs have been implicated in development and SA-JA interactions in Arabidopsis (Li et al. 2009; Ndamukong et al. 2007). While the existence of S-glutathionylated plant proteins was established over two decades ago (Butt and Ohlrogge 1991), less than a handful of protein targets have been identified to date. In these cases, glutathionylation negatively regulates protein function (Michelet et al. 2005; Palmieri et al. 2010; Zaffagnini et al. 2007). GSH can also be bound to NO to form GSNO, which can also cause S-glutathionylation of Cys residues as well as S-nitrosylation

(Noctor et al. 2012). In particular, GSNO has been implicated in pathogen and insect defense (discussed below).

With its diverse roles in redox homeostasis, ROS and xenobiotic detoxification, cells require relatively high GSH levels in virtually all cellular compartments. To determine if plants can benefit from enhanced levels of GSH, enzymes involved in GSH biosynthesis have been ectopically expressed in plants. Over-accumulation of GSH to ~3-4-fold higher than WT had no phenotypic effect on unstressed Indian mustard (*Brassica juncea*) or poplar (Noctor et al. 1998; Zhu et al. 1999); however, other studies have shown that similar increases in γ–ECS activity and GSH accumulation leads to lesion formation in *N. tabacum* or early leaf senescence in poplar (Creissen et al. 1999; Herschbach et al. 2010). This counter-intuitive result may suggest that artificially high accumulation of GSH or its precursor γ–EC can sometimes result in disruption in normal redox-sensing and signaling processes (Herschbach et al. 2010).

While GSH biosynthesis in plants is fairly well characterized, GSH catabolism in plants remains elusive. At least four classes enzymes have been identified that could be involved in initial GSH turnover (Noctor et al. 2012). These include: i) carboxypeptidases whose activity has been demonstrated in barley to remove the Gly from the C-terminus of GSH (Wolf et al. 1996); ii) phytochelatin synthase, which may have a role during heavy metal stress (Blum et al. 2007) iii) γ–glutamyl cyclotransferase (GGC), which has been proposed to be the major GSH degradation enzyme in Arabidopsis (Ohkama-Ohtsu et al. 2008); and iv) γ–glutamyl transpeptidase (GGT), which has been shown to have active homologues in several species including Arabidopsis, maize, barley, tobacco, and tomato (Ferretti et al. 2009; Martin and Slovin 2000; Masi et al. 2007; Ohkama-Ohtsu et al. 2007a; Ohkama-Ohtsu et al. 2007b; Storozhenko et al. 2002).

In Arabidopsis, cytosolic GGC is proposed to convert the γ -Glu of GSH to 5-oxoproline (5-OP) and release a Cys-Gly dipeptide (Ohkama-Ohtsu et al. 2008). 5-OP is then metabolized by 5-OPase to release free Glu, while the Cys-Gly is processed by a yet unidentified plant dipeptidase. While GGC activity has been detected in Arabidopsis and *N. tabacum* (Ohkama-

Ohtsu et al. 2008; Steinkamp and Rennenberg 1985; Steinkamp et al. 1987), no obvious homologues of the human GGC have been identified in plants (Oakley et al. 2008).

GGT produces a γ–glutamyl peptide and also releases Cys-Gly (Noctor et al. 2012). The γ–glutamyl peptide is then further processed by GGC and 5-OPase. GGT activity has been primarily detected in the apoplast, while one Arabidopsis GGT (GGT3) is vacuolar localized. While in Arabidopsis, GGC is the predominant enzyme involved in initial GSH breakdown and turnover, no studies have been performed in other species to determine which pathway is preferred. For instance, another pathway must be utilized to breakdown GSH in tobacco since its GGC homologue was unable to utilize GSH directly (Steinkamp et al. 1987).

Of all the potential steps involved in GSH degradation in plants, only Cys-Gly hydrolysis has not been studied. However, Cys-Gly hydrolysis has been studied in other kingdoms. Cys-Gly dipeptidase activity is important not only important for GSH catabolism to release free Cys and potential recycle GSH (Baudouin-Cornu et al. 2012; De Donatis et al. 2010), but also for removal of excess Cys-Gly, which is a damaging oxidant (del Bello et al. 1999; Del Corso et al. 2002; Dominici et al. 1999; Enoiu et al. 2007). In pig kidney, rat kidney, and rat neurons, the M1 aminopeptidase M (identical to aminopeptidase N; EC 3.4.11.2) has been shown to have Cys-Gly dipeptidase activity (Dringen et al. 2001; Grau et al. 1979; Mcintyre and Curthoys 1982; Rankin et al. 1980). An additional membrane-bound M19 dipeptidase (EC 3.4.13.19) has also shown Cys-Gly dipeptidase activity in pig kidneys (Robinson et al. 1953). In rat liver, bovine lens, and the bacterium *Treponema denticola*, a M17 leucine aminopeptidase (LAP) is the major or only Cys-Gly dipeptidase (Cappiello et al. 2004; Chu et al. 2008; Jösch et al. 2003). In *E. coli*, the multifunctional M17 LAP (PepA) is able to cleave Cys-Gly, however, other peptidases have Cys-Gly dipeptidase activity as well (Suzuki et al. 2001). Interestingly, in *Saccharomyces cerevisiae*, which lacks an M17 LAP, an M20A peptidase Dug1p is the only Cys-Gly dipeptidase (Kaur et al. 2009). This study also showed that Dug1p is the Cys-Gly dipeptidase in *Saccharomyces pombe*, which also has a M17 LAP homolog. Additionally, humans, which have an M17 LAP, also have a

Dug1p homolog called carnosinase-like dipeptidase (CNDP2). CNDP2 has Cys-Gly dipeptidase activity *in vitro* and can complement a *S. cerevisiae dug1Δ* strain.

Glutathione and Defense

Many studies have shown that glutathione is essential for defense. GSH-S mutants *cad2* and *apx1-1*, as well as a GSH plastid-transport mutant (*CLT*), show increases in pathogen susceptibility, while a *gr1* mutant accumulated lower levels of SA and *PR1* expression in response to pathogen infection (Ball et al. 2004; Maughan et al. 2010; Mhamdi et al. 2010). In addition, the GSH-S mutant *pad2* also shows decreased resistance to insect larvae feeding (Schlaeppi et al. 2008). However, in Arabidopsis, GSH defense against pathogens and pests is due to GSH's role as a precursor for the phytoalexin camalexin and toxic glucosinolates, respectively (Parisy et al. 2007; Schlaeppi et al. 2008). These defenses are unique to the order Capparales (Halkier and Gershenzon 2006; Su et al. 2011); however, sulfur-induced resistance is present in a wide range of plants (Bloem et al. 2007). In particular, sulfur levels, in part in the form of GSH and Cys, rise in resistant tomato in response to the fungus *Verticillium dahlia* (Williams et al. 2002). While the exact mechanism of action has not been confirmed, evidence points to elemental S rather than GSH or Cys as the anti-microbial agent (Bloem et al. 2007).

GSH may also regulate defenses through its role in SA-JA antagonism. NPR1 is a key central regulator of SA and JA responses (Mou et al. 2003). NPR1's quaternary structure and sub-cellular localization is regulated by redox. Upon pathogen attack, disulfide bridges are reduced, which allows NPR1 oligomers to become monomers and localize to the nucleus. This allows NPR1 to interact with transcription factors, such as TGAs, to mediate SA-induced gene expression (Despres et al. 2003; Kesarwani et al. 2007). A recent study has suggested that this monomerization is regulated by thioredoxin (Tada et al. 2008). However, given the interconnected nature of redox metabolism and the fact that GSH is known to induce *PR-1* expression, the role of GSH in NPR1 monomerization has not been ruled out (Noctor et al. 2012). On the other hand, role for cytosolic NPR1 in SA-dependent JA antagonism has been demonstrated and GSH was shown to be essential for this function (Koornneef et al. 2008; Spoel et al. 2003). Therefore,

cytosolic GSH promotes SA defenses either through helping to reduce NPR1 and induce SA responses or by acting with cytosolic NPR1 through an unknown pathway to suppress JA defense.

Finally, GSH may have an indirect role in defense through its role in NO turnover. S-nitroglutathione reductase (GSNOR) hydrolyzes NO-GSH conjugates to release GSSG and NH_3, thus eliminating the reactive NO signal (Wilson et al. 2008). GSNOR has been shown to positively regulate pathogen defense in Arabidopsis, as well as insect defenses in *N. attenuata* (Feechan et al. 2005; Wunsche et al. 2011). The role of GSNOR in pathogen defense is in part due to GSNO-mediated S-nitrosylation of NPR1, which inhibits NPR1's ability to become a monomer (Tada et al. 2008); while GSNOR's role in insect defense may be due in part to GNOR's role in catabolizing NO, which negatively regulates JA defenses (Orozco-Cárdenas and Ryan 2002).

Direct and Indirect Defenses Against Insect Herbivores

Plants defend themselves against insects either directly through interfering with insect performance or indirectly through emission of volatile organic compounds to attract natural enemies to insect-infested plants. Direct defenses include those that limit herbivore access to nutrients, reduce the nutritional value of plant material ingested, or are directly toxic to the insect. In response to insect feeding, plants will increase their number of trichomes in emerging leaves and induce genes to fortify their cell walls, both of which provide physical barriers to further insect feeding (Boughton et al. 2005; Divol et al. 2005; Liu et al. 2007a). Glandular trichomes also have the added benefit of releasing compounds that are toxic or entrap insects (Farag and Paré 2002; Kennedy 2003; Simmons and Gurr 2005). Similar to pathogen defense, plants can limit nutrient access to insects with fixed feeding sites by inducing localized cell death through the hypersensitive response (reviewed in (Chen 2008)). Fortified cell walls and HR also have the added benefit of protecting wounded plants from opportunistic pathogens (Bostock and Stermer 1989; Kahl 1982). Some plants also release volatile compounds that actively deter potential herbivorous insects, thus limiting the number of feeders (Unsicker et al. 2009).

Plants limit nutrient value in the lepidopteran midgut through the production of anti-nutritive and/or toxic enzymes. These include amino acid degrading enzymes such as arginase and threonine deaminase (TD), which are active in the insect midgut and thought to reduce availability of essential arginine and threonine, respectively (Chen et al. 2007; Chen et al. 2005; Kang and Baldwin 2006). Asparginase has also been shown to be induced in resistant wheat plants (Liu et al. 2007a). Proteases such as a 33-kD cysteine protease in maize and leucine aminopeptidase (LAP) in some Solanaceous species are also up-regulated and stable in the midgut (Chao et al. 2000a; Dammann et al. 1997; Fowler et al. 2009; Gu et al. 1996a; Pechan et al. 2000). While cysteine protease in maize acts through directly damaging the peritrophic matrix in caterpillars (Pechan et al. 2000), the role of LAP is still being elucidated (discussed below).

Plants also produce a wide range of proteinase and amylase inhibitors (Chen 2008; Haq et al. 2004). Proteinase inhibitors (PINs) were the first discovered and best characterized defensive proteins (Green and Ryan 1972; Haq et al. 2004).While initially PINs were believed to act directly through reduction of proteolysis, PINs' toxic effects are due to a compensatory hyperaccumulation of PIN-resistant proteases produced by the insect gut (Broadway 1995; Broadway and Duffey 1986; 1988). These PIN-resistant proteases are thought to release excessive free amino acids, which then can be processed by the plant's arginase and TD, thus working together to deprive the insect of essential amino acids.

Another set of toxic and anti-nutritive enzymes are oxidation enzymes. A classic example is PPO, which catalyzes the oxidation of phenolics to quinones (Felton et al. 1989). Quinones in turn bind to nucleophilic side chains of proteins and amino acids, thus limiting nutrient access as well as being toxic. Lectins are a diverse group of proteins that bind to carbohydrates and in plants are toxic to a wide variety of insect species (Janzen et al. 1976; Murdock et al. 1990; Peumans and Vandamme 1995; Powell et al. 1993; Rahbe et al. 1995; Shukle and Murdock 1983). Finally, plants produce a wide array of compounds that are toxic to the insect. Each plant species produces a unique array of secondary metabolic products that either toxic or repellent to insects and can include phenolics, terpenoids, and nitrogen-containing compounds (Chen 2008).

Plants also have a wide array of constitutive and inducible volatile compounds which primarily are involved in insect defense (Dudareva et al. 2006; Unsicker et al. 2009). Volatiles are primarily studied in the context of indirect defense due to their roles in priming neighboring plants to induce defense responses faster in response to attack and in attracting parasitic or predatory arthropods (tritrophic interactions) (Dicke 1999; Frost et al. 2008; McCormick et al. 2012). However, volatiles can also have more direct toxic or repellent properties (Bleeker et al. 2009; Hildebrand et al. 1993; Kessler and Baldwin 2001; Vancanneyt et al. 2001). Volatiles are also involved in plant-pollinator interactions (Lucas-Barbosa et al. 2011). While the major emphasis has been placed on the role of aerial volatiles, it is important to note that root volatiles have also been implicated in insect deterrence, tritrophic interactions, and allelopathic interactions with other plants (Dudareva et al. 2006; Rasmann et al. 2005; Romagni et al. 2000; Singh et al. 2002).

The plant volatiles comprise a very large and structurally diverse group of small molecules. To date, over 1700 volatiles from over 90 plant families have been discovered, with many individual species containing over 200 compounds (Dicke and van Loon 2000; Knudsen 2006). These compounds are synthesized through many different pathways in the cell including the shikimic pathway (phenylpropanoids and benzenoids), the lipoxygenase pathway (C_6 aldehydes, alchohols, and esters; green leafy volatiles; GLVs) and the isoprenoid pathway (isoprene, monoterpenes, sesquiterpenes, homoterpnes, carotenoid derivatives, and indoles) (Dudareva et al. 2006). There is also a wealth of volatiles that are derived from amino acids such as Ala, Val, Leu, Ile, and Met.

Many of the studies of volatiles in plant-insect interactions have been performed in tomato. These studies have shown that tomatoes release primarily monoterpenes and GLVs after herbivory and wounding, but tomatoes can also release sesquiterpine and phenolics (Andersson et al. 1980; Buttery et al. 1987; Dicke et al. 1998; Farag and Paré 2002; Kant et al. 2004; Thaler et al. 2002a). While many tomato volatile blends are similar, mechanical damage and *Spodoptera exigua* larvae feeding do not appear to induce MeSA or the homoterpene 4,8,12-trimethyltrideca-1,3,7,11- tetraene (TMTT), while spider mite (*Tetranychus urticae*), *S. littoralis* and *M. sexta*

feeding do induce MeSA and TMTT (Degenhardt et al. 2010; Dicke et al. 1998; Farag and Paré 2002; Kant et al. 2004; Thaler et al. 2002a). In the case of spider mite feeding, the induction of MeSA has been shown to be important for attraction of the predatory mite *Phytoseiulus persimilis* (Ament et al. 2010). MeSA in Arabidopsis and potato has also been shown to be important in plant priming (Manosalva et al. 2010; Park et al. 2007). Other roles for tomato volatiles include a role for terpenoids and flavanoids in direct defense against *B. tabaci*, *M. sexta*, the flea beetle (*Epitrix cucumeris*), and the Colorado potato beetle (*Leptinotarsa decemlineata*) (Bleeker et al. 2009; Kang et al. 2010a; Kang et al. 2010b) and a role for GLVs in reducing aphid fecundity (Hildebrand et al. 1993).

Aminopeptidases: Homeostasis and Defense

Proteases and proteolysis are a ubiquitous part of cell life and homeostasis. Enzymes involved in protein and peptide turnover include peptidases that hydrolyze internal peptide bonds (endoprotease) or at the N or C terminus (aminopeptidase or carboxypeptidase, respectively) (Barrett et al. 2004). Other paths of protein degradation include the 26S proteasome, which degrades proteins tagged by poly-ubiquitination (Vierstra 2009). Classic roles for proteolysis include those involved in protein turnover and nutrient recycling during seed germination, leaf senescence, and inflorescence growth and development. However, in recent years, a plethora of studies have shown that proteolysis has a regulatory role in nearly every aspect of plant development including embryogenesis, inflorescence and trichome development, programmed cell death, and circadian rhythms as well as roles in phytohormone signaling and abiotic and biotic stress responses (Kelley and Estelle 2012; Schaller 2004; van der Hoorn 2008). A large part of this research has focused on the ubiquitin-proteasome system (UPS), which has been shown to be significant part of the signaling pathways of almost all hormones (Delaure et al. 2008; Kelley and Estelle 2012). E3 ubiquitin ligase, which are members of the UPS, have been implicated in gene-for-gene resistance outside of the core SA signaling pathway (Delaure et al. 2008). In addition selected endoproteases have regulatory roles in development, as well as

regulatory and direct roles in defense (Chen et al. 2008; Schaller 2004; van der Hoorn 2008; van der Hoorn and Jones 2004).

Aminopeptidases are a subset of the peptidases that are ubiquitous all living organisms (Barrett et al. 2004). In *Arabidopsis* alone there are over 600 peptidases, 28 of which are identified as aminopeptidases (Walling 2006). In addition to the terminus that they act on, peptidases were initially named based on the amino acid residues they intially prefer to hydrolyze (Barrett et al. 2004). This resulted in a confusing peptidase nomenclature that was not based on the peptidase's biological function and its true *in vivo* substrate preferences. In addition, since much of this work was performed before the age of 'omics', many proteins were named by more than one research group based on the initial substrate studied and this had led to confusion within the field. In contrast with endoproteases and the UPS, peptidases have mainly been regarded as housekeeping proteins. Only relatively few recent discoveries in the past decade, have shown that aminopeptidases have far more diverse roles. While aminopeptidases are essential in the degradation of damaged, misfolded, or incomplete proteins, they can have more direct roles in the regulation of plant cell growth, development, homeostasis, and stress response (Barrett et al. 2004; Walling 2006).

Aminopeptidases have essential roles in general protein metabolism including N-terminal Met removal, and protein maturation and turnover (Meinnel et al. 2006; Rock et al. 2010; van Endert 2011). Aminopeptidases are also involved in the generation or catabolism of bioactive peptides, which have roles in a variety of physiological processes. In comparison to animals, there are few vacuolar-localized and membrane-bound aminopeptidases within the plant kingdom (Peer 2011; Walling 2006). However, like animals, roles for plants aminopeptidases beyond housekeeping have been demonstrated including wound signaling (Fowler et al. 2009), meiotic recombination (Sánchez-Morán et al. 2004), cell cycle progression (Peer et al. 2009), and embryonic and seedling development (Peer et al. 2009; Ross et al. 2005).

Aminopeptidases influence protein metabolism posttranslationally by revealing penultimate residues that might be substrates for N-terminal transferases or N-end-rule machinery (Graciet

and Wellmer 2010; Tasaki et al. 2012; Varshavsky 2011; Walling 2006). Modifications due to N-terminal transferases have been shown to influence protein stability, localization and activity. In addition, it has been demonstrated that protein stability is regulated in part by a protein's N terminal residues. N-end specific ubiquitin ligases recognize specific degrons on proteins and then mediate their ubiquitination and targeting for proteolysis by the 26S proteasome. Proteins with small side chain amino acids (M, G, V, S) at their N-terminus are not recognized and therefore have longer half-lives, while those with charged, bulky or polar residues are targeted for more rapid turnover. Cleavage of N-terminal residues expose penultimate residues that may be more or less stable based on the N-end rules, thus greatly affecting protein half-lives. N-end rules have been established in animals, yeast, plants, and prokaryotes (Graciet and Wellmer 2010; Tasaki et al. 2012; Varshavsky 2011). N-end rules in mitochondria and plastids are presumed to be similar to prokaryotes (Apel et al. 2010; Dougan et al. 2010; Mogk et al. 2007).

Leucyl aminopeptidases (LAPs, EC.3.4.11.1) are in the M17 peptidase family. LAPs are di-zinc metallopeptidases, which are highly conserved within plants, animals and microbes (Barrett et al. 2004). Interestingly, *S. cerevisiae* lacks an M17 LAP, even though other fungi, including the closely related *S. pombe*, contain a LAP homolog (Matsui et al. 2006). LAPs are composed of ~55-kDa subunit, which assembles into homohexamers. Mammalian and microbial LAP crystal structures have been solved and residues essential for catalysis have been identified (Huynh et al. 2009; Kale et al. 2010; McGowan et al. 2010; Sträter et al. 1999). While animal LAPs are cytosolic, most plant LAPs are localized to the plastid (Narváez-Vásquez et al. 2008; Walling 2006; Wallner et al. 1993). Only the Arabidopsis LAP1 is cytosolic within the plant kingdom (Bartling and Weiler 1992).

Animal LAPs were initially characterized for their proposed role in the turnover of oxidatively damaged proteins in the eye lens (Taylor 1985). Human LAP was also implicated in peptide processing in MHCI presentation; however, recent work has demonstrated that its role is not essential in this process (van Endert 2011). *E. coli* has a LAP (*PepA*) with the most divergent roles. PepA is an aminopeptidase that also has a DNA-binding domain. This DNA-binding domain

is able to mediate site-specific recombination in *ColE1* plasmids, as well as to act as a transcription factor to modulate the *carAB* operon (Colloms 2004). LAPs from animal and bacteria have also been shown to be Cys-Gly dipeptidases. Cys-Gly dipeptidase activity is important for removal of excess pro-oxidant Cys-Gly, as well as recycling of the antioxidant GSH, and release of free cysteine (discussed above).

The Plant LAPs and the History of LAP-A

Currently, two distinct classes of LAP proteins have been identified in plants (Chao et al. 2000a). These two classes were first classified in tomato based on their relatively neutral (LAP-N) or acidic (LAP-A) pIs (Gu et al. 1996b). LAP-A has been shown to be localized to the stroma in mesophyll plastids and LAP-N is also predicted to be localized in this subcellular compartment (Narváez-Vásquez et al. 2008; Tu et al. 2003). However, the two forms of LAP have different stabilities and chromogenic substrate preferences, with LAP-N preferring Met and Phe over Leu and LAP-A preferring Leu (Tu et al. 2003). Moreover, LAP-N and LAP-A have distinctly different expression patterns within the plant. *LapN* is a rare class transcript; however LAP-N proteins are detected by via western blotting and are constitutively expressed within the plant and not responsive to any stresses (Bartling and Nosek 1994; Chao et al. 2000a; Tu et al. 2003). LAP-N and LAP-N like proteins have been identified in all plants studied including: maize (*Zea mays*), barley (*Hordeum vulgare*), tobacco (*N. tabacum*), *Brassica napus*, soybean (*Glycine max*), Arabidopsis, tomato, potato (*Solanum tuberosum*) and nightshade (*Solanum nigrum*) (Chao et al. 2000a). This ubiquitous expression has suggested that LAP-N and LAP-N-like proteins most likely function in cell maintenance through protein turnover in plants.

In contrast, LAP-A has only been identified in a subset of solanaceous species (Chao et al. 2000a; Dammann et al. 1997; Hartl et al. 2008; Herbers et al. 1994). *LapA* RNAs accumulate during floral and fruit development and is present in high levels in mature flowers in tomato (Pautot et al. 1993; Tu et al. 2003). *LapA* RNAs also accumulate in potato tubers and during early floral development, but does not accumulate to appreciable amounts in mature flowers (Herbers et al. 1994). In addition, *LapA* RNAs have been shown to accumulate in response biotic (*M.*

sexta, *S. littoralis*, *P. parasitica*, and *P. syringeae*) and abiotic stresses (water deficit and salinity) (Chao et al. 1999; Jwa and Walling 2001; Pautot et al. 2001; Pautot et al. 1993). *LapA* has also been shown to increase during the late phase after mechanical wounding and in response to wound-response elicitors (systemin, JA, and ABA) (Chao et al. 1999; Dammann et al. 1997).

LapA transcription increases almost 200 fold after wounding (Chao et al. 1999; Chao et al. 2000a; Puthoff et al. 2010). In addition, studies have shown that changes in *LapA* transcription rates, mRNA, protein, and activity levels are well-correlated (Chao et al. 1999; Chao et al. 2000a). Therefore, these distinct, tightly regulated expression patterns suggest that LAP-A plays a key role in a wide variety stress responses and potentially floral and fruit development.

Consistent with this hypothesis, LAPs were recently shown to be important in insect deterrence in tomato and nightshade (Fowler et al. 2009; Hartl et al. 2008). Silencing of LAPs in transgenic tomatoes or transiently in nightshade plants made plants more susceptible to *M. sexta* feeding and insect masses were larger than insects grown on wild-type plants (Fowler et al. 2009; Hartl et al. 2008). Reciprocally, transgenic tomatoes that ectopically express the tomato LAP-A were more resistant to *M. sexta* feeding and delays in insect growth and development were displayed (Fowler et al. 2009).

The mechanism by which LAP-A affects insect feeding and growth is still unknown. Given that LAP-A is the most abundant protein in the lepidopteran digestive track and that LAP-A has a pH optima, which corresponds to the alkaline gut, it was proposed that LAP-A may act by degrading essential peptides within the insect midgut (Chen et al. 2007; Dow 1992; Gu et al. 1999; Pautot et al. 1991). However, preliminary data in which *M. sexta* were fed on an artificial diet supplemented with LAP-A show that LAP-A does not act alone to directly affect insect growth and development (Fowler et al. 2009). It is possible that LAP-A's peptidase activity still plays an anti-nutritive role in the midgut but relies on other wound-induced enzymes to affect insect growth. For example, LAP-A can readily hydrolyze N-terminal Arg from peptides and proteins (Gu et al. 1999). Therefore, LAP-A could act in concert with wound-induced arginase to deprive the insect of the essential Arg (Chen et al. 2005).

More interestingly, LAP-A was shown to modulate gene expression of other late wound-response genes (Fowler et al. 2009). *PPO-F*, *Pin1*, and *Pin2* transcripts accumulated to lower levels in the *LapA-SI* after wounding in comparison to WT plants. Reciprocally, these late wound-response transcripts accumulated to higher levels and remained elevated longer in the *LapA-OX* plants. In contrast, early wound-response transcript levels were similar in all three genotypes before and after wounding.

My dissertation studies have focused on elucidating the potential roles of LAP-A in tomato in wound signaling or insect defense or both. In Chapter 1, a novel function for plant LAPs as *in vitro* molecular chaperones was identified. The acidic LAP-A and the neutral LAP-N of tomato and LAP1 and LAP2 of Arabidopsis displayed *in vitro* chaperone activity. Using three assays for chaperone activity, the unique substrate preferences between the different LAP homologues was demonstrated. Moreover, LAP-A and LAP-N active site mutants that abolish peptidase activity were characterized for chaperone activity. In LAP-A, chaperone activity was shown to be independent of peptidase activity with increased activity in the peptidase mutants whose hexameric structure was disrupted. In LAP-N, chaperone dependency on the active peptidase site was more complex. These studies provide additional evidence for the distinct properties of the tomato LAP-A and LAP-N. In addition, these studies suggest that LAP-A may have multiple roles within the plant in wound signaling or within the insect midgut in defense. LAP-A's function (peptidase and/or chaperone activity) may depend on LAP-A's structural organization *in planta* and the insect gut.

Previous studies of LAP-A's role in modulating wound signaling have focused only a narrow set of core set of wound-response genes. In order to understand the range of LAP-A's influence on wound signaling microarray analysis of RNAs from wounded tomato leaves in WT and *LapA-SI* lines were performed and analyzed in Chapter 2. Surprisingly, no study to date has characterized large-scale transcriptional changes on both a temporal (early and late) and spatial (local and systemic) scale in tomato. Therefore, WT plants were wounded and microarray analysis was performed on transcripts isolated 1 and 8 hr after wounding both locally and systemically. Like

other species, tomato regulated a wide set of metabolic pathways in response to injury. These pathways and their potential roles in wound signaling, healing and defense are discussed. *LapA-SI* lines were analyzed as WT and gene expression after wounding was compared. While few genes had significantly different expression in the *LapA-SI* lines compared to WT, *LapA-SI* lines appeared to have an overall delayed response to wounding. In addition, a set of genes was identified as differentially regulated in the *LapA-SI* lines before wounding. This led to the identification of two sets of genes (*PR1* and *TAS14/Dhns*) that are regulated by LAP-A. This study demonstrated that LAP-A's role in wound signaling is more complex and expansive than previously known, being a positive and negative regulator of gene expression and affecting gene expression before and early after wounding.

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CHAPTER 1: Plant Leucine Aminopeptidases Moonlight as Molecular Chaperones to Alleviate Stress-Induced Damage

ABSTRACT

Leucine aminopeptidases (LAPs) are present in animals, plants and microbes. In plants, there are two classes of LAPs. The neutral LAPs (LAP-N and its orthologs) are constitutively expressed and detected in all plants; while the stress-induced acidic LAPs (LAP-A) are expressed only in a subset of the Solanaceae. LAPs have a role in insect defense and act as a regulator of the late branch of wound signaling in *Solanum lycopersicum* (tomato). While LAP-A's mechanism of action is unknown, it has been presumed that LAP's peptidase activity is essential for regulating wound signaling. Here we show that plant LAPs are bifunctional. Using three assays to monitor protein protection from heat-induced damage, it was shown that the tomato LAP-A and LAP-N and the *Arabidopsis thaliana* LAP1 and LAP2 are molecular chaperones. Assays using LAP-A catalytic site mutants demonstrated that LAP-A's chaperone activity was independent of its peptidase activity. Furthermore, disruption of LAP-A's hexameric structure increased chaperone activity. Together these data identify a new class of molecular chaperones and a new function for the plant LAPs, as well as suggesting new mechanisms for LAP action in the defense of Solanaceous plants against stress.

INTRODUCTION

All organisms contain a complex array of aminopeptidases that cleave N-terminal residues from proteins and peptides (Barrett et al. 1998). Aminopeptidases have important roles in N-terminal Met removal, protein turnover, protein maturation, and generation or catabolism of bioactive peptides that are important in a variety of physiological processes (Meinnel et al. 2006; van Endert 2011). Aminopeptidases may be important in exposing penultimate amino acid residues that may profoundly affect a protein's half-life as predicted by the N-end rule (Graciet and Wellmer 2010; Varshavsky 2011; Walling 2006). The plant aminopeptidase complement is distinctive, with a paucity of vacuolar-localized and membrane-bound aminopeptidases relative to animals (Peer 2011; Walling 2006). In plants, aminopeptidases modulate wound signaling (Fowler et al. 2009), meiotic recombination (Sánchez-Morán et al. 2004), cell cycle progression (Peer et al. 2009), and embryonic and seedling development (Peer et al. 2009; Ross et al. 2005).

Leucyl aminopeptidases (LAPs, EC.3.4.11.1) belong to the M17 family of peptidases. LAPs are highly conserved, di-zinc metallopeptidases found in plants, animals and microbes (Barrett et al. 1998). Animal LAPs may have a role in the turnover of oxidatively damaged proteins in the lens of the eye (Taylor 1985). Human LAP was proposed to process peptides released from the 26S proteasome for use in MHCI presentation; however, its role in this process is not essential (van Endert 2011). In contrast, the *Escherichia coli* LAP (PepA) is multifunctional. It is an aminopeptidase and a DNA-binding protein that mediates site-specific recombination in *ColE1* plasmids and acts as a transcription factor to modulate the *carAB* operon (Colloms 2004). The complement of LAPs in plants is more complex and their roles are being elucidated.

In plants, there are two classes of LAPs, which are 70-77% identical (Tu et al. 2003). The LAPs with neutral pIs (LAP-N) are detected in all plants and are constitutively expressed (Chao et al. 2000; Tu et al. 2003). The LAPs with acidic pIs (LAP-A) are found only in a subset of the Solanaceae and are induced in response to both biotic and abiotic stresses (Chao et al. 1999; Chao et al. 2000; Tu et al. 2003). Recently, LAPs were shown to be important in insect

deterrence in *Solanum lycopersicum* (tomato) and *Solanum nigrum* (nightshade) (Fowler et al. 2009; Hartl et al. 2008). Silencing of LAPs in transgenic tomatoes or transiently in nightshade plants made plants more susceptible to *Manduca sexta* feeding and insect masses were larger than insects grown on wild-type plants (Fowler et al. 2009; Hartl et al. 2008). Reciprocally, transgenic tomatoes that ectopically express the tomato LAP-A were more resistant to *M. sexta* feeding and delays in insect growth and development were displayed (Fowler et al. 2009). The tomato LAP-A modulates the late branch of wound signaling downstream of the synthesis of the defense hormone jasmonic acid. By controlling the late branch of wounding, LAP regulates the levels of critical defense proteins that deter herbivore growth and development. To date, LAP-A's in *vivo* substrates have yet to be discovered.

LAP-A resides within the plastid (Narváez-Vásquez et al. 2007), which is a dynamic compartment subject to many cellular stresses during development and biotic/abiotic stresses that can result in protein damage and aggregation. In order to prevent the accumulation of misfolded proteins, cells express a wide range of molecular chaperones (Tyedmers et al. 2010). Molecular chaperones can act as 'holdases' by binding to misfolded proteins to prevent aggregation or as 'foldases' by actively refolding misfolded proteins (Kampinga and Craig 2010; Tyedmers et al. 2010). There are five major classes of chaperones in plants (Vierling 1991; Wang et al. 2004). The most abundant and diverse class of chaperones is the 'holdase' class of small heat-shock proteins (sHSPs).

Molecular chaperone activity has also been revealed in proteins with other primary biological functions including: thioredoxin, peroxidase, elongation factors (eEF1a and EF-TU), and several protein-degrading enzymes (Hotokezaka et al. 2002; Jang et al. 2004; Lee et al. 2009a; Malki et al. 2005; Spiess et al. 1999). For example, several plastid ATP-dependent proteases are molecular chaperones (Neuwald et al. 1999; Suzuki et al. 1997). Currently, no plant aminopeptidase is known to function as a molecular chaperone. However, the tomato LAP-A has several compelling biochemical characteristics that are shared with molecular chaperones including: its stability, high temperature optimum (60-70°C), high pH optimum (9.0), and induction

by a wide range of stresses (Chao et al. 1999; Gu et al. 1999). Finally, two microbial proteins are known to possess both chaperone and aminopeptidase activity *in vitro*: the *E. coli* Hsp31 chaperone and a *Shizosaccharomyes pombe* aspartyl aminopeptidase (Lee et al. 2009b; Malki et al. 2005).

While these Hsp31 and aspartyl aminopeptidases do not share conserved protein domains with the plant M17 peptidases, the discovery of the aminopeptidase-chaperone bifunctionality in these microbial enzymes prompted investigations into the plant LAPs. Here, the *in vitro* chaperone activities of plant LAPs were studied by assaying the ability of the tomato and Arabidopsis LAPs to prevent protein unfolding, prevent protein aggregation and promote protein refolding. These assays indicated that the tomato LAP-A and LAP-N and Arabidopsis LAP1 and LAP2 form a new class of molecular chaperones in plants. Assays performed on LAP-A active site mutants also indicated that LAP-A's chaperone activity was independent of its peptidase activity and that disruption of LAP-A's hexameric structure, which is essential for its peptidase activity, increased chaperone activity. In contrast, one catalytically inactive LAP-N was impaired in chaperone activity. These data shed new light on the complexity of plant LAPs and suggest new potential roles for LAPs in defending tomato against stress.

EXPERIMENTAL PROCEDURES

Isolation of AtLAP1 and AtLAP2 cDNAs

RNA was isolated from 1-week-old seedlings of *Arabidopsis thaliana* ecotype Columbia by the hot-phenol method (Pautot et al. 2001). Total RNA (5 µg) was used to synthesize first-strand cDNA using the Smart PCR cDNA synthesis kit (Clontech, Palo Alto, CA) and an oligo-dT primer. *LAP1 (At2g24200)* and *LAP2 (At4g30920)* coding regions were cloned by RT-PCR using gene-specific primers (Table 1.1). The resulting PCR-amplified cDNA fragments included the entire coding region of *LAP1* and the mature protein of *LAP2* (excluding the plastid transit peptide) (Bartling and Weiler 1992; Walling 2006).

PCR was performed with two cycles of 30 sec at 94°C, 30 sec at 52°C and 2 min at 72°C. This was followed with 30 cycles of 2 min at 95°C, 30 sec at 65°C and 2 min at 72°C. In healthy

leaves, the *AtLAP2* RNA is present at low levels and the *LAP2* RNA was not detected after 30 PCR cycles. Therefore, 1 µl of the primary PCR product was used as a template for a second round of 30 PCR cycles. The PCR products amplified using Ex-Taq (Dakara, Madison, WI) and were cloned into pGEM T-easy (Promega, Madison, WI) to generate pGEM-LAP1 and pGEM-LAP2. Fidelity of the cDNA sequences was determined by DNA sequencing at the Genomics Core Facility at the Institute of Integrative Genome Biology (University of California, Riverside). pGEM-LAP clones were digested with corresponding restriction enzymes and cloned into the pET28 expression vector (Novagen, Darmstadt, Germany). The resulting clones, pET-LAP1, and pET-LAP2, expressed LAP proteins with N-terminal His_6 fusions (His_6-LAP1 and His_6-LAP2, respectively).

Isolation of LAP-N K357 Substitution Mutants

LAP-N K357 mutants were generated using the QuikChange Lightning Multi Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The template was the pQLapN plasmid that contains a His_6-LAP-N coding region (Tu et al. 2003). The primer used for introducing mutations in the K357 codon (in bold) was: 5'-GGTGGCTACAACATC**NNK**ACTGGACCTGGTTG-3'. Plasmids containing putative K357 mutations were sequenced to identify the residue substitution and confirm that no polymerase errors occurred at other locations in the His_6-LAP-N coding region. Twelve mutants that had a 357 residue substitution were confirmed and eight mutants (K357E, K357R, K357L, K357C, K357M, K357G, K357T, and K357P) were further characterized

Over-expression and Purification of LAP Proteins

The *E. coli* vectors that express the His_6-LAP-A, His_6-LAP-N, and the His_6-LAP-A mutants (R431A, K354R, K354E, D347N, D347R, E429R, and E429V) were previously described (Gu and Walling 2002; Tu et al. 2003). His_6-LAP fusion proteins were expressed in and purified from *E. coli* according to Gu and Walling (2000) with minor modifications (Gu and Walling 2000). Cultures were grown at 37°C overnight. Overnight cultures were diluted 1:20 and the cultures (0.1-1 L) were grown at 37°C (wild-type and mutant His_6-LAP-As) or 30°C (His_6-LAP1, His_6-LAP2,

and wild-type and mutant His_6-LAP-Ns) to an OD_{600}=0.6. At this time, cultures were induced with 0.4 mM IPTG and allowed to grow for an additional 6-18 hr at 37°C (wild-type and mutant His_6-LAP-As), 30°C (His_6-LAP1, His_6-LAP2), or 22°C (wild-type and mutant His_6-LAP-Ns). Cells were resuspended in 5 volumes of pre-chilled Buffer A (50 mM $NaPO_4$, pH 8.0, 300 mM NaCl) with 75 mM lysozyme. After 0.5 hr incubation on ice, cells were lysed using six 10-sec sonicator pulses followed by 10 sec on ice. The lysate was cleared at 10,000 g for 30 min at 4°C.

With the exception of the His_6-LAP-N mutants, His_6-LAP proteins were purified using Ni/nitrilotriacetic acid resin columns (Qiagen, Valencia, CA) as previously described (Gu and Walling 2000). For the His_6-LAP-N mutant analyses, His_6-LAP-N wild-type and mutants (K357E, K357R, K357L, K357C, K357M, K357G, K357T, and K357P) and wild-type LAP-N proteins were expressed (100 ml) and cleared lysates were prepared as described above. Cleared lysates were loaded onto 0.2-ml Ni/nitrilotriacetic acid spin resin columns (Thermo Fisher Scientific, Rockford, IL) equilibrated with Buffer A. The column was washed twice with 0.5 ml Buffer A with 20 mM imidazole and twice with 0.5 ml Buffer A with 40 mM imidazole. His_6-LAP-N proteins were eluted with Buffer A with 250 mM imidazole and collected in 0.2-ml fractions. LAP-A wild-type and mutant proteins were stored in 25 mM sodium phosphate (pH 8.0), 250 mM NaCl, 125 mM imidazole, and 50% glycerol at -20°C until use; LAP-A is stable for one year under these conditions. LAP-N wild-type and mutant proteins were used on the day they were purified, due to their limited stability (5 d) under these storage conditions (Tu et al. 2003).

Protein concentrations were determined with the Bradford method using IgG as a standard (Bio-Rad Protein Assay Kit I, Bio-Rad, Hercules, CA). To determine the molecular masses of the WT and mutant His_6-LAP-A and His_6-LAP-N complexes, purified His_6-LAP-As and His_6-LAP-Ns were fractionated on a set of four native polyacrylamide gels (7.5-12% w/v) based on the methods of Bryan (Bryan 1977; Gu et al. 1996b). The proteins used as molecular mass standards included: chicken egg albumin (45 kDa); bovine serum albumin monomer (66 kDa) and dimer (132 kDa); and tomato His_6-LAP-A hexamer (357 kDa). Protein purity was determined by SDS-PAGE and LAP masses were determined by native PAGE by staining with Coomassie Brilliant

Blue R-250 (Gu et al. 1996a). For all assays, molar amounts of the mature WT and non-disruption mutant proteins were calculated using hexameric values (LAP-A, 357 kDa; LAP-N, 365 kDa; LAP1, 327 kDa; LAP2, 330 kDa), while molar amounts for mature proteins of disruption mutants were calculated using mass of the monomer (55 kDa).

LAP Activity Assay

The peptidase activities of all LAPs were determined prior to use in chaperone assays. LAP activity was determined using the fluorescent substrate, leucine-amino methyl coumarin (Leu-AMC; Bachem, Bunderdorf, Switzerland). Purified His_6-LAPs (2160 ng) were pre-incubated in assay buffer (50 mM Tris-HCl, pH 8, 0.5 mM $MnCl_2$) in a total volume of 162 µl in a 96-well microtiter plate. Activity assays was initiated with the addition of Leu-AMC (1.58 µM) and proceeded for 30 min at 37°C. Reactions were performed in duplicate. Leu-AMC hydrolysis was quantified by measuring the fluorescence emission of AMC at 460 nm by the Victor^2 1420 Multilabel Counter (PerkinElmer Life Sciences, Waltham, MA). The extinction coefficient for AMC at 460 nm was 16,500 M^{-1} cm^{-1}. The Leu-AMC hydrolyzing activities of wild-type His_6-LAP proteins were (in µmol min^{-1} mg^{-1}): His_6-LAP-A (0.59 $\pm$ 0.01), His_6-LAP-N (0.24 $\pm$ 0.01), His_6-LAP1 (0.22 $\pm$ 0.01) and His_6-LAP2 (0.22 $\pm$ 0.01). Consistent with previous reports, the His_6-LAP-A mutant proteins were peptidase deficient with Leu-AMC hydrolyzing activities less than 0.01 µmol min^{-1} mg^{-1} (Gu and Walling 2002).

Thermal Restriction Enzyme Protection Assay

Thermal protection of the restriction enzyme NdeI was performed according to Santhoshkumar and Sharma (Santhoshkumar and Sharma 2001). The reactions contained 1 U of NdeI [New England Biolabs (NEB), Beverly, MA], 1X NEB restriction enzyme buffer 4, 4% glycerol, and of LAP (0-2 µM), *Pisum sativum* Hsp18.1 (0-2 µM), protein A (0-2 µM) (Sigma, St. Louis, MO), or lysozyme (0-48 µM) (Sigma, St. Louis, MO) in a final volume of 13 µl. PsHsp18.1 was kindly donated by Dr. Elizabeth Vierling (University of Massachusetts, Amherst). Since lysozyme (14.7 kDa) is 24 times smaller than the LAP-A hexamer (365 kDa), higher

concentrations of lysozyme were used to get equal protein amounts as a negative control. Protein A (42 kDa) is approximately the same size as the LAP monomer (~55 kDa) and therefore was used in equal molar amounts. NdeI, NdeI-LAP, NdeI-PsHsp18.1, and NdeI-lysozyme mixes were incubated for 90 min in a 43°C water bath. At this time, 140 ng of plasmid DNA (cLEX-6-H6; 2 µl) was added. Digestion was allowed to occur for 90 min at 37°C. Digested plasmid DNA was visualized by electrophoresis on a 1% agarose gel stained with ethidium bromide. NdeI digestion of plasmid DNA at 37°C (without the 43°C incubation) served as a positive control. cLEX-6-H6 is cDNA clone encoding a GID-like gibberellin receptor (SGN-E304247; Sol Genomics Network). NdeI cuts at two sites in this 4.8-kb plasmid releasing fragments 4.6 kb and 0.2 kb. His_6-LAP-A was stored in 50% glycerol to prolong its stability and for this reason, NdeI-His_6-LAP-A reactions had 4% glycerol. To assure the thermal protection provided by His_6-LAP-A was due to chaperone activity and not higher glycerol levels, His_6-LAP-A was purified and stored without glycerol. When tested in the NdeI thermal-inactivation assay, the glycerol-free and 50% glycerol His_6-LAP-A had similar levels of chaperone activity towards NdeI (Fig. 1.1).

Thermal Citrate Synthase Aggregation Assay

Aggregation assay reactions contained 300 nM citrate synthase (Sigma, St. Louis, MO), 50 mM HEPES-KOH (pH 7.5), 5% glycerol, and 0-1200 nM purified His_6-LAP in a total volume of 600 µl. The mix was placed in a plastic cuvette and heated in a 43°C water bath. Light scattering at 360 nm was measured at indicated times (0-60 min) using NanoDrop 2000c (Thermo Scientific, Rockford, IL). As a negative control, lysozyme (1200 nM) or protein A (1200 nM) was added instead of LAP protein in separate reactions. PsHsp18.1 (1200 nM) was used as a positive control.

Luciferase Refolding Assay

Prior to assays, His_6-LAP-A wild-type and mutant proteins were dialyzed against Buffer A using “V” series membranes (0.05 µM; Millipore, Billerica, MA) to remove glycerol and imidazole. LAP-N wild-type and mutant proteins were used fresh and were in glycerol-free Buffer A. Firefly luciferase (Luc) refolding was measured according to Siddique et al. with some modifications

allowing use of a 96-well format (Siddique et al. 2008). Heating reactions contained 1 μM QuantiLum Recombinant Luciferase (Promega, Madison, WI) and 0-6 μM His_6-LAP, 1 μM PsHsp18.1, 3 μM protein A or 3 μM lysozyme in 2.5 mM HEPES–KOH (pH 7.5), 5 mM $MgCl_2$, 150 mM KCl, and 2 mM dithiothreitol (DTT) (total volume of 25 μl). Samples were heated for 11 min at 42°C and chilled on ice for 5 min. One μl of heated samples was added to the reactivation mix that included 24 μl rabbit reticulocyte lysate (RRL; Promega), 25 mM HEPES-KOH (pH 7.5), 2 mM ATP, 5 mM $MgCl_2$, 10 mM KCl, and 1 mM DTT; the final volume was 40 μl and had a final concentration of 25 nM Luc. Fifty μl of Luc assay system (Promega) was added to 10-μl aliquots of the reactivation mix in a 96-well microtiter plate and incubated at 30°C. For the His_6-LAP-A (wild-type and mutants), His_6-LAP-N, His_6-LAP1, and His_6-LAP2 assays, luminescence was measured using a LUMIstar Galaxy luminometer (*BMG* Labtechnologies, Offenberg, Germany) with an integration time of 10 sec. Luminescence for the His_6-LAP-N mutant studies was measured using a TriStar LB 941 luminometer (Berthold, Oak Ridge, TN) with an integration time of 10 sec. Percent activity corresponds to the relative luminescence compared to unheated luciferase.

RESULTS

Tomato LAP-A Exhibits Chaperone Activity Towards Three Model Substrates

Three assays were used to evaluate the tomato His_6-LAP-A's chaperone activity. The ability of His_6-LAP-A to prevent protein unfolding was demonstrated in a thermal denaturation assay. The restriction enzyme NdeI was heated for 90 min at 43°C either alone, with lysozyme or protein A (negative controls), PsHsp18.1 (positive control), or with His_6-LAP-A (0.2-2.0 μM). NdeI's activity was measured by cutting of plasmid DNA. NdeI was inactivated after 90 min at 43°C (Fig. 1.2*A*). The addition of 1-2 μM His_6-LAP-A protected NdeI from thermal inactivation, while lysozyme and protein A did not (Fig. 1.2*A*, Fig 1.3*A*). PsHSP18.1 was able to protect at concentrations as low as 0.2 μM (Fig. 1.3*B*). His_6-LAP-A's chaperone activity level was similar to that reported for the bovine α-crystallins (Santhoshkumar and Sharma 2001).

The ability of His_6-LAP-A to protect the model substrate citrate synthase (CS) from heat-induced aggregation was tested. CS (300 nM) was heated for 60 min at 43°C in the presence or absence of His_6-LAP-A and CS aggregation was measured by light scattering. His_6-LAP-A protected CS from aggregation in a dose-dependent manner with activity seen with as little as 300 nM His_6-LAP-A (Fig. 1.4*A*). Neither lysozyme (1.2 μM) nor protein A (1.2 μM) prevented CS aggregation, while 1.2 μM His_6-LAP-A reduced CS aggregation by ~50% (Fig. 1.4*A*, Fig. 1.5). PsHSP18.1 was able to completely protect CS at 1.2 μM (Fig. 1.5).

The third chaperone assay assessed if His_6-LAP-A could facilitate the refolding of the heat-sensitive luciferase (Luc) to its native state (Lee and Vierling 1998). Luc (1 μM) was heated at 42^{o}C for 11 min alone or with 3-6 μM of His_6-LAP-A or 1 μM PsHsp18.1 (positive control). Luc was then allowed to refold in rabbit reticulocyte lysate (RRL) supplemented with 2 mM ATP; RRL is a rich source of ATP-dependent chaperones (HSP70 system) (Frydman et al. 1994). In the absence of a His_6-LAP-A or PsHsp18.1, less than 5% of Luc activity was detected (Fig. 1.6). His_6-LAP-A was an effective chaperone, since 3 and 6 μM His_6-LAP-A restored 17% and 35% of Luc activity, respectively. His_6-LAP-A-mediated refolding of Luc was dependent on the presence of the RRL. In comparison, 38% activity of Luc was protected by 1 μM PsHsp18.1 [Fig. 1.7*A* and as previously shown (Lee and Vierling 1998)] and protein A did not enable Luc refolding (Fig. 1.7*B*). These data indicated that His_6-LAP-A protected Luc from complete denaturation and thereby enabled its refolding by the ATP-dependent chaperones similar to well-characterized sHSPs (Fig. 1.7*A*) (Kampinga and Craig 2010; Tyedmers et al. 2010; Wang et al. 2004).

The Tomato LAP-N and Arabidopsis LAPs are Molecular Chaperones

Since the stress-inducible LAP-A of tomato displayed chaperone activity based on three independent chaperone assays, the chaperone activities of the tomato LAP-N was tested. Using the NdeI thermal-protection assay, chaperone activity was detected using as little as 0.2 μM His_6-LAP-N (Fig. 1.2*B*). His_6-LAP-N was at least 5-fold more active than His_6-LAP-A in this assay. In the CS-aggregation assay, His_6-LAP-N and His_6-LAP-A displayed similar chaperone activity levels, with 900 nM His_6-LAP-N and His_6-LAP-A preventing ~40% of CS aggregation (Fig. 1.4*B*).

In the Luc-refolding assay, His_6-LAP-N (3 μM) enabled Luc refolding. This level of chaperone activity was similar to His_6-LAP-A, with approximately 17% of Luc activity being restored in the presence of 3 μM His_6-LAP-N (Fig. 1.6).

To determine if chaperone activity was solely associated with the tomato LAPs or characteristic of plant LAPs, the chaperone activities of *Arabidopsis thaliana's* cytosolic LAP1 and plastid-localized LAP2 were tested. LAP1 and LAP2 are orthologs of the tomato LAP-N (Tu et al. 2003). His_6-LAP1 (1-2 μM) prevented thermal inactivation of NdeI; this level of chaperone activity was similar to the tomato His_6-LAP-A (Fig 1.2*A*;*C*). In contrast, His_6-LAP2's chaperone activity was detected at 0.2 μM, similar to the tomato His_6-LAP-N (Fig. 1.2*B;D*). In addition, both His_6-LAP1 (3 μM) and His_6-LAP2 (3 μM) were able to protect Luc from thermal inactivation and restored Luc activity to 17% or 19% of the unheated control, respectively. These activity levels were similar to the tomato LAPs (Fig. 1.6). However, AtLAP1 and AtLAP2 chaperone activity was not demonstrated using the CS aggregation assay. His_6-LAP1 (900 nM) was unable to protect CS from aggregation (Fig. 1.8), and the His_6-LAP2 protein aggregated on its own and could not be tested for its chaperone activity towards CS.

His_6-LAP-A's in vitro Chaperone Activity is Independent of its Peptidase Activity

A bank of mutations in four residues (Glu347, Lys354, Asp429, and Arg431) of the tomato LAP-A's reactive site were previously characterized (Gu and Walling 2002). Glu347 and Asp429 correspond to the *E. coli* and bovine LAP residues that coordinate one of the two zinc ions in the reactive site. Lys 354 and Arg431 have a role in catalysis. All mutations at these sites inactivate His_6-LAP-A's peptidase activity (Gu and Walling 2002); unexpectedly, some amino acid substitutions prevent assembly of the LAP-A hexamer (disruption mutants) and fast migrating forms are observed, while other mutant peptidases assemble into hexameric complexes (non-disruption mutants) (34; Fig. 1.9*A*). To further characterize the disruption mutants, purified His_6-LAP-As were run on a series of native polyacrylamide gels to determine the masses of the oligomeric species present. Disruption mutants had protein complexes migrating with a masses of

60, 120 and 192 kDa; these masses were consistent with the disruption mutant LAP-As being a mixture of trimers (165 kDa), dimers (110 kDa) and monomers (55kDa) (Fig. 1.9).

To determine if LAP-A's chaperone activity was dependent on its peptidase activity, four non-disruption mutant proteins were tested for their chaperone activity. The His_6-LAP-A catalytic mutants R431A and K354R and zinc ion-binding mutants D347N and E429V protected NdeI from thermal denaturation (Fig. 1.10, 1.11). Their levels of chaperone activity were similar to the wild-type His_6-LAP-A with chaperone activity displayed at 1 to 2 μM. These data indicated that LAP-A's chaperone activity was independent of its peptidase activity and its ability to bind substrate or coordinate zinc ions.

Using the CS-aggregation assay, the chaperone activities of R431A, K354R and E429V were also demonstrated. These proteins reduced CS aggregation by 40 to 60% relative to unprotected CS (Fig. 1.12*A*). In contrast, D347N did not prevent CS aggregation. The Luc refolding assay revealed that all four non-disruption mutants had chaperone activities similar to the WT LAP-A (Fig. 1.13). Three of the four non-disruption mutant proteins (K354R, E429V, D347N) restored between 17 to 18% of Luc activity; while the R431A protein had greater chaperone activity with ~22% recovery of Luc activity. Collectively the three chaperone assays indicated that LAP-A's chaperone activity was independent of its peptidase activity.

Disruption of His_6-LAP-A's Hexameric Structure Increases in vitro Chaperone Activity

Since LAP-A's peptidase activity is dependent on its hexameric structure, we tested if His_6-LAP-A's chaperone activity was also dependent on its oligomeric integrity. Three disruption mutant proteins that abolished either catalysis (K354E) or Zn-ion binding (D347R, E429R) were tested for chaperone activity (Gu and Walling 2002). In contrast to the non-disruption mutants, all three disruption mutants had increased chaperone activity towards NdeI (Fig. 1.10, 1.11). The E429R protein protected NdeI activity at 0.4 μM, while the K354E and D357R proteins protected NdeI at concentrations as low as 0.2 μM. These data were in marked contrast with oligomeric structure mutants of HSP16.6 from *Synechocystis* where the oligomeric stability of HSP16.6 is required for chaperone activity *in vitro* (Giese et al. 2005; Giese and Vierling 2002).

LAP-A disruption mutants were tested for their ability to prevent CS aggregation. Consistent with the NdeI assay, the K354E and D347R proteins had increased chaperone activity towards CS (Fig. 1.12*B*). At 900 nM, the K354E and D347R proteins completely protected CS from heat-induced aggregation compared with the WT His_6-LAP-A, which reduced CS aggregation by 40%. However, the E429R protein was unable to protect CS from aggregating. In the Luc-refolding assay, the K354E protein was the most active chaperone with only 3 μM of K354E protein refolding ~40% of Luc (Fig. 1.13*B*). The E429R and D347R proteins (3 μM) aided in the refolding of ~30% of the Luc. While, the E429R and D347R proteins were not as active as the K354E protein, all disruption mutant proteins displayed more chaperone activity than either the WT His_6-LAP-A or the four non-disruption mutants (Fig. 1.13*A*).

His_6-LAP-N's Active Site Mutation Alters Chaperone Activity and Heat Stability

Despite their strong sequence conservation in the C-terminal catalytic domain (80% identity), His_6-LAP-A and His_6-LAP-N have distinct substrate specificities (Gu and Walling 2002; Tu et al. 2003) and distinct protein stabilities suggesting that LAP-A and LAP-N may have differences in their structure that could influence the dependence/independence of LAP-N's chaperone and peptidase activities. To this end, eight substitution mutants of LAP-N's residue Lys 357 were characterized. LAP-N's K357 is equivalent to LAP-A's K354 (Tu et al. 2003). Five of His_6-LAP-N K357 mutations (K357 → E, R, M, G, T) had corresponding His_6-LAP-A K354 mutations. The His_6-LAP-N wild-type and mutant proteins were expressed in *E. coli*, purified and their peptidase activity and oligomeric states were determined.

Consistent with the peptidase deficiencies of the K354 mutants of His_6-LAP-A (Gu and Walling 2002), all eight His_6-LAP-N K357 mutants were severely impaired in their ability to cleave Leu-AMC, with $\leq$ 1.4% of wild-type His_6-LAP-N activity (Table 1.2). However, when quaternary structures were examined His_6-LAP-N and His_6-LAP-A mutants were distinct. All eight of the His_6-LAP-N K357 mutant proteins (K357 → E, R, M, G, T, C, P, L) assembled into hexamers (Fig. 1.14). This contrasts with the His_6-LAP-A mutants, where only K354R assembled into a stable

hexamer and the other mutants had full (K354 → E, G) or partial (K354 → M, T, C, P, L) disassembly of the hexamer (34; Fig. 1.9).

While tests for increases in chaperone activity in a disruption mutant could not be performed for LAP-N, K357E and K357R allowed direct comparison with His_6-LAP-A mutants K354E and K354R. Therefore, the molecular chaperone activity of the mutants K357E and K357R were compared to wild-type His_6-LAP-N to determine if the potent LAP-N chaperone activity was independent of its peptidase. In the NdeI thermal protection assay, wild-type, K357E, and K357R His_6-LAP-N proteins had similar chaperone activity levels with thermal protection observed with as little as 0.2 μM His_6-LAP-N (Fig. 1.15). Unlike the K354E His_6-LAP-A disassembly mutant, that has enhanced chaperone activity, the K357E His_6-LAP-N did not display molecular chaperone activity in the CS assay or Luc refolding assay (Figs. 1.16, 1.17). Finally, the K357R protein aggregated in the CS assay and its chaperone activity could not be assessed; furthermore, K357R was inactive in Luc refolding assay since activity levels were similar to the RRL control (Figs. 1.16, 1.17).

DISCUSSION

Based on their ability to protect proteins from thermal denaturation and aggregation, and aid in the refolding of denatured proteins *in vitro*, plant LAPs are molecular chaperones. LAPs lack ATP-binding domains (Walling 2004) and their *in vitro* chaperone activity was independent of ATP. Together these data point to plant LAPs acting like 'holdases' similar to sHSPs (Kampinga and Craig 2010; Sun and MacRae 2005; Tyedmers et al. 2010). However, LAPs share no sequence similarity with sHSPs. In particular, they lack the α-crystallin domain that is essential for sHSP chaperone activity (Tyedmers et al. 2010). Therefore, LAPs represent a new class of chaperone proteins within plants.

Each of the tomato and Arabidopsis LAPs displayed distinct activity profiles with the three model substrates *in vitro* (Table 1.3). LAP-A's chaperone activity was detected in all three assays. LAP-A's ability to protect NdeI from denaturation and CS from aggregation was similar to that reported for the chaperone α-crystallin (Santhoshkumar and Sharma 2001). While LAP-A's ability

to enable Luc refolding was approximately four-fold lower than the well characterized PsHsp18.1 (Lee and Vierling 1998; Lee et al. 2009b; Santhoshkumar and Sharma 2001). The neutral LAPs (LAP-N, LAP1, LAP2) demonstrated varied chaperone activity towards two or more model substrates. Based on the Luc-refolding assay, all three neutral LAPs and LAP-A had similar chaperone activity levels. While LAP2 protected NdeI at the same level as LAP-A (1 μM), LAP-N, LAP1 had PsHsp18.1 had increased chaperone activity towards NdeI (0.2 μM). Finally, of the neutral LAPs, only LAP-N was able to protect CS from aggregation and its activity was comparable to LAP-A. Collectively, these data indicated that the molecular chaperone activity is functionally conserved within the plant LAPs. At the present time, it is unclear why the LAP-A and the neutral LAPs (LAP-N, LAP1, and LAP2) have differences in their relative chaperone activities as measured with three model substrates *in vitro*. This may reflect differences in substrate specificity *in vivo* and may even suggest different mechanisms of action; it is not uncommon for chaperones within the same family to show such variation (Basha et al. 2010).

Study of LAP-A active-site mutants demonstrated that LAP-A's chaperone activity is independent of its peptidase activity (Table 1.4). All seven of the LAP-A active-site mutants showed chaperone activity towards at least two of the model protein substrates. This demonstrates that key residues involved in catalysis and zinc ion interaction were not involved in protein interactions required for chaperone activity. These data suggest that a different region of LAP-A is important for chaperone substrate interaction. This molecular strategy is similar to that used by plastid ATP-dependent proteases, where the peptidase and chaperone domains are independent (Sakamoto 2006).

Analyses of LAP-N's active site mutants revealed unanticipated conformational and chaperone activity differences from analogous mutations in LAP-A. LAP-N wild-type, K357E and K357R were functional chaperones in the NdeI assay. However, unlike the K354E LAP-A protein that displayed enhanced chaperone activity in all three chaperone assays, the K357E mutation reduced LAP-N's activity in the Luc refolding and eliminated LAP-N's activity in the CS aggregation assays. Furthermore, K357R LAP-N did not display chaperone activity in the Luc

assays and it aggregated spontaneously in the CS assay. These data suggest that, unlike LAP-A, mutations in LAP-N's catalytic site impacted chaperone activity. Since the chaperone substrate binding residues are not known in any LAP, it is unclear if K357 is directly involved in chaperone binding or if disruption of K357 significantly alters the structure of LAP-N. If structural changes occur, they must occur at the secondary or tertiary levels, since these mutations did not affect the oligomeric structure of LAP-N. In fact, unlike LAP-A where a majority of substitutions at K354 cause hexamer disassembly, none of the LAP-N K357 mutants disrupted the LAP-N hexamer. The sensitivity of LAP-N's chaperone to mutations in in a catalytic residue may be consistent with the facts that LAP-N is less stable *in vitro* than LAP-A (Tu et al. 2003) and LAP-N's peptidase substrate specificity is distinctive from LAP-A (Gu et al. 1999; Gu and Walling 2000; 2002; Tu et al. 2003).

The LAP-A disruption mutants demonstrated that LAP-A's chaperone activity was independent of LAP-A's oligomeric stability (Table 1.4). In fact, loss of LAP-A's hexameric structure increased LAP-A's chaperone in every assay with one exception; the E429R protein was unable to protect CS against heat-induced aggregation. Reduction of the E429R protein's chaperone activity towards CS is not simply due to a mutation at this residue, since E429V protein had chaperone activity. Therefore, specificity of chaperone activity must be due to yet undetermined conformational changes within LAP-A. The LAP-A data are in marked contrast to the *Synechocystis* HSP16.6 that requires oligomeric structure for chaperone activity (Giese et al. 2005; Giese and Vierling 2002).

Currently, it is unclear why some substitutions in the active site of LAP-A perturb the enzyme's oligomeric structure. There is no correlation of charge or hydrophobicity with residue substitutions and LAP-A disassembly (Gu and Walling 2002). It is even more intriguing that those same substitutions do not disrupt LAP-N's oligomeric structure. While animal and microbial LAP crystal structures are known (Huynh et al. 2009; Kale et al. 2010; McGowan et al. 2010; Sträter et al. 1999), they are not adequate to predict the overall structure of LAPs due to the divergence in the C-terminal catalytic domains (49-59%) and more highly diverged N-terminal domains (Tu et

al. 2003). Therefore, to determine the structural changes that occur in the disruption mutants, crystal structures of the WT and mutant LAPs of tomato will be needed.

The enhancement of LAP-A's chaperone activity upon disruption of its oligomeric structure is consistent with the exposure of new residues or domains that interact with the protein substrate(s). In this manner, LAP-A's chaperone activity resembles the sHSP class of chaperones. Current sHSP models propose that under ambient conditions, sHSPs form large oligomeric structures. During heat stress, these sHSP oligomers either increase their subunit exchange rate or dissociate into smaller complexes, which have chaperone activity (Basha et al. 2010; Haslbeck et al. 2005). These structural changes are presumed to increase exposure of hydrophobic residues, which bind to and protect the protein substrates. Consistent with the sHSP model, it is predicted that when the LAP-A's hexamer is disrupted, more hydrophobic residues will be exposed on the smaller oligomers (monomers, dimers and trimers); these residues could enable association with unfolded protein substrates.

Given that both the tomato (LAP-A and LAP-N) and Arabidopsis LAPs (LAP1 and LAP2) have substantive molecular chaperone activity *in vitro*, it is possible that LAPs may function as chaperones *in vivo*. However due to the differences in their subcellular localizations, the peptidase/chaperone substrates of the Arabidopsis LAP1, which is located in the cytosol(Bartling and Weiler 1992), and are likely to be distinct from the plastid-localized LAP-A, LAP-N and LAP2 (Narváez-Vásquez et al. 2007; Walling 2006). In addition, it is possible that the stress-induced LAP-A has a different set of peptidase/chaperone substrates from LAP-N and LAP2. While the roles of LAP-N, LAP1 and LAP2 are not known, LAP-A has an important role in plant defense with potential roles *in planta* and in caterpillar digestive tracks.

The tomato LAP-A is induced by herbivory, wounding, salinity, and water deficit and is one of the most abundant proteins in the chloroplast stroma after these stresses (Chao et al. 1999; Gu et al. 1996b); furthermore, LAP-A modulates the late branch of wound signaling downstream of JA perception (Fowler et al. 2009). LAP-A is well adapted to the alkaline environment of the stroma exhibiting maximal peptidase activity at pH of 9.0 (Gu et al. 1999). In addition to this

extreme pH environment, during herbivory there are rapid ion fluxes, changes in redox potential, increases in reactive oxygen species, and often desiccation at the wound site that also threatens protein integrity (Bostock 2005). Therefore, it is not surprising that plants up-regulate some chaperones in response to insect feeding and wounding (Halitschke et al. 2003; Lawrence et al. 2008; Pautot et al. 1993; Reymond et al. 2000).

To dissect the roles of LAP peptidase and chaperone activities, transgenic tomatoes expressing mutant LAP proteins are being analyzed to reveal if LAP-A acts as a chaperone within the leaf and whether its chaperone activity, peptidase activity, or both are important for LAP-A's role in defense in tomato. Of particular importance will be understanding if LAP-A undergoes transitions in oligomeric forms *in vivo* (i.e., monomer/dimer/trimer vs. hexamer), which might regulate relative chaperone and peptidase activities *in planta*. Therefore, LAP-A may represent a new mechanism of stress adaptation (to alkaline environments). LAP-A could act like other chaperones that switch from their primary activity to chaperone activity in response to changes in their environments, such as low pH, temperature extremes and reactive oxygen species (Hong et al. 2005; Jang et al. 2004; Lee et al. 2009a; Spiess et al. 1999; Tapley et al. 2010).

Many defense proteins exert their effects within the insect gut by inhibiting the activity of digestive enzymes or reducing the quality of the leaf diet by removing essential amino acids or cross-linking proteins (Chen et al. 2005; Felton et al. 1989; Johnson et al. 1989; Kang et al. 2006). Lepidopteran midguts are alkaline (Dow 1992) and due to LAP-A's hyper-stability within insect digestive track and frass and its alkaline pH optima (Chen et al. 2007; Gu et al. 1999), it has been proposed that LAP-A may degrade peptides within the insect midgut (Gu et al. 1999; Pautot et al. 2001). While preliminary data indicate that artificial diets supplemented with LAP-A do not affect insect growth and development (Fowler et al. 2009), it is also possible that LAP-A works in cooperation with other anti-nutritive enzymes. For example, since LAP-A can also readily hydrolyze N-terminal Arg, it is possible that LAP-A could act in concert with wound-induced arginase to deplete essential Arg from the insect diet (Chen et al. 2005). However given the discovery of LAP-A's chaperone activity, it is also possible that LAP-A protects defense proteins

that are poorly adapted to the alkaline environment of the insect midgut. Alternatively, LAP-A may aid in maintaining the conformation, and thereby assuring the maximal activity, of defense proteins with alkaline pH optima, such as threonine deaminase and arginase, which deplete essential amino acids in the insect midgut (Chen et al. 2007; Chen et al. 2005).

The discovery that LAP-A moonlights as a molecular chaperone in vivo may have important ramifications for plant defense and this will need to be tested genetically once the chaperone domains are identified and LAP-A chaperone mutants can be created. In addition, understanding the identity *in vivo* peptidase and chaperone substrates will determine if these diverse functions target overlapping or unique processes. This study also demonstrates that the *in vitro* molecular chaperone activity is conserved within the plant LAPs suggesting the novel roles for the neutral LAPs (LAP-N, LAP-1 and LAP-2) may be revealed. Interestingly, bacterial and archaeal orthologs of the Arabidopsis LAP2 and LAP3 have been predicted to have been co-inherited with HSP70s (AraNet, Score=2.05), suggesting that LAP chaperone function may have an ancient evolutionary origin. Future studies will determine if chaperone activity is an evolutionary conserved function displayed in LAPs from other kingdoms. Since two microbial enzymes, with structures distinct from LAPs, have dual aminopeptidase and chaperone activities (Lee et al. 2009b; Malki et al. 2005), it is intriguing to speculate that aminopeptidase and chaperone activities co-evolved. If confirmed, LAPs will add to the growing diversity of multifunctional aminopeptidases.

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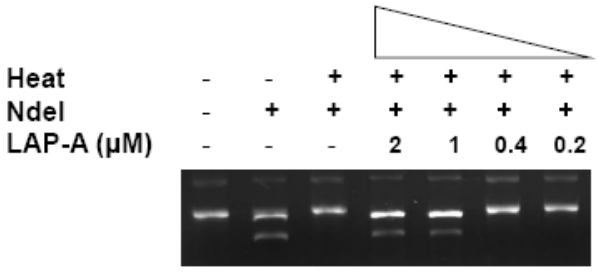

Figure 1.1 LAP-A protects NdeI from thermal inactivation in the absence of glycerol. His_6-LAP-A was isolated and stored in the absence of glycerol. Restriction enzyme NdeI (1 U) was incubated in 1X restriction enzyme buffer 4 with or without His_6-LAP-A (0-2 μM) for 90 min at 43°C. After thermal deactivation, 140 ng of plasmid DNA was added and digested for 90 min at 37°C. Control lanes show plasmid DNA only with supercoiled (SC) monomer and multimers and DNA after digestion with unheated NdeI. NdeI released a 4.6-kb and 0.2 kb fragment (not shown).

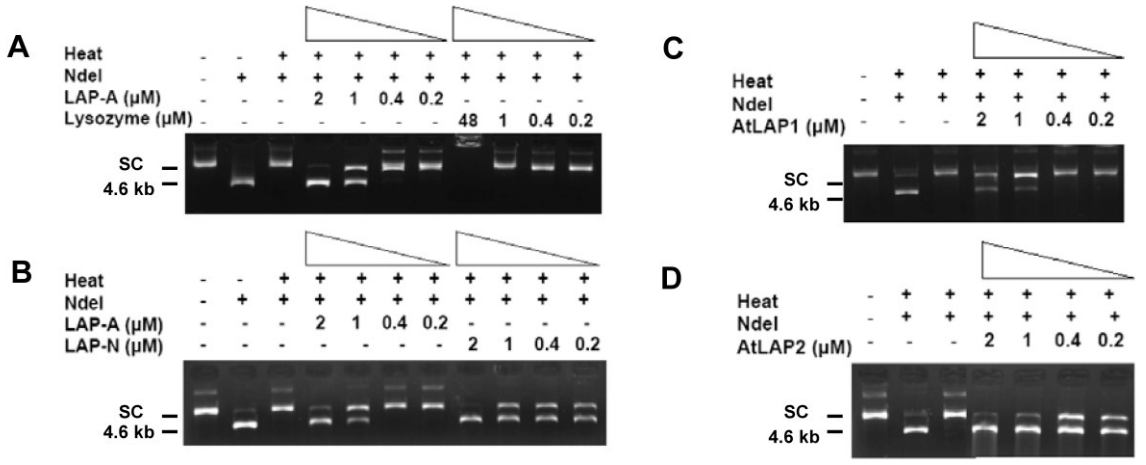

Figure 1.2Tomato and Arabidopsis LAPs protect NdeI from thermal inactivation. NdeI (1 U) was incubated in buffer with 4% glycerol (*A*-*D*) in the presence or absence of His_6-LAP-A (0.2-2 μM; *A*-*B*), lysozyme (0.2-48 μM; *A*), His_6-LAP-N (0.2-2 μM; *B*), His_6-LAP1 (0.2-2 μM; *C*) or His_6-LAP2 (0.2-2 μM; *D*) for 90 min at 43°C. At this time, 140 ng of plasmid DNA was added and digested for 90 min at 37°C. Control lanes show plasmid DNA only and DNA after digestion with unheated NdeI. NdeI cuts at two sites in the 4.8-kb plasmid releasing fragments 4.6 kb and 0.2 kb; only the 4.6-kb fragment is shown on these gels. The monomeric supercoiled plasmid (SC) and multimeric supercoils are observed in undigested DNA samples.

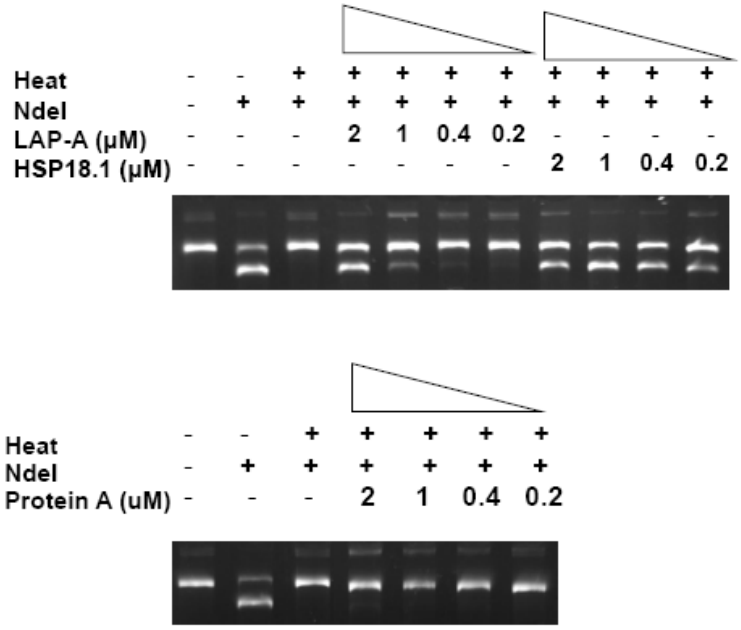

Figure 1.3 *Pisum sativum* Hsp18.1 protects NdeI from thermal inactivation. Restriction enzyme NdeI (1 U) was incubated alone or with 0.2-2 μM His_6-LAP-A or PsHSP18.1 (Panel *A*) or protein A (Panel *B*). NdeI was heat denatured at 43°C and plasmid DNA was digested as described in Fig. 1.1. Control lanes show plasmid DNA only and DNA after digestion with unheated NdeI.

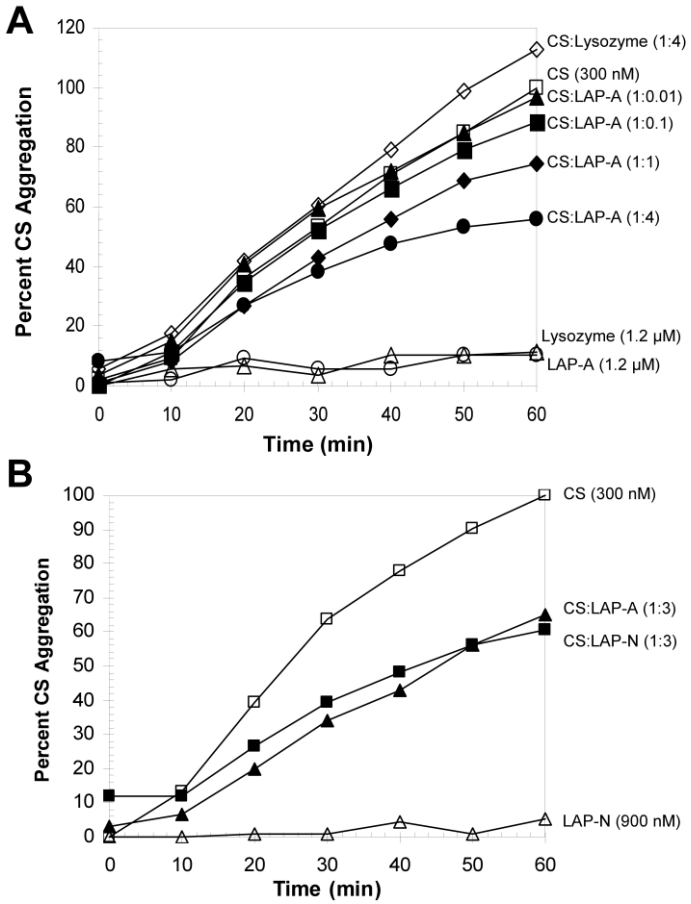

Figure 1.4 Tomato LAP-A and LAP-N protect CS from thermal aggregation. *A,* CS (300 nM) was incubated in 50 mM HEPES-KOH (pH 7.5), 5% glycerol, and His_6-LAP-A or lysozyme (1200 nM; ◊) at 43°C for 60 min. The LAP-A concentrations of 0 (□), 3 (▲), 30 (■), 300 (♦), or 1200 (●) nM corresponded to CS:LAP-A ratios of 1:0.01, 1:0.1, 1:1, and 1:4, respectively. Neither His_6-LAP-A (○) nor lysozyme (Δ) aggregated on their own. *B*, CS was incubated with 900 nM His_6-LAP-A (■) or His_6-LAP-N (▲). His_6-LAP-N did not aggregate on its own (Δ). Aggregation of CS was determined by measuring light scattering at 360 nm. After 60 min at 43°C, aggregation of 300 nM CS reached an absorbance of 0.8-1. Data shown is representative of two or more independent experiments. His_6-LAP-A (900 nM) reduction of CS aggregation varied in independent assays from 40-60%.

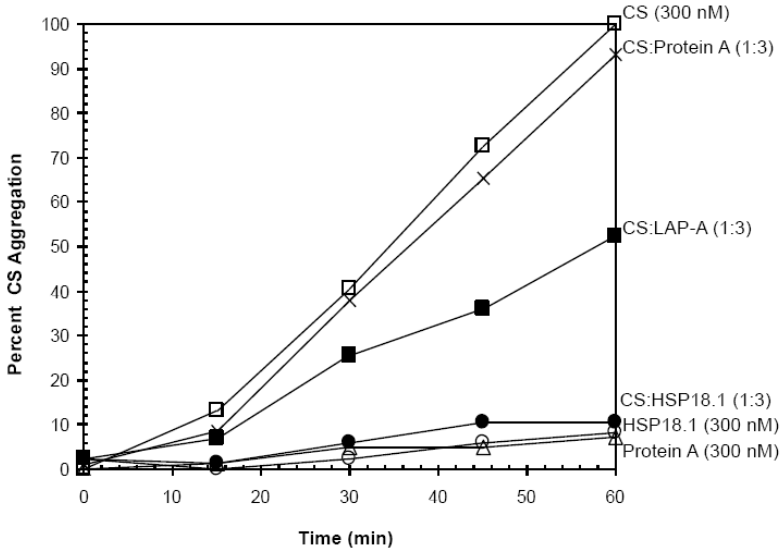

Figure 1.5 PsHsp18.1 protects citrate synthase from thermal aggregation. Citrate synthase (300 nM; CS) was incubated in 50 mM HEPES-KOH (pH 7.5), 5% glycerol, and 0 (□) or 900 nM His_6-LAP-A (■), PsHSP18.1 (●), or protein A (x) at 43°C for 60 min. Aggregation of CS was determined by measuring light scattering at 360 nm. Neither PsHSP18.1 (○) nor protein A (Δ) aggregated on their own. Data shown is representative of two independent experiments.

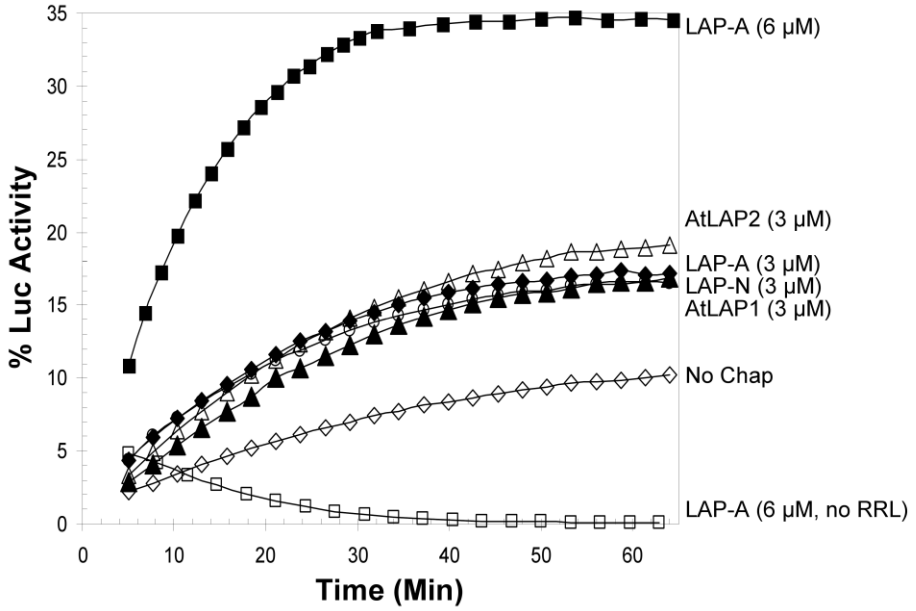

Figure 1.6 Tomato and Arabidopsis LAPs aid refolding of Luc. Luc (1 μM) was heated for 11 min at 42°C with 3 μM (♦) or 6 μM His_6-LAP-A (■), 3 μM His_6-LAP-N (▲), 3 μM His_6-LAP1 (○), 3 μM His_6-LAP2 (Δ), or no chaperone (◊). Luc was allowed to refold in the presence of rabbit reticulocyte lysate (RRL) supplemented with 2 mM ATP. Luc was also heated in the presence of 6 μM His_6-LAP-A allowed to refold without RRL (□). Percent activity corresponds to the relative luminescence compared to unheated luciferase. Measurements were taken for three technical replicates. Data is representative of two or more independent experiments. The degree of His_6-LAP-A (3 μM) protection of Luc varied in independent experiments, ranging from 14-20%. The ability of Luc to refold in the absence of ATP-independent chaperone varied in independent experiments, ranging from 2-10%.

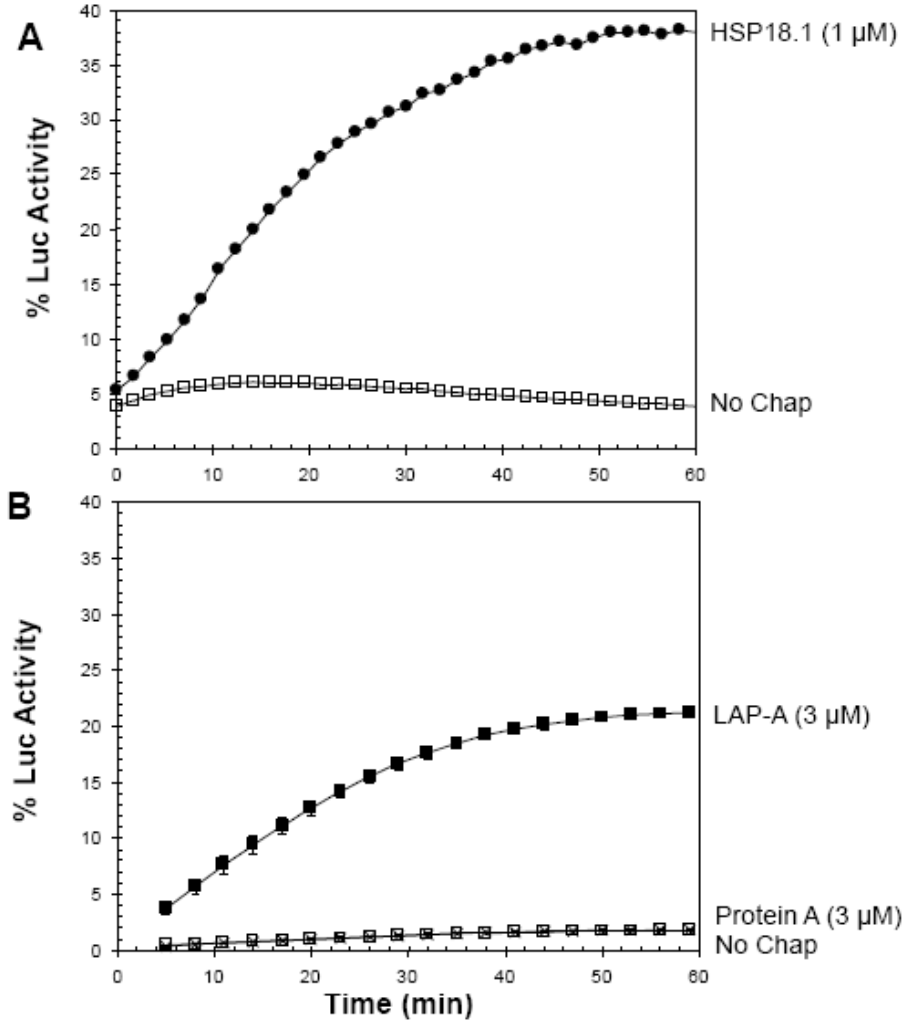

Figure 1.7 *Pisum sativum* Hsp18.1 aids in refolding of firefly luciferase. Luc (1 μM) was heated at 42°C for 11 min with 1 μM PsHsp 18.1 (●, Panel *A*), 3 μM His_6-LAP-A (■, Panel *B*), 3 μM protein A (x, Panel *B*), or no chaperone (□, Panels *A*-*B*) and then allowed to refold in rabbit reticulocyte lysate (RRL) in refolding buffer supplemented with 2 mM ATP. Percent activity corresponds to the relative luminescence compared to unheated luciferase. Measurements were taken for three technical replicates. Data is representative of at least 2 independent experiments.

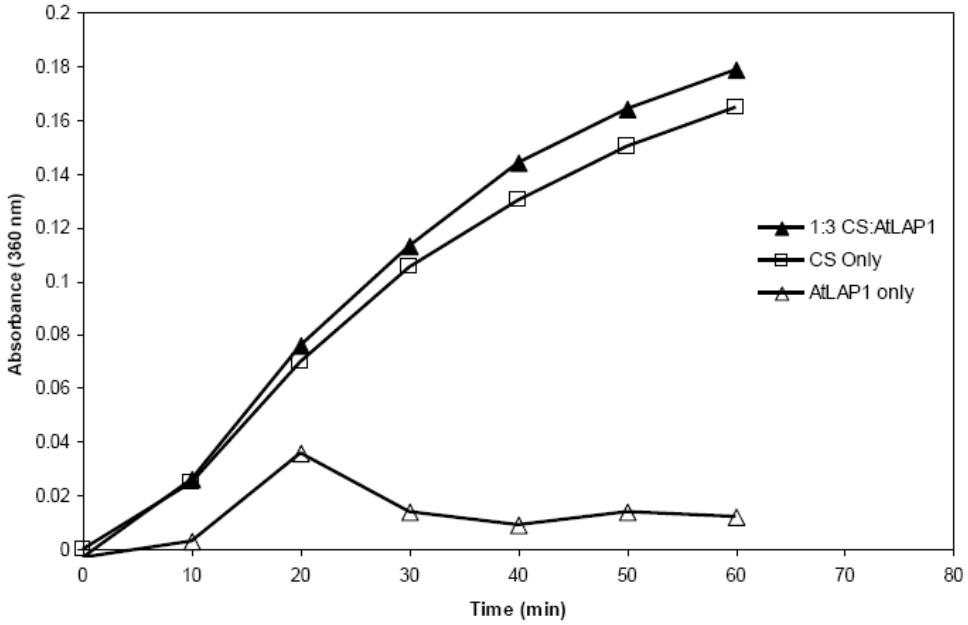

Figure 1.8 LAP1 does not protect citrate synthase from thermal aggregation. Citrate synthase (300 nM; CS) was incubated in 50 mM HEPES-KOH (pH 7.5), 5% glycerol, and 0 (▲) or 900 (□) nM His_6-LAP1 at 43°C for 60 min. Aggregation of CS was determined by measuring light scattering at 360 nm. LAP1 (Δ) did not aggregate on its own. Data shown is representative of two independent experiments.

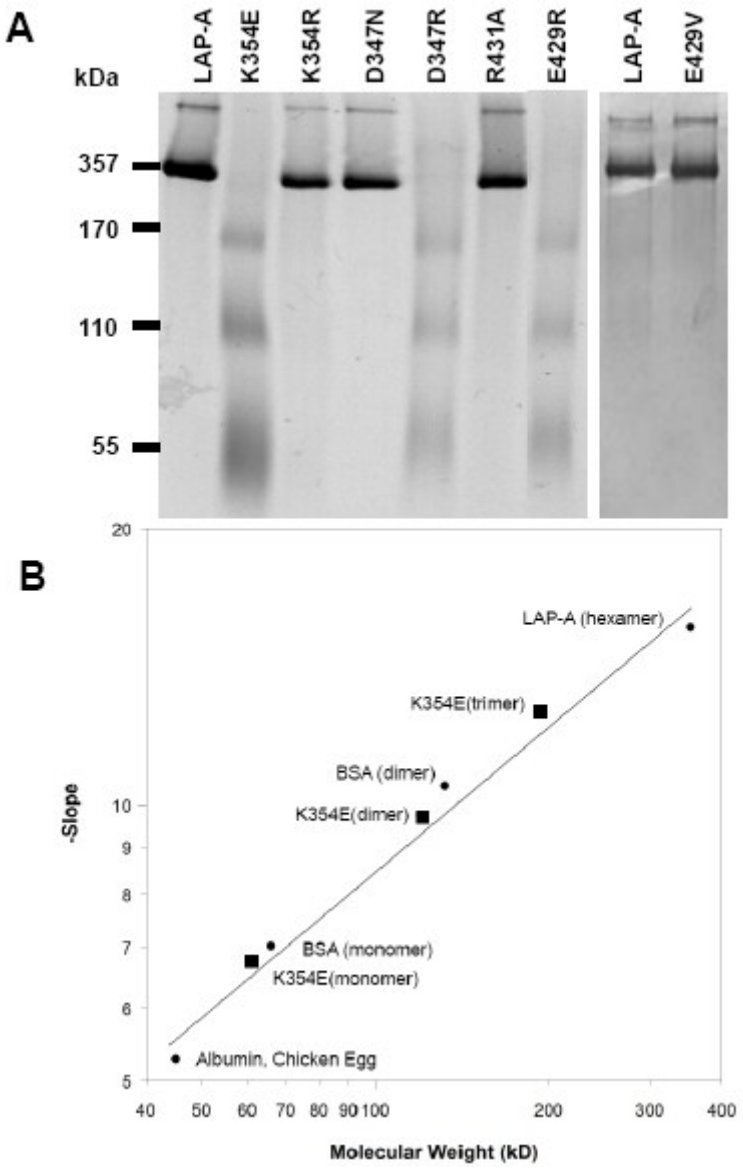

Figure 1.9 Oligomeric structure of LAP-A mutants. *A,* Purified His_6-LAP-As (10 µg) were fractionated on a native polyacrylamide gel (9% w/v). Protein bands were visualized by staining with Coomassie Brilliant Blue R-250. Masses of each multimer were determined as in Panel *B*. *B,* Purified wild-type and mutant His_6-LAP-As (10 µg) were fractionated on a set of four native polyacrylamide gels (7.5-12% w/v). Molecular mass standards were chicken egg albumin (45 kDa); bovine serum albumin monomer (66 kDa) and dimer (132 kDa); and tomato His_6-LAP-A hexamer (357 kDa). LAP oligomer species masses were determined by their relative mobility and retardation coefficient as previously described (Bryan, 1977).

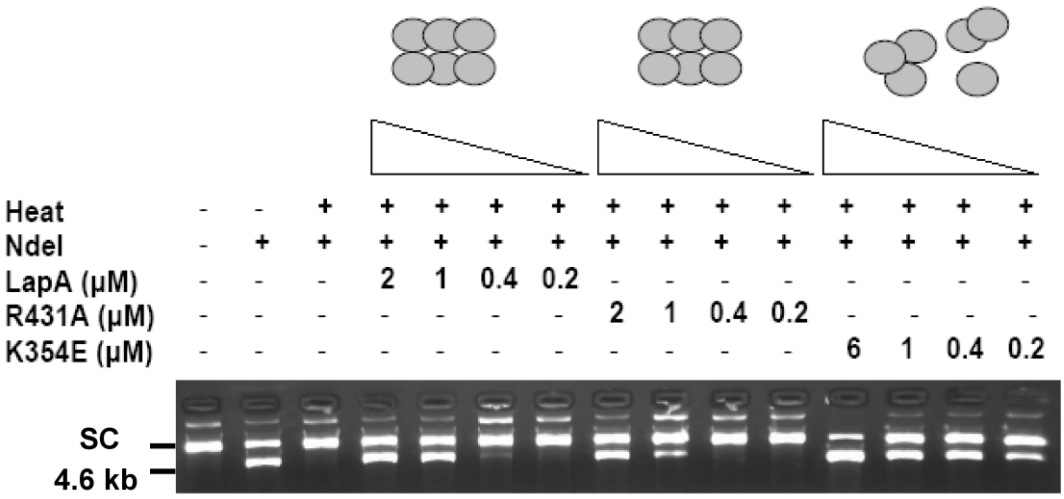

Figure 1.10 LAP-A mutants protect NdeI from thermal inactivation. NdeI (1 U) was incubated alone or with 0.2-2 μM His_6-LAP-A (wild-type), R431A (peptidase-deficient, non-disruption mutant), or K354E (peptidase-deficient, disruption mutant) as described in Fig. 1.1. Control lanes show supercoiled (SC) plasmid DNA only and DNA after digestion with unheated NdeI (4.6 kb).

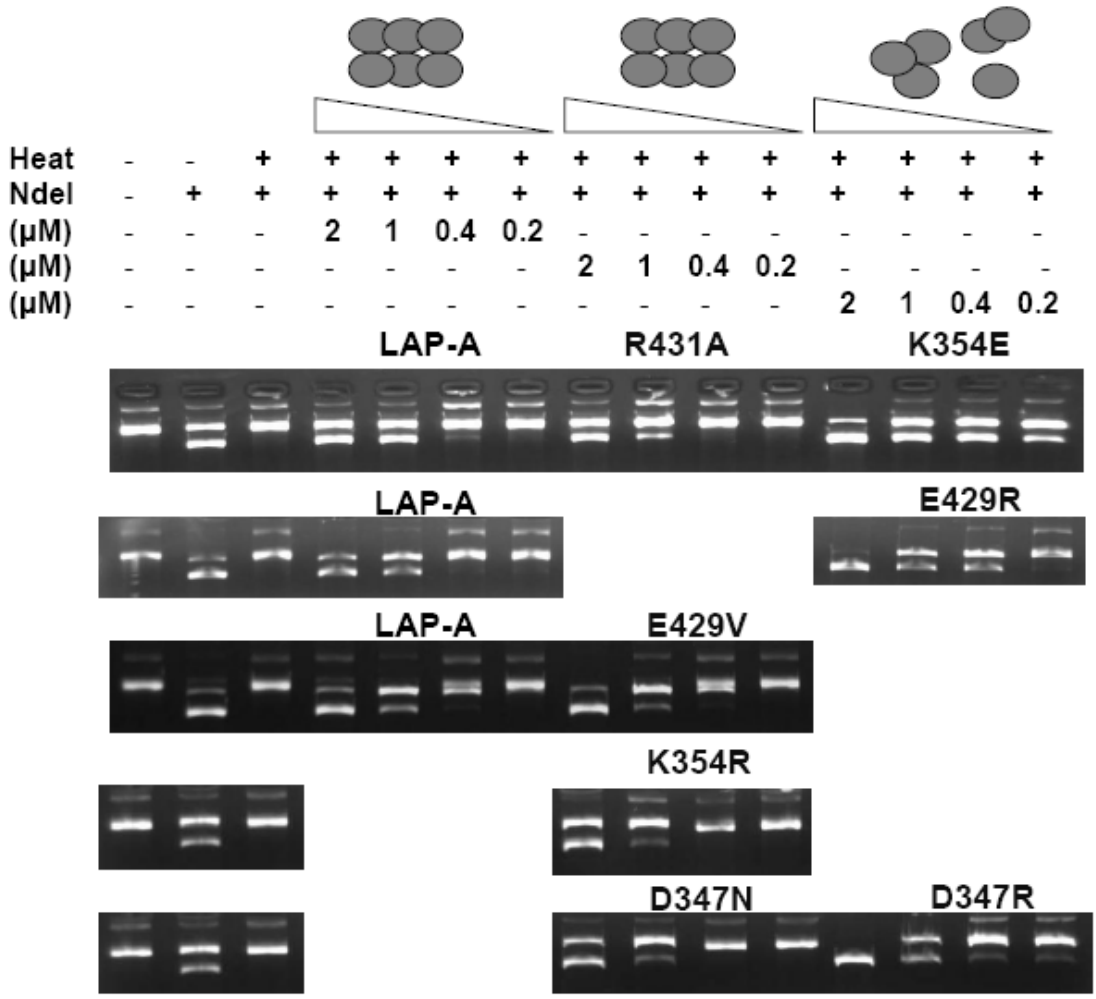

Figure 1.11 LAP-A active site point mutants protect a NdeI from thermal inactivation. Restriction enzyme NdeI (1 U) was incubated alone or with 0.2-2 μM His_6-LAP-A, K354E, R431A, E429V, E429R, K354R, D347N, or D347R. NdeI was heat denatured and plasmid DNA was digested as described in Fig. 1.1. Control lanes show plasmid DNA only and DNA after digestion with unheated NdeI.

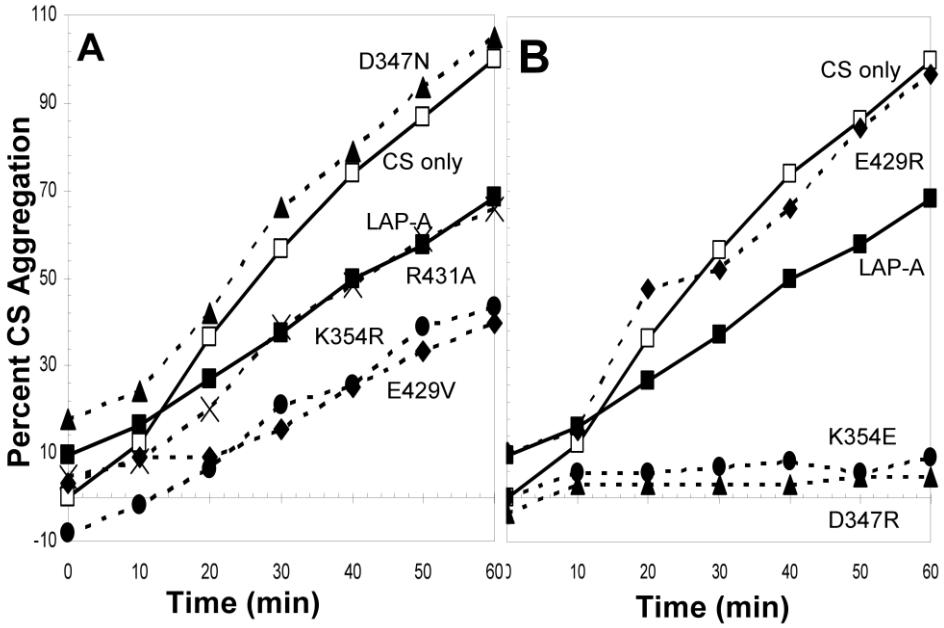

Figure 1.12 LAP-A mutants protect CS from thermal aggregation. CS aggregation assays were performed as described in Fig. 1.2. *A*, CS was heated at 43°C with 900 nM His_6-LAP-A (■) or the non-disruption mutant proteins E429V (♦), D347N (▲), K354R (●), R431A (x) or alone (□). *B*, CS was heated with 900 nM His_6-LAP-A (■) or the disruption mutant proteins E429R (♦), D347R (▲), K354E (●), or alone (□). Data shown is representative of at least two independent experiments.

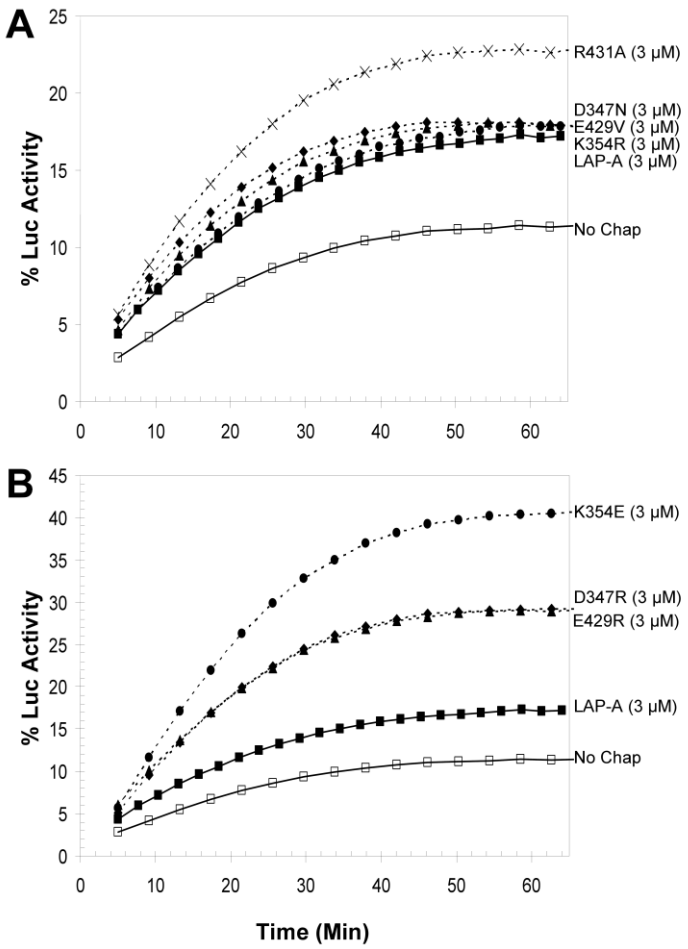

Figure 1.13 LAP-A mutants aid refolding of Luc. *A*, Luc (1 μM) was heated for 11 min at 42°C with 3 μM His_6-LAP-A (■) or one of the non-disruption mutants E429V (♦), D347N (▲), K354R (●), R431A (x), or alone (□). *B*, Luc (1 μM) was heated for 11 min with 3 μM His_6-LAP-A (■) or one of the disruption mutants E429R (♦), D347R (▲), K354E (●), or alone (□). Luc was allowed to refold and its activity was measured as described in Fig. 1.6. Data shown is representative of at least two independent experiments.

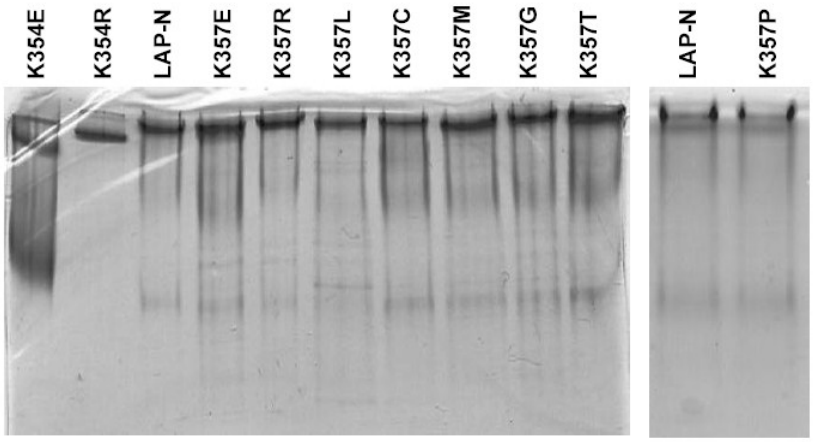

Figure 1.14 Oligomeric structure of LAP-N mutants. Purified His_6-LAP-Ns (10 µg) were fractionated on a native polyacrylamide gel (9% w/v). Protein bands were visualized by staining with Coomassie Brilliant Blue R-250.

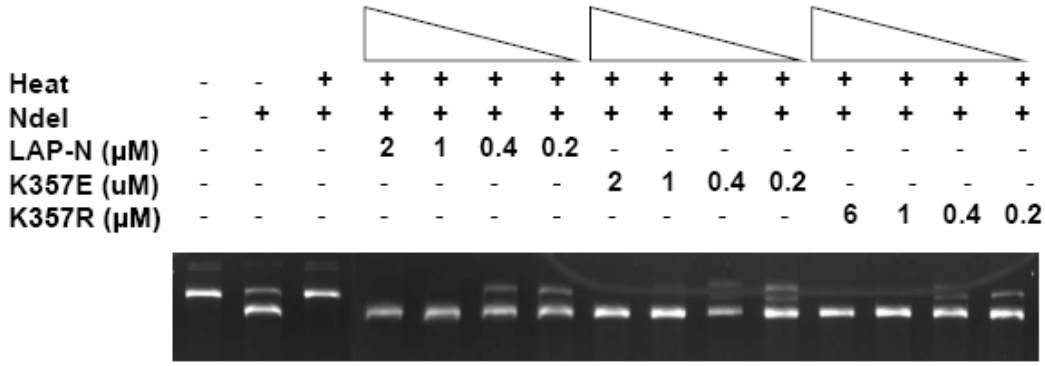

Figure 1.15 LAP-N mutants protect NdeI from thermal inactivation. Restriction enzyme NdeI (1 U) was incubated alone or with 0.2-2 μM His_6-LAP-N, K357E, or K357R. NdeI was heat denatured at 43°C and plasmid DNA was digested as described in Fig. 1.1. Control lanes show plasmid DNA only and DNA after digestion with unheated NdeI.

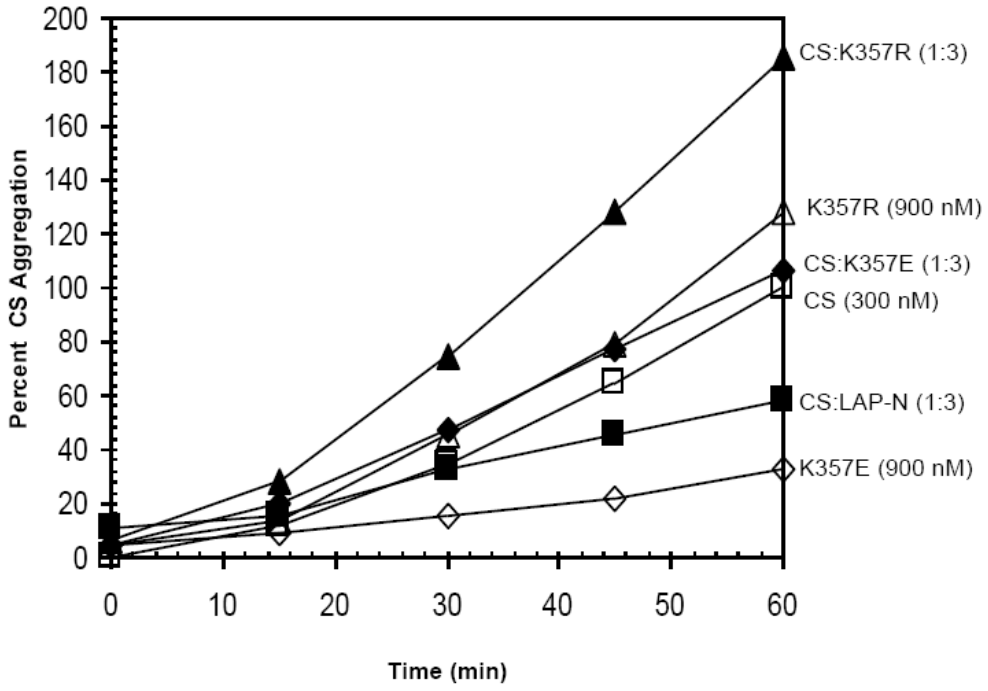

Figure 1.16 LAP-N mutants do not protect CS from thermal aggregation. Citrate synthase (300 nM; CS) was incubated in 50 mM HEPES-KOH (pH 7.5), 5% glycerol, and 0 (□) or 900 nM His_6-LAP-N (■), K357E (♦), or K357R (▲) at 43°C for 60 min. Aggregation of CS was determined by measuring light scattering at 360 nm. K357E (◊) had a small amount of aggregation on its own while K357R (Δ) highly aggregated its own. Data shown is representative of two independent experiments.

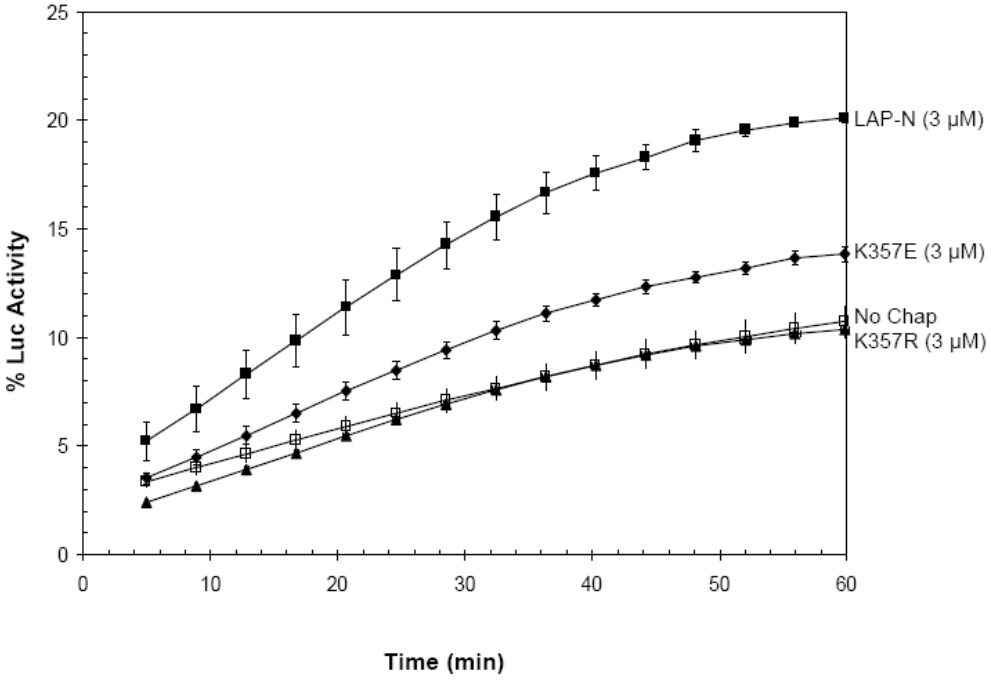

Figure 1.17 LAP-N mutants have lower chaperone activity based on the Luc refolding assay. Luc (1 μM) was heated for 11 min at 42°C with 3 μM His_6-LAP-N (■), K357E (♦), or K357R (▲), or no chaperone (□) and then allowed to refold in rabbit reticulocyte lysate (RRL) in refolding buffer supplemented with 2 mM ATP. Percent activity corresponds to the relative luminescence compared to unheated luciferase. Measurements were taken for three technical replicates. Data is representative of at least two independent experiments.

Table 1.1 Primers used for cloning of *AtLAP* cDNAs.

Locus	Gene	Primer name	Primer sequence[A]
At2g24200	*AtLAP1*	LAP1-F	5'-AGCATATGATGGCTCACACTCYCGGT-3'
		LAP1-R	5'-ATGCGGCCGCTCACGAAGATGAATTCTTC-3'
At4g30920	*AtLAP2*	LAP2-F	5'-GCATATGGC- TCATACAATCTCACACGC-3'
		LAP2-R	5'-GCTCGAGTTAAGAAGAAGAATGGTTCTGT-3'

[A] Restriction enzyme sites for cloning of *AtLAP* cDNAs were incorporated into the forward (F) and reverse (R) primers. These sites are underlined and correspond to NdeI (LAP1-F, LAP2-F), NotI (LAP1-R), and XhoI (LAP2-R) sites.

Table 1.2 Percent Wild-type His_6-LAP-N enzyme activity on Leu-AMC substrate

His_6-LAP-N enzyme	% Wild-type His_6-LAP-N enzyme activity
Wild-Type	100
K357E	0.35 ± 0.03
K357R	0.93 ± 0.07
K357L	0.7 ± 0.09
K357C	0.26 ± 0.11
K357M	1.47 ± 0.04
K357G	0.84 ± 0.07
K357T	0.28 ± 0.03
K357P	0.34 ± 0.13

Purified His_6-LAPs (2160 ng) were assayed for activity towards Leu-AMC (1.58 µM) in assay buffer (50 mM Tris-HCl, pH 8, 0.5 mM $MnCl_2$) in a total volume of 162 µl. Activity assays proceeded for 30 min at 37°C. Rate of hydrolysis was determined and activity expressed as percent of wild-type His_6-LAP-N activity which was 0.24 ± 0.01 µmol mg^{-1} min^{-1} of protein (± SD).

Table 1.3 Summary of Chaperone Assays for Plant LAPs.

	CS Protection at 1:3	Lowest conc (μM) of NdeI Protection	%Luc Recovering at 1:3
LAP-A	40-60%	1	14-20%
LAP-N	40-60%	0.2	17%
AtLAP1	0%	1	16%
AtLAP2	N.D.	0.2	18-19%

Table 1.4 Summary of Chaperone Assays for Tomato LAP-A and LAP-N mutants.

		CS Protection at 1:3	Lowest conc (μM) of NdeI Protection	% Luc Recovering at 1:3	Site Disruption	Hexamer Disruption
LAP-A Mutants	D347N	0%	1	17-18%	Zinc ion binding	no
	D347R	100%	0.2	~30%	Zinc ion binding	yes
	E429R	0%	0.4	~30%	Zinc ion binding	yes
	E429V	40-60%	1	17-18%	Zinc ion binding	no
	K354E	100%	0.2	40%	Catalysis	yes
	K354R	40-60%	1	17-18%	Catalysis	no
	R431A	40-60%	1	~22%	Catalysis	no
LAP-N Mutants	K357E	0%	0.2	13%	Catalysis	no
	K357R	N.D.	0.2	0%	Catalysis	no

CHAPTER 2: Microarray Analysis of the Early and Late Wound Response Reveals New Regulatory Targets for the Tomato Leucine Aminopeptidase A

ABSTRACT

Wounding due to mechanical injury or insect feeding causes a wide array of damage to the cell including cell disruption, desiccation, metabolite oxidation, and disruption of primary metabolism especially within the plastid. In response, plants regulate a variety of genes and metabolic pathways to cope with injury. Tomato (*Solanum lycopersicum)* has long been established as a model for wound responses in plants. However, to date, no study has looked at the comprehensive gene expression response in tomato in response to injury. Therefore, the TIGR potato 10-K cDNA array was utilized to analyze large scale temporal (early and late) and spatial (locally and systemically) responses to mechanical wounding in tomato leaves. Analyses demonstrated that tomato regulates many primary and secondary metabolic pathways and this regulation is dependent on timing and location. In addition, recent studies have identified leucine aminopeptidase A (LAP-A) as a modulator of a subset of tomato wound responses. Therefore, microarray analysis was performed on *LapA* silenced (*LapA-SI*) lines after wounding to determine if LAP-A modulates gene expression beyond the core defense genes. While most of the wound responses in the *LapA-SI* lines were similar to WT, overall defenses were delayed in the *LapA-SI* lines. Moreover, two new sets of genes [two *basic pathogenesis-related 1* (*PR-1*) genes and two *dehydrins* (*Dhns*)] were modulated by LAP-A. Together this study has shown that tomato wound responses are complex and that LAP-A's role in modulation extends beyond the late-wound gene expression.

INTRODUCTION

In nature, plants must cope with multitude of stresses individually and simultaneously. Many of the abiotic (rain, hail, wind) and biotic stresses (herbivory) breach cellular integrity causing membrane disruption, desiccation, lipid and protein oxidation, and protein aggregation (Bostock 2005). This damage can range from mild (responses to phloem-feeding whiteflies, psyllids and aphids) to extreme (responses to pruning, hail or herbivores that chew and tear plant tissues) (Walling 2000). A plant's ability to rapidly respond to its injured status is integral to activating and modulating the pathways to promote cellular healing, limit pathogen ingression into wound sites and interfere with herbivore success (Heil 2009; Howe and Jander 2008; Walling 2009). Plants recognize damage-associated molecular patterns such as plant cell wall fragments (oligogalacturonides) and respond to membrane depolarization to activate defense signaling (Heil 2009; Maffei et al. 2007).

At the core of the wound responses are the defenses activated by oxylipins (Farmer et al. 2003; Ryan 2000; Wasternack 2007). Synthesized by the octadecanoid pathway, jasmonic acid (JA), its bioactive isoleucine conjugate (JA-Ile), and JA-biosynthetic intermediates induce defense-response genes. Many of the JA-regulated genes encode proteins that directly interfere with insect performance by increasing anti-nutritive proteins and chemicals or are involved in the emission of volatile organic compounds to attract natural enemies to hervivore-infested plants (Chen 2008; Hare 2011). While oxylipin-regulated defenses are often the dominant response to damage, this signaling pathway is integrated into a complex and dynamic defense-signaling network that involves salicylic acid (SA), abscisic acid (ABA), ethylene (ET), gibberellic acid (GA), brassinosteriods (BR), cytokinins (CK), as well as reactive oxygen species and redox changes (Erb et al. 2012; Pieterse et al. 2009; Robert-Seilaniantz et al. 2011) These responses are also influenced by herbivore elicitors and effectors to stimulate or suppress defenses (Felton and Tumlinson 2008; Heil 2009; Hogenhout and Bos 2011; Howe and Jander 2008; Schmelz et al. 2009; Walling 2009).

Insights into the dynamics and specificity of damage-induced responses has been gleaned from small and large scale microarray studies of wounding and attack by tissue-damaging herbivores in Arabidopsis and poplar (Bilgin et al. 2010; Cheong et al. 2002; Major and Constabel 2006; Reymond et al. 2000; Walley et al. 2007). In addition to the core JA-regulated wound-response genes, a wide variety of genes influencing primary and secondary metabolism, as well as photosynthesis, are differentially regulated in response to wounding in plants. Many of these genes are involved in protecting the cell against the stress-induced damage and/or are co-regulated by other hormone- and stress-response pathways (Bilgin et al. 2010; Cheong et al. 2002; Reymond et al. 2000).

While *Solanum lycopersicum* (tomato) and *Nicotiana attenuata* have served as model organisms to understand wound responses in the Solanaceae (Green and Ryan 1972; Ryan 2000; Sun et al. 2011), large-scale microarray studies focused on understanding recognition of self-damage are few. Microarray studies of the Solanaceae have primarily used small-scale oligonucleotide arrays focused on a narrow set of core defense genes to study herbivory, wounding, or methyl jasmonate (MeJA) treatments (Halitschke et al. 2003; Li et al. 2004; Strassner et al. 2002). Two studies have used large-scale arrays to study herbivore defense in Solanaceous plants. Lawrence et al. (2008) used the TIGR 10K potato (*Solanum tuberosum*) cDNA array to study potato responses to the oral secretions (regurgitant) of the Colorado potato beetle [*Leptinotarsa decemlineata* (Say)]. In this study, regurgitant and buffer were applied to wound sites; however, comparisons to undamaged leaves were not performed and therefore wound-induced changes in transcript levels were not determined. A second study by Uppalapati et al. (2005) used the 13,440-element tomato cDNA array (Tom1) to study the changes tomato transcripts 12 hr after treatment with MeJA, JA's biosynthetic precursors or coronatine (a JA-Ile mimic).

While there is substantive overlap between JA-induced defenses and injury, there may be critical distinctions plant responses to mechanical damage including JA-independent responses (Bilgin et al. 2010; Böttcher and Pollmann 2009; Howe 2004). Furthermore, given tomato's

seminal role in understanding JA-signaling and identifying a core of defenses associated with herbivory, it is timely to examine changes RNAs that occur when plants perceive injury. Based on studies with sentinel wound-response genes, damaging tomato leaves results in temporal (early and late) and spatial (locally and systemically) changes in gene expression (Ryan 2000). The early wound-response genes are up-regulated 0.5 to 2 hr after wounding and primarily involved in amplification of the octadecanoid pathway. The late wound-response gene RNA levels increase from 4 to 24 hr and primarily consist of genes important for insect deterrence. Examples of tomato late-wound response genes include *Polyphenol Oxidase* (*PPO*), *serine Proteinase inhibitors* (*PinI* and *PinII*), *Arginase*, and *Thr deaminase*, which are known to encode proteins that have anti-nutritive roles (Chen et al. 2005; Felton et al. 1989; Green and Ryan 1972; Howe 2004).

The acidic leucine aminopeptidase (LAP-A) is a late wound-response protein present in a subset of the Solanaceae (Chao et al. 2000). Recently, LAP-A was shown to have an important role in insect defense (Fowler et al. 2009; Hartl et al. 2008). Transiently silencing *Lap* in *Solanum nigrum* (nightshade) using viral-induced gene silencing leads to increases in insect mass (Hartl et al. 2008). Moreover, transgenic tomato lines that have silenced *Lap* genes (*LapA-SI)* are more susceptible to *Manduca sexta* feeding and insect mass was larger than insects grown on wild-type (WT) plants (Fowler et al. 2009). Reciprocally, transgenic tomatoes that ectopically express the acidic tomato *LapA* (*LapA-OX*) are more resistant to *M. sexta* feeding and delays in insect growth and development were observed.

LAP-A may affect insect feeding and growth either directly or indirectly. LAP-A could have a direct anti-nutritive role like those of other late wound-induced enzymes (Chen et al. 2005; Felton et al. 1989; Howe 2004; Johnson et al. 1989; Kang and Baldwin 2006). However, when *M. sexta* fed on an artificial diet supplemented with LAP-A, there was no impact on insect growth or development indicating that LAP-A does not act directly (Fowler et al. 2009). Alternatively, LAP-A may work in conjunction with other plant-derived anti-nutritive enzymes within the insect gut to enable their post-ingestive effects. Interestingly, LAP-A has a role in signal transduction. LAP-A acts downstream of JA biosynthesis and perception to modulate the late branch, but not the early

branch, of wound signaling in tomato (Fowler et al. 2009). After wounding *PPO-F*, *PinI*, and *PinII* transcripts accumulate to lower levels in *LapA-SI* relative to WT plants. Reciprocally, these late wound-response transcripts accumulate to higher levels and remain elevated longer in the *LapA-OX* plants. Early wound-response transcript levels are similar in all three genotypes before and after wounding.

Unlike the early JA biosynthesis genes and prosystemin, which are expressed in the vascular bundle cells, late wound-response genes, including *LapA,* are expressed in mesophyll cells (Hause et al. 2003; Madureira et al. 2006; Moura et al. 2001; Narvaez-Vasquez and Ryan 2004; Narváez-Vásquez et al. 2008; Stenzel et al. 2003). Tomato LAP-A is localized to the plastids and therefore must act through a signaling network in order to affect late wound-response gene expression in the nucleus (Fowler et al. 2009; Narváez-Vásquez et al. 2008). Retrograde signals involved in stress and defense response are beginning to emerge, but a complete signaling pathway has yet to be characterized (Maruta et al. 2012; Pfannschmidt 2010). LAP-A's peptidase function has been well-characterized *in vitro* (Gu et al. 1999; Gu and Walling 2000) and it has been presumed that LAP-A acts on a peptide or protein within the stroma that contributes to this retrograde-signaling pathway (Fowler et al. 2009). Recently it was shown that LAP-A is also a molecular chaperone *in vitro* (Scranton et al. 2012). It is currently unclear if one or both of these biochemical functions is essential for LAP-A's role in the wound response and insect defense.

To investigate LAP-A's role in the regulation of the late branch of wound signaling, changes in tomato transcripts were assessed in WT and *LapA-SI* tomato plants at 0, 1, and 8 hr after wounding using cDNA microarrays. These studies provided the first insights into the wide spectrum of tomato genes influenced by wounding expanding our knowledge beyond the core jasmonate pathway. Array analysis demonstrated that tomato wound-response gene regulation is complex and, similar to other plants leads to the regulation of wide range of genes involved in primary and secondary metabolism. Analysis of *LapA-SI* gene expression after wounding revealed that while overall gene regulation was similar to WT, *LapA-SI* responses were delayed after wounding. In addition, two new *LapA*-regulated gene sets (basic *PR-1s* and *Dhns*) were

identified. Together this study demonstrates that LAP-A's role in stress response extends beyond the core late-wound signaling pathway.

RESULTS AND DISCUSSION

Functional Annotation of Wound-Responsive DEGs: An Overview

In order to identify transcriptome changes that occurred after injury in tomato, cDNA microarray analyses were performed using RNAs isolated from wild-type (WT) tomato leaves at 0, 1 and 8 hr after wounding. Changes in the RNAs that accumulated in the wounded leaves (local) and apical, non-wounded leaves (systemic) were assessed. The potato 10-K array contains 11,412 confirmed clones thus covering approximately a third of the 34,727 and 35,004 genes predicted in tomato and potato, respectively (Consortium 2012). Potato arrays were used because potato and tomato have similar genomes with respect to gene content, genome organization and nucleotide sequence conservation (Consortium 2011; Consortium 2012; Iovene et al. 2008; Rensink et al. 2005; Schlueter et al. 2004; Xu et al. 2011). Furthermore, cross-species hybridization (CSH) strategies within the Solanaceae have been informative (Bagnaresi et al. 2008; Bar-Or et al. 2006; Bar-Or et al. 2007a; Bar-Or et al. 2007b; Moore et al. 2005).

Using a reference RNA strategy (Dobbin and Simon 2002), the levels of RNAs of mechanically wounded leaves relative to non-injured leaves was determined. Both temporal and spatial changes in gene expression were determined. Differentially expressed genes (DEGs) were defined as up or down regulated based on a log2-fold change ($|FC| \geq 0.8$) and their statistical significance ($p>0.05$). Changes in the tomato transcriptome after wounding were rapid. One hr after wounding, 330 DEGs were detected in the damaged leaves (Figure 2.1). Approximately 48% (158 DEG RNAs) of the genes that responded at 1 hr were transiently expressed; their RNAs had returned to pre-damage levels by 8 hr after wounding (Figure 2.1). At 1 hr after wounding, only four DEG RNAs were detected in the systemic leaves at 1 hr post wounding. These DEGs included three genes of unknown function, as well as Ethylene-Responsive (ER5) late embryogenesis abundant protein (Zegzouti et al. 1997). *ER5* is one of the most highly expressed genes early in the wound response (discussed below).

By 8 hr, there was a 1.6-fold increase in the number of genes expressed locally (531 DEGs); 362 of these RNAs were only detected 8 hr after wounding (Figure 2.1). At this time, there was a substantial increase in the number of DEGs detected systemically with 289 DEGs in the non-wounded, apical leaves. Most of 8-hr systemic DEGs (83%) were also differentially expressed in 8-hr damaged leaves. Finally 8 hr after wounding, a small number of DEGs (24 up and 16 down) that were unique to the apical, undamaged leaves were detected; these data suggested that defense signaling in the systemic leaves may have unique features that are yet to be revealed.

To investigate the types of genes responding to damage, microarray data was annotated using MapMan software (Thimm et al. 2004; Usadel et al. 2005). ESTs present on the TIGR 10-K potato cDNA array have been annotated and assigned to 35 categories based on their molecular function (BINs; Rotter et al. 2007). After wounding, only two MapMan BINs lacked DEGs; these were BINs associated with sulfur assimilation (BIN14) and biodegradation of xenobiotics (BIN24) (Table 2.1). The lack of DEGs in these BINS could be due to the fact that BIN14 and BIN24 had a small number of cDNAs represented on the array (11 and 9, respectively); alternatively, these functions were not regulated in response to injury in tomato.

DEGs with a wide variety of molecular functions were identified at both 1 hr and 8 hr after wounding ($|FC| \geq 0.8$; $p<0.05$) (Table 2.1). While the number of DEGs increased to 1.6-fold by 8 hr after wounding (Figure 2.1), types of genes and their distribution in the different BINs regulated both locally and systemically at 1 and 8 hr after wounding were similar (Table 2.1). These DEGs were preferentially clustered in ten MapMan BINs. Five BINs (stress, photosynthesis, amino acid metabolism, tetrapyrole biosynthesis, and protein) and five subBINs (within the BINs for cell wall, RNA, lipid, and secondary and polyamine metabolism) were identified as differentially regulated after wounding (Table 2.1). Differentially regulated MapMan BINs/subBINs guide further dissection of the tomato response to wounding discussed below. Since only a small number of DEGs were statistically significant at 1 hr systemically and since 83% of the 8-hr systemic and local DEGs were the same (Figure 2.1), detailed gene expression analysis was focused on the local wound response.

Regulation of Stress-Responsive Genes After Wounding

Consistent with previous studies, up-regulation of wound- and stress-responsive genes was a substantive component of the response to leaf injury (7% of total DEGs) (Tables 2.1-2; Cheong et al. 2002; Major and Constabel 2006; Reymond et al. 2000; Strassner et al. 2002). Approximately 14% of the stress-related genes (BIN 20) on the array were differentially regulated after wounding. Of the 61 DEGs in this class, only 11 genes were down-regulated (Table 2.2). A majority of the down-regulated genes code for proteins associated with abiotic stress responses and have proposed functions in the regulation of water balance (putative major intrinsic proteins including aquaporins and tonoplast intrinsic proteins), protein folding (homologues to *HSP83*, *HSP80*, *HSP81-1*, *DnaJ*), and tolerance to desiccation (a dehydrin and *RD22*) (Table 2.2). Down regulation of water stress-responsive genes was surprising given the proposed role for dehydration in the wound response. The breaches in tissue and cellular integrity caused by injury can lead to local dehydration at the site of wounding (Bostock and Stermer 1989). In response, plants increase levels of the water-stress hormone abscisic acid (ABA), which is essential for a robust wound response (Chao et al. 1999; Peña-Cortés et al. 1996; Peña-Cortés et al. 1989). In addition, transcript regulation in response to mechanical wounding and dehydration overlaps substantially in Arabidopsis (Reymond et al. 2000). Therefore the decline of water stress-responsive gene RNAs at 8 hr may reflect the restoration of cellular homeostasis at the RNA level; this does not exclude further regulation of water-stress response proteins at other levels of gene expression.

The majority of DEGs that encode stress-related proteins were up-regulated and both spatial and temporal regulation was observed (Table 2.2). Fourteen of the 1-hr up-regulated DEGs were transiently induced (1-hr only). DEGs with this pattern of expression included genes associated with defense against pathogens and pests. Several of these genes were associated with defense signal transduction including three WRKY proteins (homologues of AtWRKY33, AtWRKY40, and AtWRKY75), putative leucine rich-repeat (LRR) receptor-like kinase, a whitefly-induced NAPDH oxidase (gp91-phox), and a Hin-1 like protein that has been associated with PAMP signaling

(POTHR-1) (Eulgem and Somssich 2007; Keller et al. 1998; Zhen-Dong et al. 2003). Other genes solely expressed at the 1-hr time point included a universal stress-related protein, stress-activated protein kinase and two *late embryogenesis-abundant* (*LEA*) genes (*ER5* and *Dhn2*; Table 2.2). The up-regulation of ethylene-responsive *ER5* was consistent with other studies that show that *ER5* is rapidly and transiently induced by ethylene, drought, ABA, and wounding in tomato leaves (Zegzouti et al. 1997).

Another set of stress-induced RNAs that accumulated early (1 hr) persisted until 8 hr after wounding. Several of these genes encoded proteins associated with defense against microbial pathogens including three PR proteins (PR-1c, PR-6, PR-10), three chitinases, and a fungal-specific endoglucanase inhibitor (Table 2.2). Chitinases break down fungal cell walls, lowering fungal fitness and generating chitin-derived elicitors to promote pathogen defense (Grover 2012). The endoglucanase inhibitor counteracts pathogen xyloglucanases that degrade hemicelluloses in plant cell walls (Misas-Villamil and van der Hoorn 2008). Therefore, the increased levels of *Chitinases*, *PR* proteins and *Xyloglucan Endoglucanase Inhibitor* RNAs may reduce the ability of pathogens to establish at the sites of injury (Miedes and Lorences 2007; van Loon et al. 2006).

Several exopeptidases (LAP-A, Ser carboxypeptidase) and a cathepsin B-like Cys proteinase were also expressed at both 1 and 8 hr after wounding. The role of cathepsin B in wounding is not well understood. Cathepsin B is a member of the Cys proteinase superfamily. Several cysteine proteases (Mir1 of maize and papain in the latex of papaya) strongly impede lepidopteran larval growth (Konno et al. 2004; Li et al. 2009; Pechan et al. 2000); this is well correlated with insects expressing Cys-protease inhibitors as a counter-measure. It is also known that plant cathepsin B has an important role in modulating the hypersensitive response to pathogens (Gilroy et al. 2007), but its role in hindering insect growth has not been established. Interestingly, insect cathepsin B-like proteases are known to have an important role in insect development and some are midgut localized (Thie and Houseman 1990). Finally, in the set of DEGs expressed at both 1 and 8 hr, there were a number of jasmonate-induced proteins (JIPs)

known to be induced after injury and herbivory including polygalacturonase, polyphenol oxidases, and LAP-A.

A substantive number of stress-related genes (29 DEGs) were only detected at 8 hr after injury (Table 2.2). The 8-hr induced DEGs encoded additional JIPs, such as polyphenol oxidase B, PinI, PinII, Cys protease inhibitors (Capthesin D inhibitor and Cystatin), and Polygalacturonase Inhibitor (PGIP). PGIPs are primarily known for their ability to inactivate pathogen-derived cell wall-degrading enzymes (Cantu et al. 2008); although, some plant PGIPs are also induced in response to wounding and insect feeding and inhibit polygalacturonases from heteropteran species (D'Ovidio et al. 2004; Reymond et al. 2000). Other well-studied JIPs such as threonine deaminase and arginase, with known anti-nutritive effects, were not present on the array (Chen et al. 2004; Samach et al. 1991).

Surprisingly transcripts for several well-characterized genes encoding “early” wound-inducible response genes (*Lipoxygenase A* (*LoxA*), *Phospholipase A1*, and *Prosystemin*) were only detected at 8 hr after injury (Ryan 2000). Although the rapid increase in tomato’s phospholipase A activity after wounding is well established (Narvaez-Vasquez et al. 1999), this array provides the first report of its RNA increasing after injury in tomato. This is consistent with changes in Arabidopsis *Phospholipase A* RNAs, which accumulate after wounding and in response to several abiotic stresses (Rietz et al. 2004). Previous studies have shown that *Prosystemin* and *LoxA* RNAs are detected in healthy leaves, increase as early as 0.5 hr after wounding, and peak levels are attained by 6-8 hr (McGurl et al. 1992; Ryan 2000). The inability to detect these RNAs at 1 hr using the arrays may be due to the relative insensitivity of CSH cDNA microarray analysis, which might not detect the subtle increases of the early wound-response gene transcripts that were readily detected by more sensitive techniques such as RNA blot analysis (Bar-Or et al. 2007a; Bar-Or et al. 2007b). Alternatively, these differences could be due to differences in the wounding methods or tomato genotypes used in these studies.

Regulation of Photosynthesis and Reactive Oxygen Species Metabolism

Although some exceptions are known (Kerchev et al. 2012), the global down-regulation of photosynthesis gene expression appears to be a response that has been subject to evolutionary selection, since it occurs in many plant-attacker interactions (Bilgin et al. 2010; Kempema et al. 2007; Kerchev et al. 2012; Little et al. 2007; Schroder et al. 2005; Velikova et al. 2010). Consistent with these findings, the most dramatic change in tomato gene expression after wounding was the down regulation of the nuclear genes encoding components of the photosynthetic machinery. Genes encoding proteins for tetrapyrrole metabolism (BIN 19) and photosynthetic complexes (BIN 1), including PSI, PSII, light harvesting complexes and the oxygen evolving complex, and carbon fixation (BIN 1), accounted for approximately 10-12% of the DEGs at 1 and 8 hr after wounding (Table 2.1; Table 2.4). While the impact was most dramatic in the injured leaves, the decline in some BIN 1 and BIN 19 RNAs was also observed systemically.

Consistent with a down-regulation of nuclear-encoded photosynthetic genes was the down regulation of *Sigma factor 1* (*SIG1*) (BIN 27.2) (Table 2.5); SIG1 interacts with the plastid-encoded RNA polymerase (Lysenko 2007). Although many responses to light quality and intensity are regulated post-transcriptionally, *SIG1* is light regulated and modulates plastid gene transcription to facilitate a rapid adjustment of photosystem stoichiometry and activity to compensate for changes in light intensity (Lerbs-Mache 2011). Therefore, the decline in *SIG1* RNAs may result is substantive changes in the plastid-encoded photosynthetic RNAs and proteins. Consistent with a role for *SIG1* in translational control of photosynthesis, 6 of 50 (12%) chloroplast ribosomal protein transcripts on the array (BIN 29.2.1) were down-regulated in damaged leaves by 8 hr; this contrasts to the minimal impact of wounding on the transcripts encoding cytosolic ribosomal proteins (3 of 213; 1.4%; BIN 29.2.2; Table 2.5). *SIG2* regulates transcription of tRNA genes that control chlorophyll and photosynthetic/photosynthesis-related protein accumulation; however, *SIG2* RNA levels were not influenced by wounding and other *SIG* genes were not represented on the array. The down-regulation of transcripts impacting plastid-

targeted functions described above was consistent with previous studies that showed cytosolic transcripts encoding plastid-imported proteins declined preferentially in response to biotic stress (Bilgin et al. 2010).

Plastid-specific gene regulation was also seen in the reactive oxygen species (ROS) metabolism genes. In this current study of tomato wounding, 13% of the ROS metabolism genes (BIN21) on the array were differentially regulated after wounding (Table 2.6). Previous studies have shown that H_2O_2 and other ROS are signals essential for wound-induced gene regulation in tomato (Orozco-Cárdenas et al. 2001). However, ROS are toxic at higher concentrations and therefore must quickly be metabolized. Surprisingly, almost half of DEGs encoding ROS catabolism proteins were down-regulated after injury suggesting there is a complex regulation of ROS metabolism during the tomato wound response, perhaps involving temporal and/or spatial regulation of different ROS species. Interestingly, three of the four down-regulated ROS metabolism genes were localized to the plastid, while none of the up-regulated ROS genes were plastid-localized. Again, this is consistent with previous studies, which showed that the localization rather than function, determined regulation of ROS metabolism genes after stress (Bilgin et al. 2010). The reduced levels of ROS catabolism RNAs may reflect the need for plastid-generated ROS, which can serve as an anti-microbial agent or a retrograde signal to enhance or modify wound responses (Bonaventure and Baldwin 2010; Fernández and Strand 2008; Maruta et al. 2012).

Regulate the Regulators: RNA and Protein Metabolism

A large portion of spots on the array represented genes involved in modulating gene expression at a variety of levels from transcriptional to post-translational regulation [BIN 27 (RNA; 1000 spots) and BIN29 (Protein; 1453 spots), respectively]. Accordingly, a substantive number of DEGs were involved in regulation of gene expression. Approximately 14% of the DEGs at 1 and 8 hr after wounding were involved in RNA metabolism (BIN 27), which includes transcription factors and their accessory proteins, as well as RNA-binding proteins (Table 2.1; Table 2.5). Forty seven of the 801 putative transcription factors on the array (4.8%) were differentially regulated after

wounding (Table 2.5). Only eight of these DEGs were regulated at both time points, suggesting that early and late responses were distinct. Twenty six transcriptional regulators were identified as DEGs at 1 hr, including three WRKYs, which modulate myriad defense responses (Table 2.2; Table 2.5; Eulgem and Somssich 2007). By 8 hr, twenty-seven transcription regulators were detected as DEGs, including two *AP2* transcription factor genes, a *TGA2* transcription factor gene, and two *AUX/IAA* genes; these proteins have been implicated in hormone signaling and response (discussed below; Chen et al. 2009; Dargeviciute et al. 1998; Nakano et al. 2006).

Like RNA biogenesis, a substantive number of genes (56 genes) involved with protein metabolism (BIN 29: protein synthesis, modification, degradation, or sorting) were regulated after injury. In particular several ribosomal protein RNAs were down-regulated (BIN 29.2), possibly negatively impacting overall protein synthesis. The wound response also impacted genes important in protein folding (BIN 29.6), as well as proteins involved with post-translational modifications of proteins. For example, nine kinases and phosphatases that could impact the phosphorylation status of proteins were DEGs and may regulate transcriptional cascades, ER to nucleus signaling (*IRE1-like* gene), or protein turnover (BIN 29.4; Koizumi et al. 2001; Mithoe and Menke 2011).

The majority of protein metabolism DEGs were involved in protein degradation (BIN 29.5); these 28 DEGs were almost exclusively up-regulated and doubled in number by 8 hr (Table 2.2, Table 2.5). The protein degradation DEGs included *Polyubiquitin*, a putative *F-box family member*, *Cysteine Proteinases*, as well as a putative *Aspartyl Protease* and *Serine Carboxypeptidase* genes. Proteases may have multiple roles in the wound response. These enzymes may function in general protein turnover and amino acid recycling, which can be particularly important in wounded cells where proteins are particularly vulnerable to damage and cellular and tissue healing is going on (Schaller 2004). However, some proteases have anti-nutritive properties and therefore play an important direct role in insect defense as mentioned earlier (Schaller 2004; van der Hoorn and Jones 2004).

Strengthening Cell Walls

The composition of cell walls is dynamic and cell wall strengthening is a common response to both biotic and abiotic stresses (Sasidharan et al. 2011). These fortified cell walls provide a physical barrier against opportunistic pathogens that attempt to invade wounded cells (Bostock and Stermer 1989; Kahl 1982). Therefore, it is not surprising to see that at both 1 and 8 hr after wounding approximately 12% of the DEGs were involved in cell wall (BIN 10) or secondary metabolism (BIN 16) (Table 2.6). One major class of secondary metabolites are phenylpropanoids, which strengthen the cell wall as cell-wall bound phenolics, lignins, suberin, and cuticle-associated phenolics (Bernards and Båstrup-Spohr 2008). In addition, phenylpropanoids can be oxidized by wound-induced PPOs to form toxic quinones and thereby have anti-feedant and toxic effects on insects (Felton et al. 1989).

Many enzymes involved in phenolic biosynthesis are controlled at several levels, including at the transcript level (Bernards and Båstrup-Spohr 2008). In the current study in tomato, genes encoding enzymes for general phenylpropanoid biosynthesis (BIN 16) were up-regulated, such as *Cinnamic acid 4-Hydroxylase* (*C4H*) and *4-Coumarate:coenzyme A Ligase* (*C4L*) (Figure 2.2; Table 2.6). Some phenylpropanoids are channeled to synthesis of monolignols or flavonoids, which are precursors for lignin and the anti-oxidant anthocyanins, respectively (Bernards and Båstrup-Spohr 2008). In response to wounding in tomato, several genes involved with monolignol biosynthesis were up regulated, while the rate-limiting flavonoid *Chalcone Synthase* (*CHS*) gene was down regulated (Table 2.6, Figure 2.2). Moreover, there was a down-regulation of cell wall-degrading and -remodeling enzyme gene RNAs (BIN 10) at both 1 hr and 8 hr after wounding (Table 2.6); this included genes such as *Xyloglucan Endotransglucosylase-hydrolases*, *β-D-Xylosidases*, and *Pectin Lyases* (Itai et al. 2003; Oh et al. 1998). Collectively the DEGs in the cell wall and phenylpropanoid/flavanoid BINs predict that after wounding in tomato, there is increased lignification and fortification of the cell wall at the expense of cell wall flexibility and expansion and production of anti-oxidant flavonoids, consistent with previous studies (Brisson et al. 1994; Sasidharan et al. 2011).

Lipid and Jasmonate Metabolism and Signaling

Lipid metabolism genes were also significantly regulated after wounding (BIN 11). Of importance was the differential regulation of *Fatty Acid Desaturase* (*FAD*) genes (BIN 11.2). FADs catalyze the formation of double bonds in the lipid tails of fatty acids. In Arabidopsis FAD5 and FAD6 are plastid localized and provide precursors for defense-related oxylipins and JA (Kachroo and Kachroo 2009). FAD5 is involved with the synthesis a 16:3 fatty acid (a precursor of dinor-OPDA) that increases after wounding (Weber et al. 1997). FAD6 works with FAD7/8 in the conversion of the 18:1 and 18:2 fatty acids to the 18:3 fatty acid linolenic acid, which is the precursor of OPDA and JA (Kachroo and Kachroo 2009). The tomato *FAD7* mutant *spr2* has no detectable 16:3 fatty acids and has reduced levels of 18:3 oxylipins (Li et al. 2003) suggesting that the role of FAD5 in 16:3 biosynthesis may be more minor in this species.

The tomato and Arabidopsis *FAD7* genes are stress induced (Li et al. 2003; Reymond et al. 2000); however *FAD7* was not represented on the array. To date the roles of the tomato *FAD5* and *FAD6* in defense have not been studied. Here we show that *FAD5* and *FAD6* RNA levels decreased 8 hr after wounding of tomato leaves, which is inconsistent with the need for oxylipin precursors but consistent with down-regulation of genes encoding plastid-localized proteins, (Table 2.6). If this translates to FAD activity, this may be important mechanism for restoring JA and other lipid levels to non-stress levels.

FAD2 is located in the ER and involved in defense-independent lipid metabolism. Two *FAD2* genes were differentially regulated after wounding (Table 2.6). *FAD2.1-like* RNAs increased early after wounding, while *FAD2.2-like* was down-regulated 8 hr after wounding. *FAD2.1-like* RNAs also increase in response to *L. decemlineata* regurgitant in potato (Lawrence et al. 2008). Although FAD2 is not involved in JA biosynthesis and does not respond to wounding in Arabidopsis, *AtFAD2* RNAs are regulated in response JA treatments and whitefly feeding (Jung et al. 2007; Kempema et al. 2007; Reymond et al. 2000).

Several other genes involved in JA biosynthesis were represented on the array and regulated after injury including lipoxygenase (*Lox*) genes. Following the release of linolenic acid from plastid

membranes by phospholipases, molecular oxygen is attached to linolenic by either 13-Lipoxygenase (LOX-C, LOX-D) or 9-LOX (LOX-A, LOX-B) (Feussner and Wasternack 2002). 13-LOX-derived lipids are precursors for JA (Kachroo and Kachroo 2009) and while the role for 9-LOX-derived oxylipins is less well-characterized, they have also been implicated in defense (Leon-Morcillo et al. 2012; Lopez et al. 2011). Both *LoxA* and *LoxD* genes are induced by MeJA, pathogen or wounding (Beaudoin and Rothstein 1997; Fowler et al. 2009; Li et al. 2004; Zhao et al. 2003). While *LoxA* RNAs increased after 8 hr of injury, changes in *LoxD* transcripts were not detected at any time (Table 2.2). This is consistent with the tomato *LoxA* encoding a more abundant transcript than *LoxD* in response to MeJA (Li et al. 2004). Therefore, it is possible that the CSH array did not allow the detection of the tomato *LoxD* RNAs. Other wound-regulated, JA-biosynthesis genes including *LoxB* and *LoxC* and those responsible for conversion of 13(S)-hydroperoxy linolenic acid to OPDA [*Allene Oxide Synthase* (*AOS*), *Allene Oxide Cyclase* (*AOC*) and *Acyl-CoA Oxidase* (*ACX1A*)] were not represented on the array (Li et al. 2004; Strassner et al. 2002; Wasternack et al. 2006). Finally, the tomato genome has three *12-OPDA Reductase* genes (*OPR1-3*) and only *OPR3* is wound-induced in tomato (Strassner et al. 2002). *OPR3* was not present on the array and neither *OPR1* nor *OPR2* transcript levels changed in response to injury consistent with Strassner et al. (2002).

cDNAs for the wound-induced *JAR1* that forms the bioactive JA-Ile was not present on the array (Suza et al. 2010); but cDNAs for *JA Methyl Transferase* (*JMT*) were present. JMT produces the volatile MeJA, which can prime or trigger JA-mediated responses in neighboring plants (Tamogami et al. 2008). While J*MT* RNAs were elevated in 1-hr local and systemic leaves, this regulation was not at a significant level (BIN 17.7; STMCL31).

The linolenic acid is also used to produce green leaf volatiles (GLV), which have both direct and indirect roles in plant-herbivore interactions (Dudareva et al. 2006) and divinyl ether fatty acids, which have been implicated in pathogen defense (Fammartino et al. 2007; Prost et al. 2005; Weber et al. 1999). In tomato GLV biosynthesis involves the activities of the rate-limiting hydroperoxide lyase (HPL) and alcohol dehydrogenase (ADH2), as well as isomerization factors

and acylases (Hatanaka et al. 1987; Matsui 2006; Strommer 2011). While the isomerization factors and acylases were not on the array, *HPL* (STMCD19, BIN 20)*, ADH2* (STMDZ95, BIN 5) and *Divinyl Ether Synthase* (*DES;* STMHH09, BIN 17.7) were and their RNAs were not injury induced. These data are consistent with previous studies in Solanaceous species, where *HPL* and *DES* transcripts do not increase in response to mechanical wounding or increased at later times after insect feeding (Halitschke et al. 2001; Howe et al. 2000; Strassner et al. 2002). This contrasts with *HPL* regulation in Arabidopsis*,* where *HPL* transcripts and activity increase after wounding and insect feeding (Matsui 2006; Reymond et al. 2000). While *ADH2* transcripts have been shown to be regulated in response to stress in tomato fruit (Bai et al. 2011), *ADH2* has not been characterized as wound- or stress-induced in tomato leaves.

Defense Modulators ET and SA

Ethylene (ET) has an integral role in defense signaling and its exact role in defense is species dependent (Adie et al. 2007; Broekaert et al. 2006; Erb et al. 2012). In Arabidopsis, ET acts as a positive regulator of JA-regulated defense responses to pathogens and a negative regulator of JA-dependent wound and insect feeding responses (Adie et al. 2007). In tomato, ET treatments regulate the levels of many defense-induced *PR* genes but do not induce nor suppress the canonical wound-response gene *PinII* (Chao et al. 1999; Van Kan et al. 1995; Van Kan et al. 1992). However, studies with the ET-perception mutant *Neverripe* (*NR*) and pharmacological studies show that ET is essential for a robust wound-response in tomato (O'Donnell 1996).

The Bilgin et al (2010) meta-analysis showed that biotic stress most often led to a decline in ET biosynthesis gene transcripts and increases in ET-response gene RNAs. Down-regulation of ET biosynthesis or signaling genes is proposed as part of a negative feedback loop after stress (von Dahl et al. 2007). Somewhat surprisingly, of the eight ET biosynthesis genes and 22 ET-response genes on the array, only four were DEGs and all were up-regulated. This include genes encoded Aminocyclopropane-1-Carboxylate Oxidase (ACO) and ER5 (BIN 17.5, Table 2.7; Figure 2.3), as well as the ET-response transcription factors similar to ABR1 and RAP2.7 (Table

2.6; Nakano et al. 2006). These data suggested that a few ET-biosynthesis and -response genes were modulated after injury; this contrast with Arabidopsis, where *ACO* RNAs increase in response to wounding and insect feeding (Reymond et al. 2000).

Another key hormone involved in the cross-talk of defense networks is salicylic acid (SA). Plants synthesize SA via one of two pathways (the isochorismate and phenylalanine ammonia-lyase pathways) using chorismate as a building block (Figure 2.2; Dempsey et al. 2011). In Arabidopsis, *Nicotiana benthamiana*, and tomato, stress-induced SA is primarily synthesized via the IC pathway within the plastid (Catinot et al. 2008; Dempsey et al. 2011; Sticher et al. 1997; Uppalapati et al. 2007; Wildermuth et al. 2001). After synthesis, SA can be methylated or conjugated to glucose or amino acids producing volatile (MeSA) or non-volatile storage forms of SA used to sequester SA and reduce its toxic effects (Dempsey et al. 2011). Release of SA from these storage forms is a controlled process and can activate defense signaling (Hennig et al. 1993).

While over a dozen genes have been reported to be involved with SA biosynthesis or modification (Dempsey et al. 2011), there was a paucity of SA biosynthesis/modification genes on the 10-K array (BIN 17.8). Notably, the key gene in stress-responsive SA synthesis, *ICS* was not present. *PAL* was present on the array but was not induced in response to wounding (Figure 2.2); this is consistent with small increases in *PAL* RNAs in response to MeJA treatments (Puthoff et al. 2010). An SA methylesterase-like protein and a SA glucosyltransferase gene were the only other SA metabolism genes on the array and neither of these RNAs changed in response to injury. With such a scarcity of genes representing SA metabolism on the array, it was hard to determine if SA metabolism was regulated at the transcript level after injury.

Finally, many of the key genes involved with SA perception (*NPR3, NPR4*) and signaling transduction (*NPR1*) were not present on the potato array. A NPR1-interactor protein gene *TGA2* was present on the array (Fan and Dong 2002) and its RNAs increased locally 8 hr after wounding (Table 2.7). TGA2 has a well-established role in controlling SA-mediated defenses and innate immunity in tomato (Chen et al. 2009). It is presumed that activation of TGA2-mediated

defenses helps to protect the cells at the wound portal from opportunistic pathogens (Table 2.2) (Cheong et al. 2002; Durrant et al. 2000; Reymond et al. 2000; Walley et al. 2007).

Isoprenoid Hormones in Wound Response

The isoprenoid pathway is responsible for the synthesis a large and functionally and structurally diverse set of metabolites (Vranova et al. 2012). The isoprenoid pathway begins with the synthesis of the precursor isopentenyl diphosphate (IPP). IPP can be synthesized via the cytoplasmic mevalonate (MVA) pathway, which provides IPP for the synthesis of cytoplasmic or mitochondrial isoprenoids that include sesquiterpenes and sterols, such as brassinosteroids (BRs). Alternatively, IPP can be synthesized via the plastid-localized 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway; here IPP serves as a precursor for monoterpenes, antioxidant carotenoids, and photosynthetic metabolites, as well as ABA, cytokinins (CK), and gibberellic acid (GA).

3-hydroxy-3- methylglutaryl-coenzyme A reductase (HMGR) and 1-deoxy-D-xylulose 5-phosphate synthase (DXS2) are the rate limiting enzymes for MVA and MEP pathways, respectively, and these RNAs increase after MeJA treatments or wounding in potato and herbivory in tomato (Choi et al. 1994; Korth and Dixon 1997; Sanchez-Hernandez et al. 2006). Based on the 10-K array data neither *HMGR* nor *DXS2*, nor four MVA-pathway genes (*Mevalonate Kinase, Phosphomevalonate Kinase, Mevalonate Disphosphate Decarboxylase, Acetoacetyl-Coenzyme A Thiolase*), nor the MEP-pathway gene (*LYTB*) were induced by wounding (BIN16). In addition, of the seven genes involved in sesquiterpenoid biosynthesis in the cytosol and monoterpene biosynthesis in the plastid that were on the array, and only the *Sesquiterpene Synthase 1* was a DEG. Its RNAs declined after injury (Table 2.6). Lack of transcript regulation of the core isoprenoid pathway genes and terpenoid biosynthesis genes was surprising since levels both monoterpenes and sesquiterpenes increase after wounding/herbivory in tomato (Kang et al. 2010; Sanchez-Hernandez et al. 2006). These data suggest that regulation at other levels of gene expression occurs (Vranova et al. 2012) or the CSH array was not able to detect these changes.

Two genes involved in long chain isoprenoid synthesis were DEGs. The plastid-localized *IPP Isomerase* (*IPI2*) RNAs increased 8 hr after wounding (Table 2.6; Sun et al. 2010). IPIs regulate the interconversion of IPP and dimethylallyl diphosphate (DMAPP) (Vranova et al. 2012), which is the base molecule for longer chain isoprenoids, as well as isoprene and CKs. In contrast, the cytosolic *Farnesyl Pyrophosphate Synthase* RNAs declined 1 hr after wounding (Table 2.6; Gaffe et al. 2000).

Three phytohormones ABA, GA, and CK are isoprenoid derived and initiate their biosynthesis within the plastid; all three molecules influence wound signaling in Solanaceous plants (Erb et al. 2012; Peña-Cortés et al. 1996; Peña-Cortés et al. 1989; Peña-Cortés et al. 1991). Like ethylene, the ability to perceive CK allows plants to produce both SA and JA (Seo et al. 1997). Therefore, it was somewhat surprising to see that of the 14 genes involved in CK biosynthesis or signaling/response on the array (BIN 17.4), none were DEGs. This contrasts with observations for genes in the plastidial ABA and GA pathways and cytosolic sterols and BR pathways (see below).

Within the plastid, isoprenoid-derived carotenoids are used to synthesize ABA (Nambara and Marion-Poll 2005). ABA levels increase after injury and are essential for a robust wound response in tomato (Peña-Cortés et al. 1996; Peña-Cortés et al. 1989; Peña-Cortés et al. 1991). Surprisingly RNAs for two carotenoid biosynthesis genes (*Phytoene Synthase 1* and *β-Carotene Hydroxylase*) decreased after wounding (BIN17.1, Table 2.6). These data suggested that injured tomato plants may have a limited ability produce these important antioxidants that provide a resistance to high light, high temperature, and lipid peroxidation (Jaleel et al. 2009); as noted earlier, a similar trend was observed with the plastid-localized enzyme genes that are important in catabolizing ROS.

Only two genes (*NCED1* and *NCED4)* dedicated to ABA biosynthesis were present on the array (BIN17.1). These genes encode plastid-localized 9-cis-epoxy-carotenoid dioxygenase (NCED) proteins, which is the rate limiting enzyme for ABA biosynthesis (Thompson et al. 2000). Although the *NCED4*-like RNAs did not change, increases in *NCED1* RNAs were detected in

damaged leaves by 1 hr (Table 2.7) and this is well correlated with increases in ABA levels after wounding (Herde et al. 1999; Thompson et al. 2004).

The phytohormone GA is also derived from plastid isoprenoids. In tomato, GA antagonizes wound signaling (Peña-Cortés et al. 1991). The array contained 17 genes that were involved with GA biosynthesis or GA signaling/ responses (BIN 17.6). Only two of these genes were DEGs. The *Ent-Kaeurenoic Acid Oxidase* (*KAO*) RNAs increased locally (1 and 8 hr) and systemically (8 hr). KAO synthesizes GA_{12} that is then acted upon by other oxidases to form various GAs, including the bioactive forms (GA_1, GA_3, and GA_4; Yamaguchi 2008). While regulation of GA synthesis occurs at the transcript level, *GA oxidase* rather than *KAO* transcripts are typically regulated (Yamaguchi 2008); however, there was no evidence for changes in the tomato *GA oxidase* RNAs after injury (BIN 17.6).

The second GA-associated DEG was *GAST1* (*GA-Regulated Transcript 1*). *GAST1* transcription is induced by GA and suppressed by ABA in tomato leaves (Shi and Olszewski 1998). In the present analysis, *GAST1* RNA levels declined 1 hr and 8 hr after leaf injury implying low levels of GA and elevated levels of ABA at these times. The function for the tomato GAST1 is not established, but a GAST1 homolog in Arabidopsis has a role in redox and light signaling (Rubinovich and Weiss 2010). Surprisingly, *GAST1* RNAs are up-regulated in response to herbivory in potato and tomato (Kant et al. 2004; Lawrence et al. 2008) suggesting that herbivore elicitors may influence *GAST1* gene expression and the complexities of GA's relationship with wound signaling in tomato have yet to be revealed.

Finally, BR is derived from cytosolic isoprenoids and is a defense regulator in tomato; BR negatively regulates JA-defense responses in tomato (Campos et al. 2009). Squalene is the precursor of all cyclic triterpenoids including: sterols, BR, and non-steroidal triterpenoids (Vranova et al. 2012). Seven genes associated with sterol and BR biosynthesis were present on the array (Bin 17.3); five were DEGs (Table 2.6). cDNAs for squalene synthase, an activity that increases in response to wounding in potato, was not present on the array (Zook and Kuc 1991). The first committed step for production of sterols and BR from squalene is catalyzed by squalene

monooxygenase/epoxidase; this gene was down-regulated 8 hr after wounding (Table 2.7). In contrast, RNAs for a gene encoding a rating-limiting enzyme, C-8,7 sterol isomerase, and genes encoding proteins important for BR biosynthesis [DWARF1, DWARF1-like, steroid 5-α-reductase (DET2)] increased at 1 hr or 8 hr after injury (Table 2.7). Up-regulation of *DET2* at the RNA level suggests that there are low BR levels after wounding; this is based on the fact that *DET2* and other BR-biosynthesis genes are negatively regulated by BR levels in Arabidopsis (Tanaka et al. 2005). While these data suggest that injury could influence both steroid and perhaps preferentially impact BR levels, it should be noted that none of the 11 BR-response or -signaling genes on the array were DEGs (BIN 17.3).

Amino Acid-Derived Signaling Molecules in Defense (IAA, Polyamines, GABA)

MapMan identified the amino acid metabolism BIN (BIN13) as differentially regulated after wounding (Table 2.1, Table 2.6). BIN13 includes several genes involved in Glu and Ala metabolism. Genes encoding alanine aminotransferase (Glu $\leftrightarrow$ Ala) and alanine glyoxylate transaminase (Ala $\leftrightarrow$ Gly) were down-regulated DEGs (Table 2.6). In contrast, critical genes associated with Trp and Glu metabolism (*Anthranilate Synthase*, *Acetylornithine Transaminase, Gaba Transaminase*) and synthesis of S-adenosyl methionine from Met (*S-Adenosylmethionine Synthase*) were up-regulated DEGs (Table 2.6). Changes in amino acid synthesis can influence the pool of amino acids available for protein synthesis and synthesis auxin, polyamines and γ-aminobutyrate (GABA), which are known to contribute to plant defense.

In tomato, Trp is a precursor to auxin and indole alkaloids (Maeda and Dudareva 2012; Schneider et al. 1972). The conversion of chorismate to anthranilate by anthranilate synthase (AS) is the first committed step to Trp biosynthesis. *AS1* RNAs increased at 8 hr after injury (Table 2.6). While the array did not provide evidence for changes in the levels of other auxin biosynthetic gene RNAs (BIN 17.2), five indole acetic acid (IAA) metabolic genes were DEGs (Table 4). IAA is stored as amino acid conjugates, which are formed by IAA-amido synthetases (IAS), and conjugates are hydrolyzed by IAA-amino acid hydrolases (Ludwig-Muller 2011).

Although *IAS* genes are often up-regulated in response to IAA and stress (Bari and Jones 2009; Kumar et al. 2012), there was no evidence for increases in the five tomato *IAS* transcripts (Figure 2.2). In contrast, three *IAA-Amino Acid Hydrolase* gene RNAs accumulated in damaged leaves at 1 hr (Table 2.7). This is in marked contrast to 12-hr MeJA treatments, which do not induce *IAA-Amino Acid Hydrolase* RNAs (Uppalapati et al. 2005). Finally, studies in rice leaves and tomato roots suggest that IAA can be a positive regulator of JA signaling and IAA-amido conjugate accumulation leads to a reduced JA response (Bari and Jones 2009; Ding et al. 2008; Taylor et al. 1993). Therefore, IAA-amino acid hydrolases may free active IAA to enhance JA signaling. Consistent with this theory, two negative regulators of auxin signaling (*AUX/IAA* genes*: IAA2.3-like* and *IAA28-like*) were down-regulated late after wounding (Table 2.7; Dargeviciute et al. 1998).

One of the most prominent changes in secondary metabolism gene expression after injury was seen in the polyamine biosynthesis genes (BIN 22; Table 2.6). Polyamines are small aliphatic compounds with roles in development, senescence and abiotic/ biotic stress responses in plants (Kusano et al. 2008). The array contained 21 genes involved with polyamine biosynthesis and >50% of these genes were up-regulated after wounding (Figure 2.3). Both Met and Arg are key substrates for polyamine biosynthesis (Figure 2.3). In fact, some of the most strongly up-regulated genes after wounding were those involved in S-adenosylmethionine (SAM) decarboxylation (BIN 22.1.2; Figure 2.3; Table 2.7). SAM is synthesized from Met by SAM synthetase and can be converted to ET or decarboxylated to SAMDC, which is used as a substrate for polyamine biosynthesis (Kusano et al. 2008). While there is little evidence for the regulation of ET biosynthesis genes after injury (see above), the polyamine biosynthesis gene RNAs encoding arginine decarboxylase and spermidine synthase were detected 1 hr after wounding and the *SAMDC* and *Orthinine Decarboxylase* RNAs that increased by 8 hr after injury (Table 2.7; Figure 2.3). Both SAMDC activity and transcript levels have been correlated with polyamine levels (Cheng et al. 2009; Groppa and Benavides 2008; Kusano et al. 2008; Mehta et

al. 2002). Thus, based on transcript levels, polyamine biosynthesis appears to be favored over ethylene biosynthesis after wounding in tomato.

The 10-K array data is consistent previous studies that show that polyamine biosynthesis and phenylpropanoid pathway gene RNAs increase after MeJA treatments and *Pseudomonas syringae* pv. *tomato* DC3000 infection (Chen et al. 2006; Uppalapati et al. 2008). Polyamines generate H_2O_2, which strengthens cell walls and serves as a mobile defense signaling molecule, and toxic phenylpropanoid-polyamine conjugates (PPC; Cona et al. 2006; Edreva et al. 2007). PPCs slow insect growth, have a direct antimicrobial role or an indirect impact due to their ability to strengthen the plant cell wall (Edreva et al. 2007; Kaur et al. 2010).

The Glu and the polyamine pathways also feed into the biosynthesis of GABA (Figure 2.3) (Bown et al. 2006; Huang et al. 2011). GABA antagonizes insect growth and increases resistance to herbivory in several plant species. GABA levels rapidly increase in response to mechanical wounding, insect footsteps and herbivore feeding (Bown et al. 2002; Ramputh and Bown 1996; Steinbrenner et al. 2011; Wallace et al. 1984). Increases in GABA is mediated by elevated glutamate decarboxylase (GAD) activity in response to damage-induced pH changes and Ca^{2+} influx (Bown et al. 2006). Consistent with these findings, the 10-K array analyses indicated that the tomato *GAD* RNAs did not increase in response to mechanical wounding (BIN 13). However, RNAs encoding GABA transaminase, which catabolizes GABA, increased at 1 and 8 hr after wounding (Figure 2.3; Table 2.6). These data are consistent with a rapid (1-5 min) post-transcriptional up-regulation of GABA biosynthesis and a catabolism that is mediated by wound-induction of *GABA-T*.

LapA-SI has Delayed Responses After Wounding

LAP-A is critical for mounting an effective defense against chewing insects (Fowler et al. 2009; Hartl et al. 2008). By monitoring the temporal and spatial accumulation of early and late wound-response RNAs and using JA-complementation studies, it was shown that LAP-A controls the abundance and persistence of late wound-response RNAs, such as *PinI*, *PinII*, and *PPO-F* (Fowler et al. 2009). To broaden our understanding of the scope of LAP-A's impact on tomato's

gene expression programs, the local and systemic injury responses in WT and *LapA-SI* plants were compared at 0, 1, and 8 hr after wounding using the potato cDNA arrays. RNA levels relative to the WT 0-hr control (FC) were determined and statistical analysis was performed as before.

The local and systemic gene expression trends at 1 hr and 8 hr after injury in WT versus *LapA-SI* leaves are displayed in Figure 2.4. Overall gene expression was correlated between the genotypes in all samples. The strongest correlation between WT and *LapA-SI* DEGs was in systemic leaves at 8-hr after wounding (R^2= 0.8016). Only a few genes were differentially regulated ($p<0.05$, $|FC| \geq 0.8$) in the *LapA-SI* line after wounding relative to WT plants (genotype DEGS or gDEGs). As expected, *Lap* transcripts were absent in the *LapA-SI* line (Figure 2.4; open triangles). Surprisingly, none of the previously characterized wound-responsive genes (*PinI, PinII* and *PPO*) that were strongly *LapA* dependent in previous studies were statistically different in the *LapA-SI* lines at 1 and 8 hr after wounding. This is likely due to the fact that LAP-A had largest impacts at 12 to 24 hr after wounding (Fowler et al. 2009).

Fourteen other genes were identified as gDEGs at 1 and/or 8 hr after wounding (open circles; Figure 2.4; Table 2.8). Two genes impacting ion transport (a vacuolar proton pump and chloride channel protein) were suppressed 1 hr after injury in *LapA-SI* leaves relative to WT leaves. By 8 hr, a new set of gDEGs were expressed locally and systemically. Many of the gDEGs encoded proteins involved in stress (BIN 20) and/or protein metabolism (BIN 29) including HSP80, a DnaJ-like protein, ClpP, and cathepsin B. While both up and down-regulated gDEGs were identified, seven of the ten gDEGs identified in injured leaves at 8-hr were up-regulated in *LapA-SI* relative to WT. This indicated that LAP-A's impact on responses to injury is broader than previously recognized; our previous study solely examined a subset of early and late wound-response genes and LAP-A enhanced the accumulation of the late wound-response gene RNAs (Fowler et al. 2009).

While a small number of gDEGs were detected after injury of leaves, the overall responsiveness of the *LapA-SI* lines to wounding was delayed. For example, the number of

DEGs (FC| ≥ 0.8) identified in *LapA-SI* leaves 1 hr after wounding (149 DEGs) was less than half of those in the WT plants (329 DEGs) (Table 2.1). In addition, there was a trend of lower expression of stress-related DEGs (BIN20) in the *LapA-SI* lines compared to WT (Table 2.2); this was most striking at 1 hr.

Therefore, to identify the genes that were differentially regulated in the *LapA-SI* plants at this early time point, the top 100 genes with the largest fold differences (|FC|=0.72-1.66) between *LapA-SI* and WT at 1 hr in damaged leaves were compared (Table 2.9). A majority of these genes were not identified as significantly different due to high variation associated with cross array comparisons. However, this analysis showed a strong trend. A majority of these RNAs (78%) were at lower levels in the *LapA-SI* relative to WT leaves. Interestingly, when the top 30 most suppressed genes were viewed, 12 of the cDNAs encoded proteins involved in stress responses (BIN 20), while no other BIN, other than BIN35 (Not Assigned), had more than three genes in the top 30. No enrichment of gene classes were seen in the 22 genes that showed an up-regulation trend in *LapA-SI* plants (Table 2.9).

By 8 hr after wounding, the number of local and systemic DEGs in *LapA-SI* vs WT plants was similar (Table 2.1). However, inspection of the top 100 most differentially expressed genes on the array at 8 hr after injury (|FC|=0.72-3.93) in the *LapA-SI* vs WT plants indicated that the majority of genes (74%) had lower RNA levels in *LapA-SI* than WT (Table 2.10). Different BINs were preferentially represented in the top 30 suppressed and induced genes in *LapA-SI* plants at 8-hr than at 1 hr. Only six of the top 30 suppressed genes were stress-related (BIN 20). In contrast, ten of the 26 genes that were up-regulated were in this BIN 20. These genes encoded proteases (ClpP, cathepsin B), abiotic stress-response proteins (HSP90, TAS14) and PR proteins (PR-1b, class II chitinase, and glucan endo-1,3-β-glucosidase). Again no other BINs had more than 2 genes in the top 30 most suppressed or up-regulated genes in *LapA-SI* at 8 hr. While subject to high variability, together these trends suggests that LAP-A modulation of stress responses is more complex and occurs earlier after injury than previously known.

LAP-A Impacts Gene Expression of PR-1 and Late Wound-Induced Dehydrins

While a small number of DEGs were identified in the *LapA-SI* line compared to WT after mechanical wounding (Table 2.8), there was a set of 49 genes that were predicted to be differentially regulated by LAP-A before injury (0-hr gDEGs) (Figure 2.5; Table 2.11). All but three of these putative 0-hr gDEGs were up-regulated in the *LapA-SI* line. In addition, the microarray indicated that these putative 0-hr gDEGs were largely down-regulated in the *LapA-SI* line 1 hr and 8 hr after wounding (Figure 2.6; Table 2.11).

To assess the expression programs of the putative 0-hr gDEGs, tomato homologs of seven potato ESTs were identified (see *Experimental Procedures*). They encoded three transcription factors (BEL-1-related protein, WRKY-like protein, MYBR29-like protein), a putative gibberellin receptor (GID1), a basic PR-1, subunit D of the vacuolar ATPase, and TAS14 (a LEA protein). In the case of the basic *PR-1, GID1,* and *BEL-1*, several tomato genes had high nucleotide sequence identity to the potato EST and therefore the array hybridization signals might reflect the expression of one or more of these genes. For this reason, multiple genes were screened for their expression after wounding (Table 2.12). In contrast, single tomato genes had compelling identities with the *SlWRKY42* and *MYBR29-like* potato ESTs, despite the fact that these transcription factors are members of large gene families (Table 2.12; Coker et al. 2003; Feller et al. 2011; Huang et al. 2012).

The levels of these candidate gDEG RNAs were determined at 0, 1 and 8 hr after wounding in WT, *LapA-SI* and *LapA-OX* leaves by RT-PCR or qPCR. Unfortunately, none of the tomato genes studied were verified as differentially expressed in *LapA-SI* plants at 0 hr. However, these studies did show that these genes were grouped into three expression categories and some displayed *LapA* genotype-dependent expression. First, *SlWRKY42, MYBR29-like, Vaculolar ATPase, BEL1-like,* and *BEL1-like3* RNAs were not abundant, did not respond significantly to wounding, and were not different between the genotypes at 0, 1 or 8 hr (data not shown). This may be due to the fact that the genes were not highly expressed and sensitive to biological variation.

Second, of the five putative tomato *GID* family members that were identified, three of these genes (*GID1, GID-like2, GID-like3*) had a strong nucleotide identity with the potato EST (Table 2.12). While none of the *GID* or *GID-like* genes were a 0-hr gDEG, the tomato *GID1* RNA was wound induced (1 hr) as predicted by the microarray (Table 2.11; data not shown). Due to the low levels of *GID* and *GID-like* RNAs in both control and wounded leaves, these samples were sensitive to biological variation making it difficult to identify the differentially regulated tomato *GID* homolog at 0 hr.

Finally, genotype-dependent expression patterns were observed when the tomato homologs to the potato basic *PR-1* and *TAS14* genes were studied (Figure 2.7). In tomato, the *PR-1* family includes five genes encoding basic PR-1 proteins (*PR-1a, PR-1a1, PR-1a2, PR-1b,* and *PR-1c*; see Table 2.12). *PR-1c*, *PR-1a1*, and *PR-1a2* were the most closely related to the basic *PR-1* EST on the potato array (Table 2.12; Niderman et al. 1995; Tornero et al. 1997). *PR-1b* is the best characterized and is highly induced in response SA, ET and MeJA (Chao et al. 1999; Tornero et al. 1994; Tornero et al. 1997). While *PR-1a* and *PR-1a2* are closely related to *PR-1b*, neither is regulated by ET or SA. Less is known about *PR-1c* and the related *PR-1a1*, which are more diverged from *PR-1b*. *PR-1a1* encodes a low abundance RNA that is not responsive to ET or SA (Tornero et al. 1997) and *PR-1c* is induced in response to *Phytophthora infestans* and, like PR-1a and PR-1b, has anti-fungal activity (Niderman et al. 1995).

RT-PCR studies confirmed that *PR-1a1* RNAs were at very low levels in tomato leaves prior to and after wounding. Therefore, the *PR-1a1* gene expression pattern did not match the changes in RNAs detected by the potato *PR-1* EST; for this reason *PR-1a1* was not studied further (Table 2.12; data not shown). qPCR analysis was performed for *PR-1c* and *PR-1a2*. *PR-1a2* was not induced in response to wounding in WT plants (Figure 2.7). However, its RNAs significantly increased at 1 and 8 hr after wounding and declined to control levels by 24 hr in both *LapA-SI* and *LapA-OX* plants. This genotype dependent pattern of *PR-1a2* RNA accumulation was surprising, since late-wound response genes show reciprocal responses in *LapA-SI* and *LapA-OX* lines (Fowler et al. 2009).

Temporal differences in gene expression were noted for *PR-1c* in WT, *LapA-OX* and *LapA-SI* plants. In WT leaves, the tomato *PR-1c* transcripts increased slightly in response to wounding by 8 hr and were 100-fold more abundant at 24 hr (Figure 2.7). In the *LapA-OX* line, *PR-1c* RNAs accumulated more rapidly than in the WT leaves; based on 24-hr *PR-1c* RNA levels, these RNAs either declined more rapidly or did not reach the levels observed in WT leaves. In contrast, in *LapA-SI* leaves *PR-1c* RNAs appeared to accumulate more slowly than in WT plants. These RNAs decreased at 1 hr after injury and then increased at 8 and 24 hr. Unlike late-wound response genes and similar to *PR-1a2,* reciprocal trends in RNA patterns were not seen in the *LapA-SI* and *LapA-OX* lines. The reasons for this unanticipated pattern of *PR-1c* and *PR-1a1* gene expression could have one of two explanations. First, since both *LapA* and *LapN* are silenced in the *LapA-SI* lines and only LAP-A is over-expressed in the *LapA-OX* lines, the non-wound induced, rare class *Lap-N* may contribute to these RNA patterns. Alternatively, differences between the genotypes may suggest multiple or more complex roles for LAP-A in the defense signaling network that controls *PR-1a2* and *PR-1c* expression after wounding. Further work needs to be done to identify defense cues that regulate *PR-1A2* and *PR-1c* in order to understand how LAP-A may be modulating their expression.

TAS14 (le4) is a well-characterized dehydrin from tomato that is regulated by water deficit, salinity, ABA, and mannitol (Birkenmeier and Ryan 1998; Chao et al. 1999; Godoy et al. 1990). TAS14 is also a late embryogenesis abundant (LEA) protein. *TAS14* RNA levels in WT, *LapA-SI* and *LapA-OX* lines before and after wounding were measured by qPCR. The microarray data predicted *TAS14* to have elevated transcript levels by 8 hr after wounding and be a 0-hr gDEG (Table 2.11). Although *TAS14* was not verified as a 0-hr gDEG, it displayed a genotype-dependent pattern of RNA accumulation (Figure 2.7). In WT plants, *TAS14* RNAs peaked 8 hr after wounding in tomato and declined to 0-hr levels by 24 hr (Figure 2.7); this is consistent with previous findings (Birkenmeier and Ryan 1998; Chao et al. 1999; Godoy et al. 1990). In *LapA-SI* plants, *TAS14* RNAs accumulated more rapidly in the *LapA-SI* line compared to WT; reciprocally, *TAS14* transcript accumulation was delayed in the *LapA-OX* line relative to *LapA-SI* plants. These

data are consistent with the microarray analysis and indicated that *LapA-SI* line was primed to express *TAS14*.

Because *TAS14* was differentially regulated in the *LapA-SI* line, additional tomato dehydrins (*Dhn2, Dhn3, Dhn4*) and LEA (*ER5, ER5-like*) genes were identified and their wound and LAP-A regulation determined (Table 2.12; Figure 2.7; *Experimental Procedures*). *TAS14* and *ER5* are currently the only characterized LEA genes in tomato (Cohen et al. 1991; Godoy et al. 1990; Zegzouti et al. 1997). Like *TAS14*, a potato *Dhn2* (C17), *ER5*, and *LEA-like* cDNAs were on the array (Table 2.2).

RT-PCR showed that *Dhn4* RNAs were not detected in healthy or wounded tomato leaves (Table 2.12, data not shown). Consistent with the microarray data (Table 2.2), qPCR analysis showed that *Dhn2* and *ER5* RNAs were most abundant 1 hr after wounding (Figure 2.7). However, there was no significant difference in RNA levels between the genotypes. The tomato LEA-like gene did not accumulate in response to wounding and there were no differences in transcript levels in the different genotypes (data not shown). However, the potato LEA-like EST on the array had similarity to the highly abundant ER5 (85% identity) and therefore cross reaction was likely.

Unlike *Dhn2* and *ER5*, *Dhn3* transcripts peaked at 24 hr after wounding in WT (Figure 2.7). Moreover, *Dhn3* accumulated significantly faster in the *LapA-SI* line, while *Dhn3* accumulated to lower levels in the *LapA-OX* line. This is consistent with LAP-A's regulation of *TAS14*, which also accumulated later after wounding. Taken together, LAP-A appears to negatively regulate Dhns, which are part of the late-wound response in tomato and not the LEAs that are induced early, consistent with LAP-A's regulation of other late wound-response genes previously identified (Fowler et al. 2009).

Plant Dhns are a distinct subgroup of LEAs that are characterized by the presence of amphiphilic K-segments (Allagulova et al. 2003). While the exact mechanism of action remains elusive, due to their protective role in osmotic stress and their amphiphilic K-segments, Dhns are proposed to bind to denaturing protein substrates as molecular chaperones. In addition, LAP-A

and LAP-N also have a secondary function as potent molecular chaperones *in vitro* (Scranton et al. 2012). It is therefore interesting that Dhns appear to be up-regulated in the *LapA-SI* line compared to the other late-wound response genes, which are repressed. Together this suggests that *TAS14* and *Dhn3*, as well as other potential chaperones, may be up-regulated to compensate in the *LapA-SI* line. These data support the hypothesis that LAPs may also function as a molecular chaperone *in vivo*.

CONCLUSION

In this study, tomato RNAs were hybridized to a TIGR potato 10-K cDNA array in order to determine differential accumulation of tomato RNAs after wounding. While cross-species hybridization (CSH) has been used in many studies, it is still regarded as a non-standard and its results should be interpreted carefully (Bar-Or et al. 2007a). Many studies have shown that CSH can lead to lower spot quality and overall signal intensity correlated strongly with the degree of sequence divergence between the probe species and transcript species orthologs (Bar-Or et al. 2007a; Bar-Or et al. 2007b). This can be seen in species with even 1% sequence divergence and has been demonstrated CSH studies between potato and tomato which have ~8% sequence divergence (Bagnaresi et al. 2008; Bar-Or et al. 2006; Bar-Or et al. 2007b; Consortium 2012; Gilad et al. 2005). Lower signals lead to fewer DEGs being identified compared to other methods such as species-specific hybridization (SSH). Secondly, CSH may be more prone to cross-hybridization by closely related gene family members; especially when the number of family members has increased for the gene of interest. For example, when Bar-Or et al. queried the potato transcriptome against the Cornell-CGEP Tomato 13-K vTOM1, they found that ~16% of the potato ESTs were homologous to more than one tomato probe (Bar-Or et al. 2006). These two factors make identification of the true target transcripts difficult for CSH experiments even in species as similar as potato and tomato.

Despite these concerns, transcript responses to injury in tomato in this study strongly correlated with previous wound and JA treatment studies (Li et al. 2004; Strassner et al. 2002; Uppalapati et al. 2005). This is the first comprehensive look at transcript regulation early after

wounding in tomato. While some of the traditional early genes were not detected until 8 hr after injury, the vast majority of core JA-dependent wound responses were significantly up-regulated after wounding including lipid and JA metabolism genes as well as JIPs. While other studies have shown core JA signaling induced early after wounding, this study demonstrated that many other stress and metabolism pathways are also regulated early and enhanced late after wounding. This is consistent with Arabidopsis, which shows rapid and early induction of a wide array of genes after wounding (Cheong et al. 2002; Walley et al. 2007). This is also the first study to comprehensively compare local and systemic transcript regulation after wounding in tomato. Consistent with other studies, systemic responses are more delayed than local (Fowler et al. 2009; Strassner et al. 2002). However, by 8 hr, there is a large overlap between local and systemic responses, suggesting that complex metabolic responses beyond core defenses occurs even in unwounded tissue.

In agreement with other studies, the most dramatic response to wounding was a down-regulation of photosynthesis, not only seen in reduced photosynthesis transcripts, but also in the repression of plastid ribosomal proteins, as well as the plastid-localized sigma factor SIG1. Mechanical wounding is the source of other stresses beyond cell damage including local dehydration and susceptibility to opportunistic pathogens. Therefore, consistent with other studies, wounding in tomato induced a wide array of signaling pathways including those involved with desiccation and pathogen defense. Pathogen defense included direct defenses such as up-regulation of genes encoding transcription factors (WRKYs and TGA2, antimicrobial PR-1s, enzymes involved in cell wall strengthening through the up-regulation of lignins and polyamines, as well as the down-regulation of genes encoding plastid-localized ROS detoxifying enzymes, which may generate antimicrobial ROS. Finally, wounding in tomato affected the expression of genes involved in multiple hormone pathways. However, the number of genes affected in these pathways was relatively small. In the case of SA, this may be due to the small number of representative genes on the array. For other hormone pathways, the effect may be due in part to

the insensitive nature of the CSH array and the fact that mechanical wounding and insect feeding typically have a less dramatic effect on gene expression (Bilgin et al. 2010).

LAP-A has been shown to modulate the expression of late-wound JIPs in tomato. This study demonstrated that LAP-A's affects wound responses earlier than previously known, showing that the *LapA-SI* line wound responses were delayed after wounding primarily affecting genes related to stress. However, since both *LapA* and *LapN* are silenced in this line, the effect of silencing the constitutively expressed *LapN* cannot be ruled out. Therefore, new RNA interference (RNAi) lines are being generated to specifically target each Lap species. In addition, future studies will utilize RNA-Seq to directly identify LAP-A targets.

In addition, two new sets of LAP-A modulated genes were identified. While two *PR-1* genes (*PR-1c* and *PR-1a1*) were regulated by *LapA*, the relationship between the *PR-1c* and *PR-1a1* expression in the *LapA-SI* and *LapA-OX* line was not reciprocal. Many PR genes are responsive to changes in SA levels, however, to date, the regulators of *PR-1c* and *PR-1a1* have yet to be identified. Therefore, it is not clear how LAP-A may modulate these *PR-1* genes. In the case of the Dhns (TAS14 and Dhn3), LAP-A regulation was correlated in the *LapA-SI* and *LapA-OX* lines, showing that LAP-A negatively regulated late-wound response Dhns. This contrasts previous findings that LAP-A is only a positive modulator of the late branch of wound signaling (ie., PinI, PinII, and PPO). Collectively these data indicate that the role of LAP-A is broader than previously anticipated. Given the recent finding that LAPs are both aminopeptidases and molecular chaperones (Scranton et al. 2012), future research will focus on identifying whether LAP-A peptidase and/or chaperone activities mediate these critical roles in defense signaling.

EXPERIMENTAL PROCEDURES

Plant Materials and Growth Conditions

Solanum lycopersicum L. UC82 (wild-type, WT), *P35S:LapA-SI*, and *P35S:LapA-OX* were previously described . Plants were grown in a growth chamber with an 18-hr (28°C)/6-hr (24°C) light (300 µE)/dark cycle as described (Chao et al. 1999).

Wound Treatments

Three- to four-week-old plants were used in the wounding time-course studies. Plants were wounded by crushing the distal end of each leaflet of one lower leaf with a pair of needle-nosed pliers. Wounded leaves (local response) and all leaflets (typically 6-8 leaflets) from apical leaves (systemic response) were collected at designated times. The leaves of five plants at each time point were pooled together for RNA extractions. This experiment was repeated three times for microarray analysis. Experiments were repeated additional three times for real-time (RT)-PCR analysis.

RNA Isolation for Microarray and Real-Time PCR Analysis

RNAs were extracted using a hot phenol method as previously described (Pautot et al. 2001). RNA for microarray analysis was further purified using the SV Total RNA Isolation System (Promega, Madison, WI USA). RNA was quantified and 260/280-nm absorbance ratios were measured using a Nano-Drop ND-1000 spectrophotometer. RNA quality was also ensured by checking for the presence of intact rRNA bands by 1.5% formaldehyde gel.

Microarray Hybridizations, Scanning and Data Acquisition

RNAs were hybridized to TIGR potato 10,000-clone version 3 cDNA microarrays (http://www.tigr.org/tdb/potato/microarray_comp.shtml). All steps of microarray processing to obtain raw data (cDNA production, cDNA labeling, microarray hybridization, data quantification) were carried out by the TIGR Expression Profiling Service according to published methods (http://www.tigr.org/tdb/potato/microarray_SOPs.shtml). A reference design hybridization strategy was used with WT (0 hr) being the reference RNA: the reference RNA was a pool from five WT 0 hr RNAs. RNAs from wounded or unwounded leaves labeled with CY3 and the reference RNA labeled with CY5 were co-hybridized to the potato cDNA array. Three dye bias experiments were performed in which WT 0 hr RNAs were labeled with CY3 and co-hybridized with the CY5-labeled reference RNA (WT 0 hr RNAs from a different pool). After normalization, no dye bias was detected on these experiments (p-value<0.05).

Image and Data Analysis

Spot data was extracted using GENEPIX (ver. 5.0 Pro: Axon Instruments, Union City, CA, USA) at TIGR. Data output obtained from GENEPIX are publicly available and can be downloaded through a database maintained at the TIGR Web site (http://www.tigr.org/tigr-scripts/sgedb/studies_SGED.pl). Using the linear model for microarray data (LIMMA: http://bioinf.wehi.edu.au/limma/) package for the statistical software R (http://www.r-project.org), data sets were print-tip loess normalized within arrays with no background correction. Background correction added variation in a biased fashion to spike-in controls and was therefore not applied to the data. Arrays were normalized between arrays using quantile normalization as described in Bolstad et al (2003). Ratio-intensity plots (also known as MA plots) between arrays and between print tips, log-fold (M) bias within and between arrays, and the distribution and density of intensities values of raw and normalized data were all assessed as measures of slide quality and array variation.

Within-array duplicate spot correlations were calculated and duplicate spots were weighted using the duplicateCorrection function of the LIMMA package (Smyth et al. 2005). Linear models were implemented to the normalized data using mean expression of three biological replicates and within-array duplicate correlations. Moderated t-statistics were calculated using empirical Bayes analysis and used to obtain *p*-values (Smyth 2004). False discovery rate (FDR) -adjusted p-values were calculated in LIMMA according to Benjamini and Hochberg (1995) (Benjamini and Hochberg 1995). Significantly differentially regulated cDNA were defined as those with log2 fold changes ($|FC| \geq 0.8$ and $p < 0.05$.

Quantitative PCR Analysis

Selected potato cDNA sequences indicated as differentially expressed in microarray analysis were aligned to the Sol Genomics Network (SGN) Lycopersicon combined tomato Unigene database (01-24-10; http://solgenomics.net/) using the Basic Local Alignment Search Tool (BLASTN) (Altschul et al. 1990). EST clones of tomato genes with high sequence similarity to the potato ESTs (expectation (E) value < 1e-30) were obtained from Boyce Thompson Institutute

(BTI) and were confirmed by DNA sequencing at the University of California Riverside's Institute of Integrative Genome Biology Genomics Core.

Total RNA was DNase treated using RQ1 RNase-Free DNase (Promega, Madison, WI). RNase H^+ iScript reverse transcriptase (Bio-rad Laboratories, Hercules, CA) was used to perform reverse transcription (RT) according to the manufacturer's instructions. cDNAs were diluted 10 fold in water for qPCR analysis. All mRNA levels determined by qPCR analysis were normalized with the tomato translation EF1a, ubiquitin (Ubi3), and housekeeping gene 4 (HKG4; a hypothetical protein) as previously described (Fowler et al. 2009). Gene-specific primers were designed to amplify unique regions of the genes of interest compared to highly related gene family members based on the sequences obtained from EST clones. Primers were designed using Primer3 (Rozen and Skaletsky 2000) and annealing temperatures and efficiencies were determined experimentally. Primer sequences, annealing temperatures, and Unigene numbers used are listed in the Table 2.13.

For preliminary screening of 0-hr gDEGs, semi-quantitative RT-PCR was performed as described in (Zarate et al. 2007). Primers were optimized using EST clone templates and confirmed by amplifying tomato gDNA templates. cDNA Templates were amplified for 23-29 cycles and normalized to eIF4 (SGN-U581466) control (26 cycles). For more quantitative measurement, qPCR reactions were performed in triplicate using iQ SYBRGreen Super-mix (Bio-Rad Laboratories) and data was analyzed using the real-time PCR miner program (Zhao and Fernald 2005) according to Fowler et al. (2009). Averaged Ct values and averaged efficiencies of replicate samples were used to calculate mRNA levels of reference genes. Individual Ct values of replicate samples and the individual efficiencies of replicate reactions were used to calculate mRNA levels of wound-inducible genes. mRNA levels of each wound-inducible gene at each time were normalized against the geometric mean of the mRNA levels of the three reference genes for each time point.

MapMan Analysis

The normalized average mean ratios obtained from the microarray analysis were imported into MapMan Software (Zhao and Fernald 2005). Annotation and functional characterization was assigned using Stu_TIGR.m02 August07 (Rotter et al. 2007). Annotation for selected genes was confirmed by aligning the potato cDNA sequence against the TIGR tomato EST database (http://www.tigr.org/) using BLAST. MapMan was used to determine metabolic pathways that were the most differentially regulated as described in (Usadel et al. 2005). Differentially expressed genes were defined as those with $|FC| \geq 0.8$.

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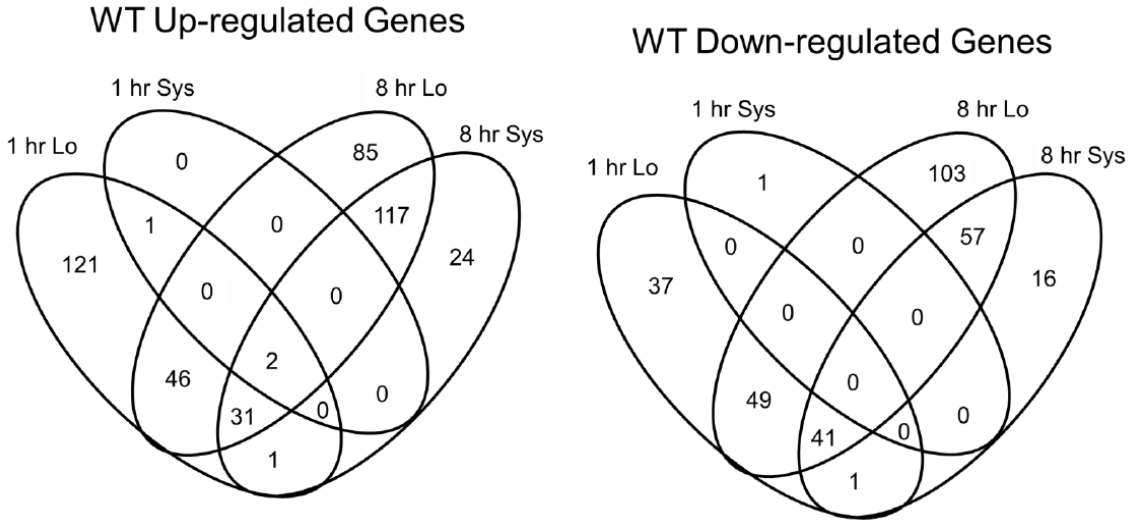

Figure 2.1 Gene expression patterns in WT plants at 1 and 8 hr after wounding. Genes that were differentially regulated in wounded (Local, Lo) and apical, non-wounded (Systemic, Sys) leaves at 1 and 8 hr after wounding were identified by analysis of the potato 10K cDNA arrays (*Materials and Methods*). Differentially expressed genes (DEGs) were defined as those with $p < 0.05$, $|FC| \geq 0.8$.

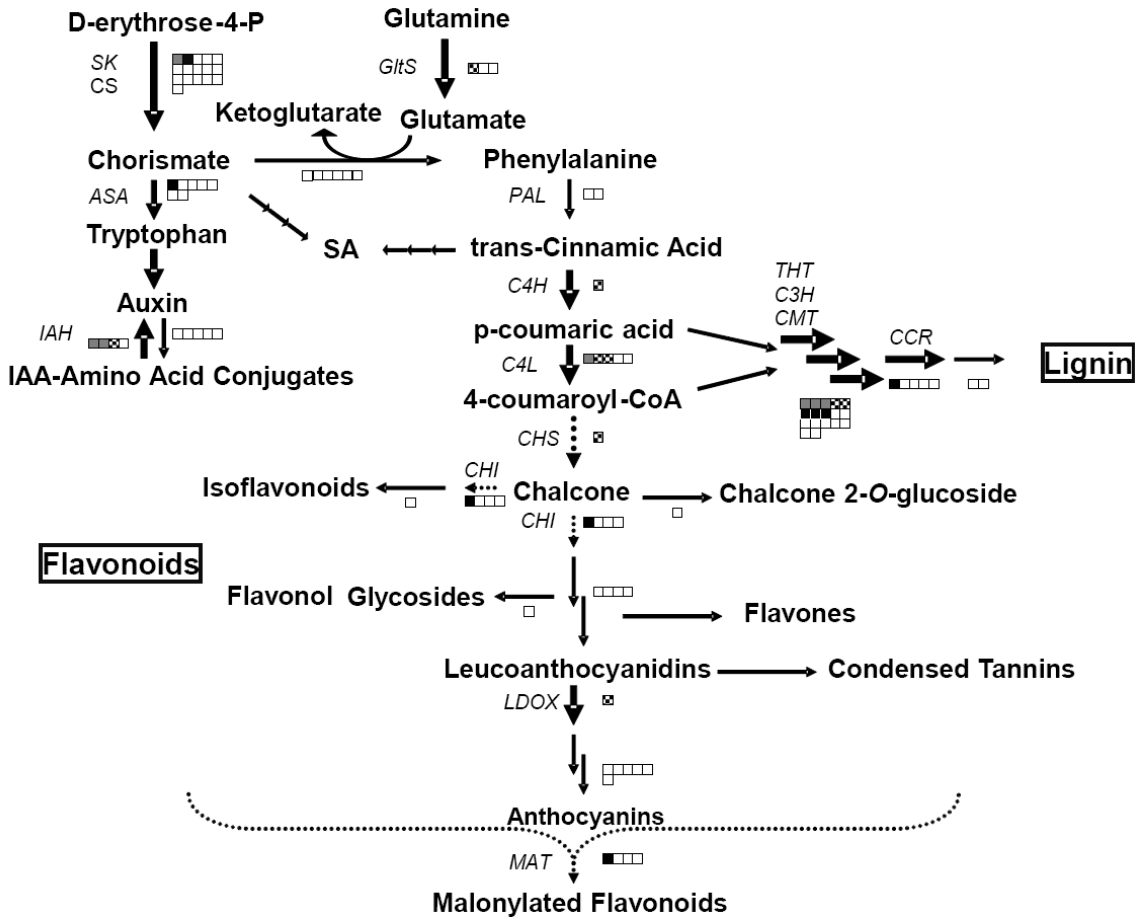

Figure 2.2 Changes in phenylpropanoid synthesis and catabolism gene RNAs after wounding. Many genes involved in phenylpropanoid metabolism were wound-regulated DEGs. For each biochemical step the number of genes regulated out of total number of clones representing those genes on the TIGR 10K (version 3) potato cDNA microarray is indicated (as a single or cluster of blocks). The colored blocks represent DEGs regulated at 1 hr (grey) and 8 hr (black) or both (checkered). Up-regulated DEGs are indicated by solid arrows and down-regulated DEGs are represented by dotted arrows. For some biochemical steps, enzymes were not represented on the array; no boxes appear at these steps. For complete pathway see Plant Metabolic Network (PMN; pmn.plantcyc.org). *PAL*- phenylalanine ammonia-lyase; *C4H*- cinnamic acid 4-hydroxylase; *C4L*- 4-coumarate--CoA ligase; *CCR*- cinnamoyl-CoA reductase; *CHI*- chalcone isomerase; *LDOX*- Leucoanthocyanidin dioxygenase; *CHS*- Chalcone synthase; *MAT*- Malonyltransferase; *ASA*- Anthranilate synthase alpha; *SK*- Shikimate kinase; *CS*- Chorismate synthase; *GltS*- Glutamate synthase; *THT*-Tyramine N-hydroxycinnamoyl transferase; *C3H*- P-coumaroyl shikimate 3'-hydroxylase; *CMT*- Caffeoyl-CoA O-methyltransferase.

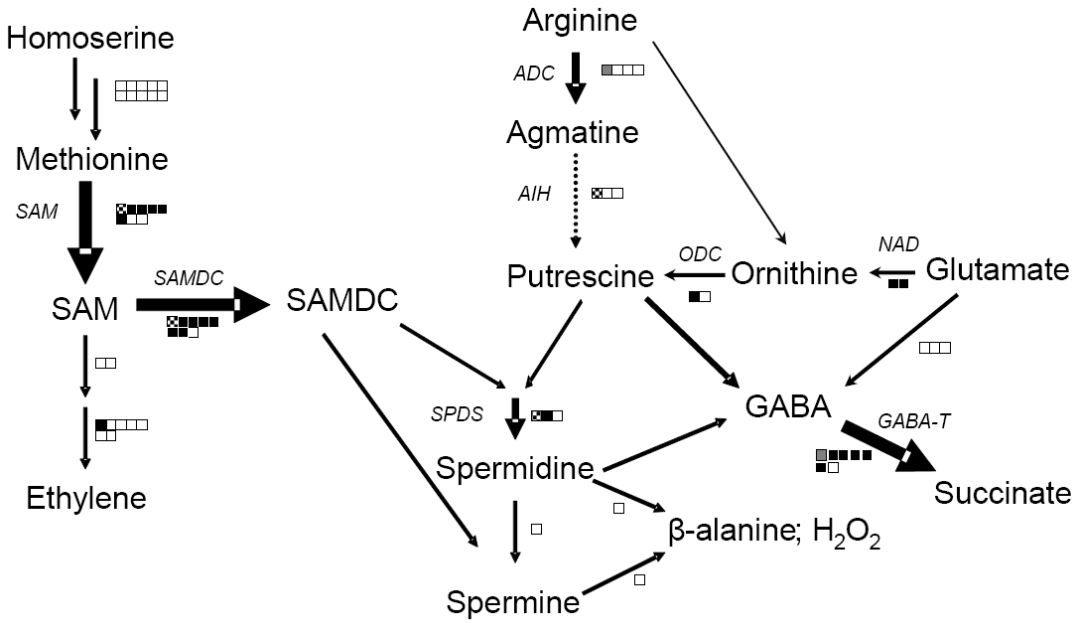

Figure 2.3 Changes in polyamine, SAM, and ethylene synthesis and catabolism gene RNAs after wounding. Many genes involved in polyamine, SAM, and ethylene metabolism had RNAs that differentially accumulated after wounding in tomato. DEGs are represented the same as in Figure 3. *SAM*- S-adenosylmethionine synthetase; *SAMDC*- S-adenosylmethionine decarboxylase; *SPDS*-Spermidine synthase; *ADC*- Arginine decarboxylase; *AIH*- Agmatine iminohydrolase; *ODC*- Ornithine decarboxylase; *NAD*- N-acetylornithine deacetylase; *GABA-T*- GABA Transaminase.

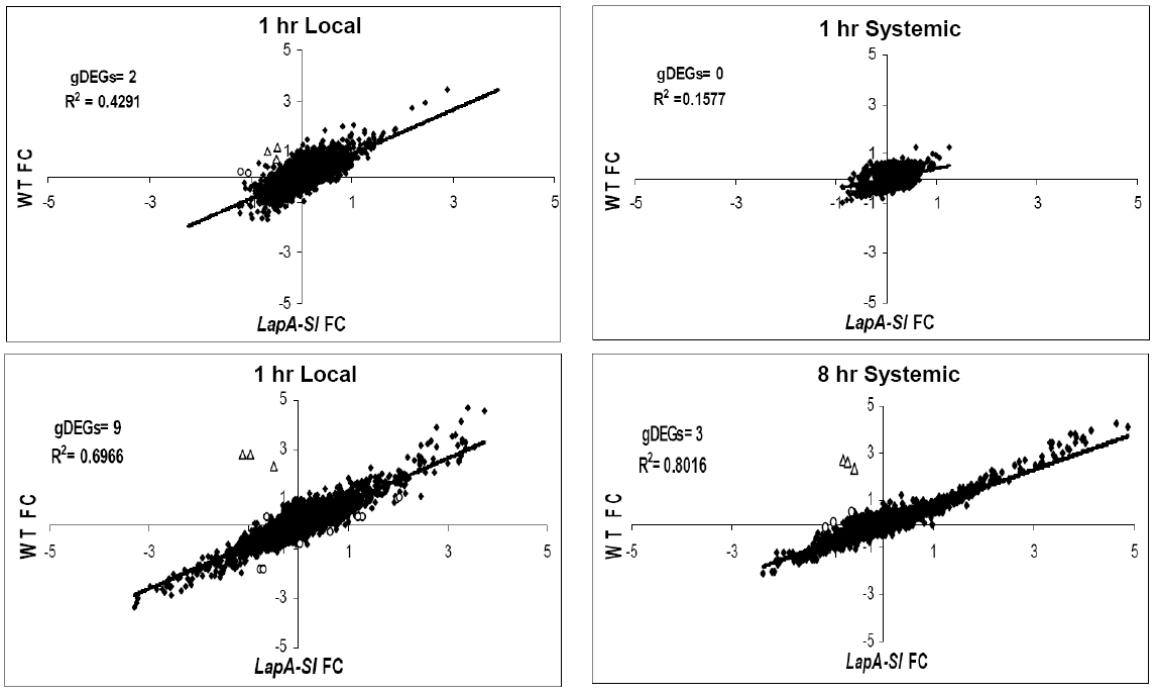

Figure 2.4 Relative RNA Fold Change in WT vs. *LapA-SI* lines 1 and 8h after wounding. Correlation of relative fold changes (FC) between WT and *LapA-SI* lines after wounding. RNAs that did not significantly differentially accumulate between the two genotypes are indicated by closed diamonds. A small number of genes differentially regulated between in *LapA-SI* compared to WT lines indicated by open circles (genotype DEGS (gDEGs); $p<0.05$, $|FC| \geq 0.8$]). *LapA* RNAs are indicated by open triangles.

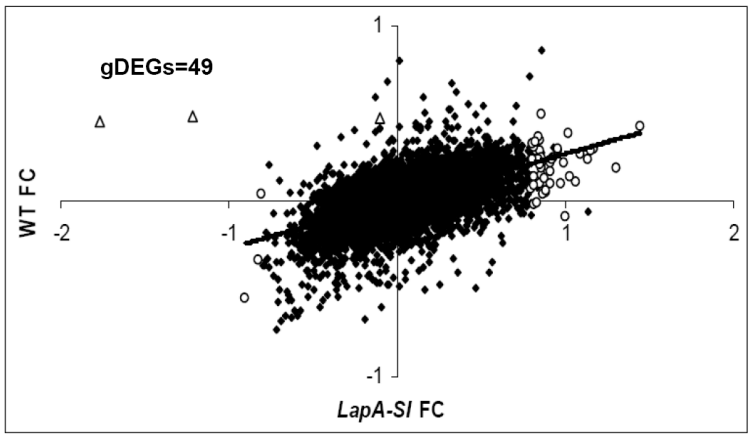

Figure 2.5 Subset of genes differentially regulated in *LapA-SI* before wounding. Most RNA levels in *LapA-SI* and WT lines before wounding did not significantly differ from WT 0 hr (closed diamonds, log2 fold change (FC) <1.5). While FC was low, there were 51 genes (g*DEG*s) whose RNAs accumulated to significantly different levels in *LapA-SI* and WT plants prior to wounding (open circles; $p<0.05$, $|FC| \geq 0.8$). Arrays confirmed that *LapA* was suppressed in *LapA-SI* lines (open triangles).

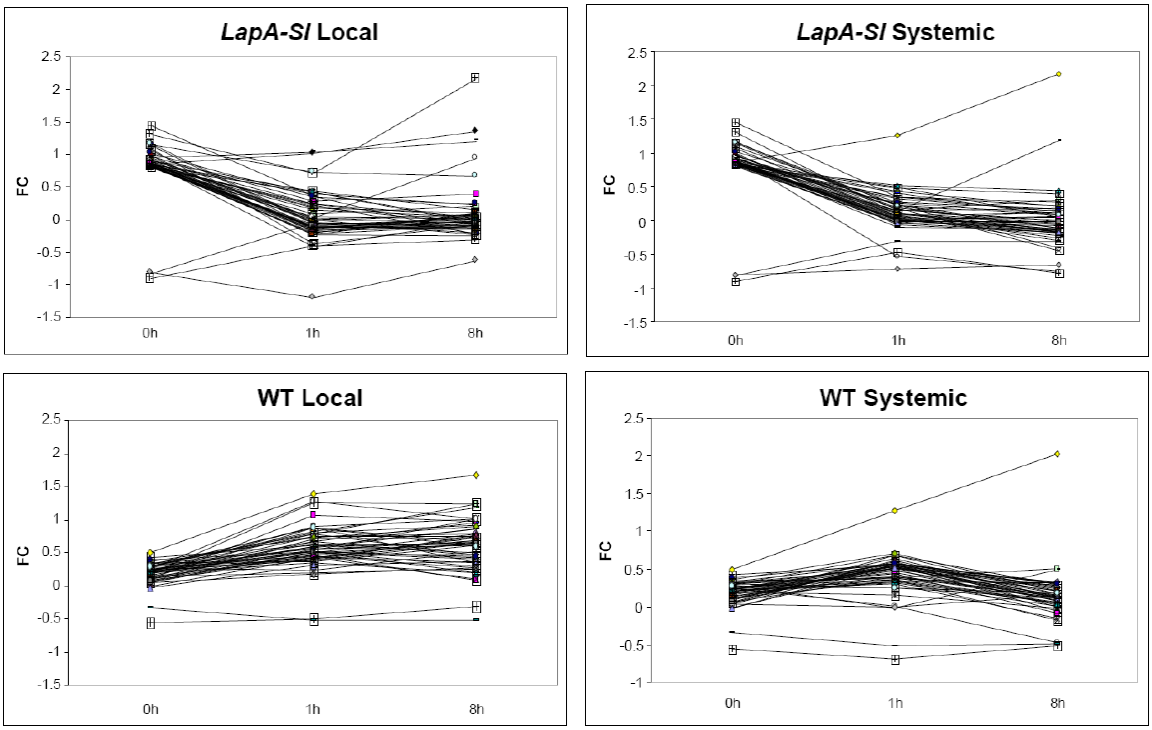

Figure 2.6 Local and systemic changes in g*DEG* RNAs after wounding. FC based on microarray analysis of *LapA-SI* 0h g*DEG* RNAs 0, 1 and 8h after wounding.

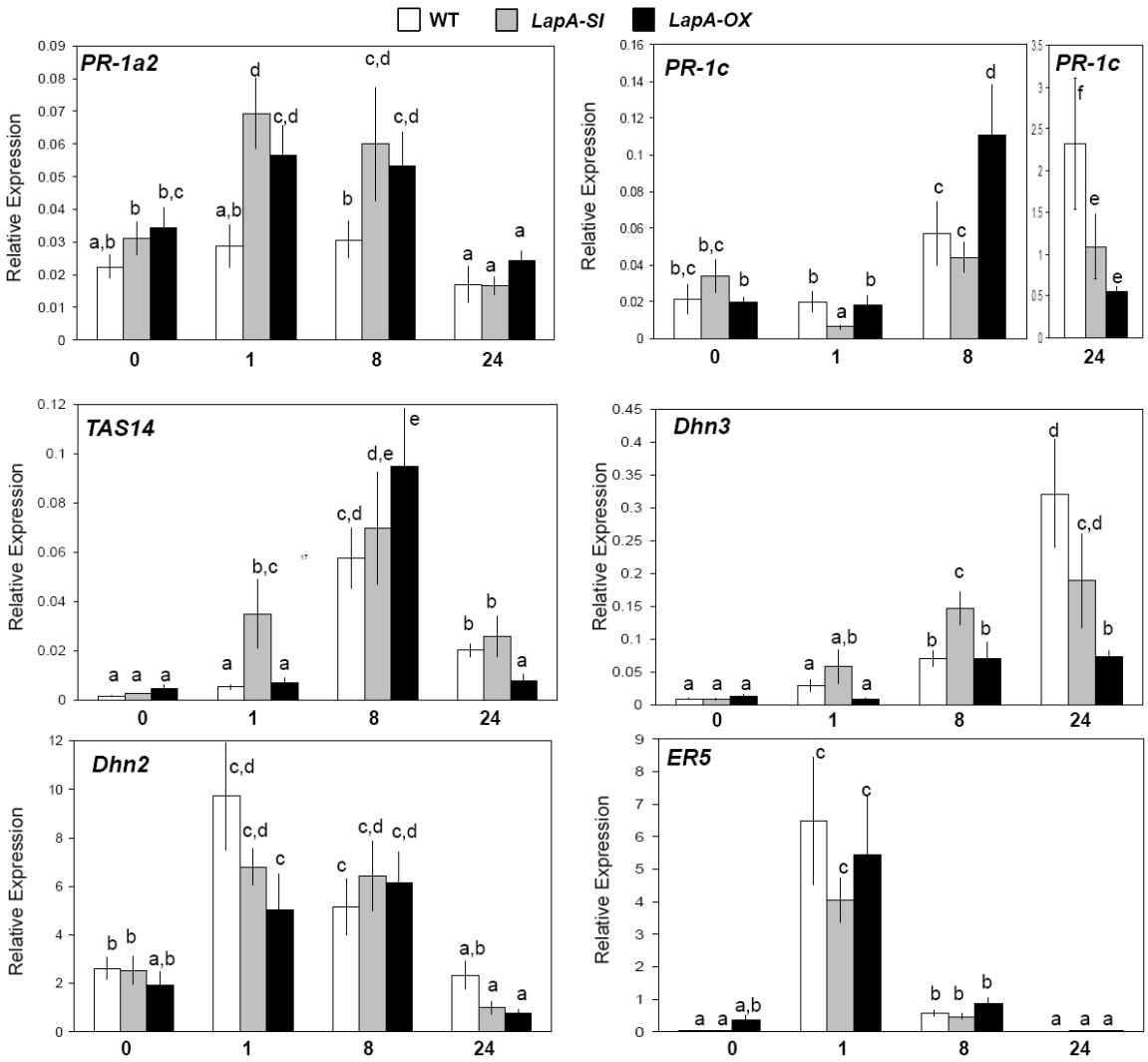

Figure 2.7 Quantitative RT-PCR analysis of selected mRNAs in leaves after wounding. Relative expression of LEA (*TAS14, Dhn3, Dhn2, ER5*) and *PR* (PR1a2, PR1c) transcripts were determined 0, 1, 8, and 24 hr after wounding in WT (white), *LapA-SI* (grey) and *LapA-OX* (black) (n=3). Significant differences between transcript accumulations was determined [ANOVA, Tukey post-hoc test ($p<0.05$)].

Table 3.1 MapMan BIN assignment of DEGs after wounding in WT and *LapA-SI* leaves.[A]

			WT					*LapA-SI*				
			0 hr	1 hr		8 hr		0 hr	1 hr		8 hr	
BIN	Name			L	S	L	S		L	S	L	S
1	Photosynthesis[C]	Up	0	1	0	0	0	0	0	0	1	1
		Down	0	23	0	51	25	0	7	0	59	26
2	Major CHO metabolism	Up	0	2	0	1	1	0	0	0	1	2
		Down	0	1	0	4	3	0	0	0	5	3
3	Minor CHO metabolism	Up	0	0	0	1	1	0	0	0	1	1
		Down	0	1	0	1	1	1	0	0	2	2
4	Glycolysis	Up	0	0	0	1	1	0	0	0	1	1
		Down	0	1	0	0	0	0	0	0	0	0
5	Fermentation	Up	0	0	0	2	0	0	0	0	1	0
		Down	0	1	0	0	0	0	0	0	0	0
6	Gluconeogenesis/ glyoxylate	Up	0	1	0	1	0	0	0	0	1	0
	cycle	Down	0	0	0	0	1	0	0	0	0	2
7	Oxidative pentose phosphate	Up	0	1	0	0	0	0	0	0	0	0
	pathway	Down	0	0	0	0	0	0	0	0	0	0
8	TCA / org. transformation	Up	0	1	0	0	0	0	0	0	0	2
		Down	0	0	0	2	1	0	0	0	3	2
9	Mitochondrial electron transport	Up	0	0	0	2	2	0	0	0	2	2
	/ ATP synthesis	Down	0	0	0	0	0	0	0	0	0	0
10	Cell wall[D]	Up	0	5	0	6	5	0	2	0	9	8
		Down	0	6	0	6	2	0	5	0	8	6
11	Lipid metabolism[D]	Up	0	9	0	10	4	2	5	0	7	7
		Down	0	0	0	8	6	0	0		10	8
12	N-metabolism	Up	0	0	0	1	0	0	0	0	1	0
		Down	0	0	0	0	0	0	0	0	0	0
13	Amino acid metabolism[C]	Up	0	11	0	12	14	2	7	0	13	17
		Down	0	2	0	2	0	0	0	0	1	4
14	S-assimilation	Up	0	0	0	0	0	0	0	0	0	0
		Down	0	0	0	0	0	0	0	0	0	0
15	Metal handling	Up	0	0	0	0	0	0	0	0	7	2
		Down	0	0	0	0	1	0	0	0	0	1
16	Secondary metabolism[D]	Up	0	13	0	20	17	0	11	1	18	22
		Down	0	6	0	10	3	0	0	0	7	6
17	Hormone metabolism	Up	0	7	0	13	9	1	7	0	12	13
		Down	0	3	0	6	2	0	1	0	5	4
18	Co-factor and vitamine	Up	0	0	0	1	1	0	0	0	1	1
	metabolism	Down	0	0	0	0	0	0	0	0	0	0
19	Tetrapyrrole synthesis[C]	Up	0	0	0	0	0	0	0	0	0	0
		Down		11	0	11	8	0	4	0	12	8
20	Stress[C]	Up	0	25	0	28	14	1	10	0	32	16
		Down	0	2	0	9	3	0	3	0	6	9
21	Redox	Up	0	2	0	5	1	0	0	0	6	4

		Down	0	0	0	3	0	0	0	0	3	1
22	Polyamine metabolism[D]	Up	0	3	0	9	9	0	0	0	9	9
		Down	0	1	0	1	0	0	0	0	1	0
23	Nucleotide metabolism	Up	0	3	0	2	2	0	2	1	1	7
		Down	0	0	0	0	0	0	0	0	1	3
24	Biodegradation of xenobiotics	Up	0	0	0	0	0	0	0	0	0	0
		Down	0	0	0	0	0	0	0	0	0	0
25	C1-metabolism	Up	0	1	0	2	2	0	0	0	2	2
		Down	0	0	0	0	0	0	0	0	0	0
26	Misc	Up	0	14	0	28	18	5	5	0	20	28
		Down	0	6	0	15	3	0	1	0	11	10
27	RNA[D]	Up	0	20	0	17	7	6	16	0	12	12
		Down	0	8	0	12	5	0	4	0	19	6
28	DNA	Up	0	0	0	0	0	1	0	0	1	1
		Down	0	3	0	2	0	0	0	0	3	2
29	Protein[C]	Up	0	17	0	29	19	3	11	1	28	25
		Down	0	8	0	17	9	3	6	0	26	19
30	Signalling	Up	0	5	0	4	4	2	3	0	7	4
		Down	0	4	0	6	3	0	1	0	10	7
31	Cell	Up	0	4	0	12	3	2	2	0	9	7
		Down	0	0	0	2	2	0	0	0	2	2
33	Development	Up	0	6	1	9	2	2	4	0	10	9
		Down	0	6	0	3	2	0	3	0	9	9
34	Transport	Up	0	7	0	8	6	0	3	1	6	16
		Down	0	3	0	9	10	1	2	0	8	8
35	Not assigned	Up	0	56	2	69	46	25	19	4	66	60
		Down	0	39	1	71	22	1	16	1	76	38
	Total Unique DEGs[B]	Up	0	202	3	281	175	49	107	8	269	251
		Down	0	127	1	250	114	5	42	1	279	183

[A] Differentially expressed genes (DEGs) are shaded in grey and defined as those genes with log2-fold change (|FC| ≥ 0.8; p< 0.05). DEGs are shown for local (L) and systemic (S) tissues.

[B] Mapman categorizes some genes into multiple functional categories (BINs). Therefore, the number of duplicated genes was subtracted from the total DEGs to yield the number of unique DEGs.

[C] The five BINs designated as differentially regulated after wounding by MAPMAP are indicated (p<0.05).

[D] The five BINs that contain subBINs that were differentially regulated after wounding according to MAPMAN are indicated (p<0.05).

Table 2.2 Differentially expressed stress-responsive genes (BIN20) after wounding of WT and *LapA-SI* leaves.[A]

			WT					*LapA -SI*				
			0 hr	1 hr		8 hr		0 hr	1 hr		8 hr	
Temporal class	Clone ID[B]	Annotation		L	S	L	S		L	S	L	S
1-hr												
	STMIW03	Similar to AtWRKY40	-0.01	**1.36**	-0.01	-0.30	-0.04	-0.29	**1.37**	0.17	-0.05	-0.02
	STMCN79	Homologue to AtWRKY33	0.16	**1.10**	0.21	0.24	0.44	0.26	0.76	0.16	-0.17	-0.59
	STMFB09	Homologue to AtWrky75/StWrky1 (potato SGN-U273670)	0.29	**0.88**	0.41	0.46	0.15	0.55	0.25	0.08	0.20	-0.01
	STMGA54	Similar to leucine rich-repeat (LRR) protien	0.09	**1.13**	0.62	0.78	0.25	0.51	0.78	0.17	0.20	0.22
	STMJA60	Homologue to POTHR-1 protein (Hin1-like protein)	0.19	**1.09**	0.13	0.17	0.21	0.13	**0.96**	-0.03	0.55	-0.13
	STMGZ63	Whitefly-induced gp91-phox (Wfi1)	-0.03	**0.94**	-0.41	0.28	0.50	-0.14	**1.20**	-0.24	0.15	0.02
	STMFB59	Similar to class IV chitinase	0.16	**1.35**	0.58	0.79	-0.04	0.65	0.62	0.16	0.75	-0.26
	STMDH09	Similar to osmotic stress-activated protein kinase	0.24	**0.95**	0.61	0.34	0.16	0.78	0.04	0.16	0.07	0.03
	STMIQ26	Late embryogenesis abundant (LEA)-like protein (ER5-like)	-0.10	**1.12**	0.47	-0.63	-0.36	-0.40	**0.87**	0.31	-0.13	**-0.84**
	STMGA34	Ethylene-responsive late embryogenesis protein (ER5)	0.13	**2.03**	**1.27**	0.36	0.16	0.40	**1.04**	0.60	0.11	-0.30
	STMEY92	Similar to universal stress protein (USP) family protein	0.08	**1.73**	0.40	0.58	0.28	-0.24	**1.25**	0.35	0.65	0.22
	STMEQ06	Major intrinsic protein 2	-0.13	**-0.96**	-0.66	-0.04	-0.54	-0.31	**-0.80**	-0.42	-0.36	-0.59
	STMIS78	Probable aquaporin NIP5.1	-0.18	**-0.87**	-0.14	-0.75	-0.09	-0.36	-0.31	-0.19	-0.68	-0.34
	STMGU90	Similar to UV-B and ozone similarly regulated protein 1 UOS1	-0.09	**-0.83**	-0.35	-0.65	-0.46	-0.27	-0.43	0.06	**-0.88**	-0.39
1-hr and 8-hr												
	STMCN84	MYC-like transcription factor (bHLB-Myc family)	0.23	**1.33**	0.62	**0.81**	0.27	0.58	**1.04**	0.20	0.02	0.24
	STMFB93*	Pathogenesis-related protein (PR1c)	0.25	**1.27**	0.01	**1.01**	-0.48	**0.93**	0.03	-0.54	**0.94**	-0.76
	STMFB60*	Pathogenesis-related leaf protein 6 precursor	-0.06	**1.97**	-0.13	**1.31**	-0.31	**1.13**	**0.82**	-0.38	**2.03**	-0.94
	STMEY55*	Similar to pathogenesis related protein 10	-0.14	**0.92**	-0.14	**1.19**	-0.39	0.32	0.24	-0.38	**1.46**	**-0.92**

	STMIS93*	Homologue to endochitinase A precursor	-0.02	**1.31**	-0.40	**1.22**	-0.63	0.27	0.40	-0.46	**1.64**	-0.78
	STMEY20	Chitinase, class II	0.06	**0.96**	0.00	**1.09**	-0.23	0.43	0.38	-0.16	**1.06**	-0.45
	STMEO91*	Acidic 26-kDa endochitinase precursor	0.08	**1.57**	-0.07	**1.12**	-0.17	0.84	**0.96**	0.16	**2.46**	0.20
	STMHY32*	Homologue to xyloglucan-specific fungal endoglucanase inhibitor	0.14	**1.00**	0.11	**1.26**	0.12	0.31	**0.82**	0.04	**1.49**	-0.16
	STMGC07*	Homologue to cathepsin B-like cysteine proteinase	-0.01	**0.86**	0.21	**1.02**	0.49	0.35	0.37	0.02	**1.45**	**0.89**
	STMCK62	Weakly similar to serine carboxypeptidase	0.08	**0.95**	0.14	**1.07**	-0.07	0.66	0.52	0.04	**1.27**	0.16
	STMEF73	Polygalacturonase family protein	0.07	**1.49**	-0.26	**1.50**	-0.60	0.61	0.12	-0.52	**1.57**	**-0.86**
	STMDO41*	Polyphenol oxidase	0.50	**0.86**	0.32	**3.57**	**3.39**	0.47	**0.83**	0.44	**3.08**	**3.49**
	STMDB46	Homologue to propolyphenol oxidase precursor	0.86	**0.90**	0.50	**4.59**	**4.29**	0.86	0.68	0.95	**3.75**	**4.63**
	STMCR61	Neutral leucine aminopeptidase preprotein precursor[C]	0.48	**1.17**	0.07	**2.35**	**2.36**	-0.10	-0.48	-0.30	-0.49	-0.56
	STMGC30	Leucine aminopeptidase A	0.49	**1.00**	0.02	**2.80**	**2.67**	**-1.21**	-0.66	-0.62	**-0.97**	-0.79
	STMIT44*	Homologue to osmotin-like protein	-0.04	**1.14**	-0.36	**1.42**	-0.28	0.35	0.64	-0.35	**1.58**	-0.44
	STMCP23*	Similar to endoplasmin homolog precursor (HSP90)	0.28	**0.84**	0.64	**1.35**	**1.40**	0.58	0.14	0.23	**1.30**	**1.62**
	STMGE38	TAS14 (Le4)- dehydrin	0.18	**1.26**	0.48	**1.24**	0.12	**1.31**	0.71	0.09	**2.16**	-0.16
8-hr												
	STMHA17	Homologue to AtWRKY13	0.19	0.16	0.15	**1.36**	**1.51**	0.04	0.06	0.02	**1.44**	**1.68**
	STMDT95	Weakly similar to NBS/LRR resistance protein	0.18	0.61	0.31	**1.09**	0.56	0.39	0.13	0.33	**1.17**	**0.83**
	STMGX36*	Lipoxygenase A	0.13	0.57	0.14	**2.53**	**0.91**	0.44	0.53	-0.03	**3.24**	**1.42**
	STMGZ27*	Homologue to Phospholipase A1	0.09	0.53	0.25	**1.28**	**0.94**	-0.24	0.22	0.27	**1.75**	**1.24**
	STMCL45	Systemin precursor	0.15	0.32	0.46	0.53	**1.16**	0.09	0.21	0.22	**1.06**	**1.33**
	STMCP56	Polyphenol oxidase B	0.71	0.71	0.60	**4.71**	**4.09**	0.78	0.59	0.74	**3.41**	**4.86**
	STMIU61*	Similar to proteinase inhibitor I	0.20	0.05	0.30	**3.28**	**3.45**	-0.41	0.28	0.27	**3.36**	**3.82**
	STMHT35*	Proteinase inhibitor II	0.59	0.20	-0.04	**2.75**	**3.14**	-0.13	0.38	-0.06	**3.27**	**3.70**
	STMCQ55	Cathepsin D inhibitor precursor	0.81	0.36	0.03	**3.18**	**3.41**	0.02	0.27	0.38	**2.47**	**4.02**
	STMIX49	Homologue to Cystatin, cysteine protease inhibitor	0.07	0.07	0.21	0.51	**1.04**	-0.22	0.20	-0.06	0.45	0.65

STMCG57	Metallocarboxypeptidase inhibitor precursor	0.28	-0.07	0.43	**1.59**	**1.86**	0.39	-0.11	0.19	**1.15**	**1.94**
STMCK43	Similar to polygalacturonase-inhibiting protein	0.05	0.75	0.44	**1.44**	**1.10**	0.11	0.79	0.50	**1.44**	**1.38**
STMCP01	Luminal-binding protein precursor, HSP70 family protein	0.08	0.36	0.22	**1.03**	**1.17**	-0.11	0.24	0.29	**1.12**	**1.62**
STMGN23	Similar to heat shock protein 70	0.10	0.08	0.05	**0.95**	**1.20**	-0.11	0.15	0.16	**1.36**	**1.73**
STMEA30	Probable aquaporin	0.03	-0.32	-0.27	**1.09**	0.70	-0.31	-0.11	0.16	**1.08**	**1.15**
STMDE88	Similar to salt tolerance protein 5-like protein	0.03	0.26	-0.04	**1.33**	**0.89**	0.01	-0.22	-0.11	**1.16**	**0.98**
STMCL06*	Homologue to 2-oxoglutarate-dependent dioxygenase (SPP2)	0.10	0.02	0.26	**1.12**	0.58	-0.19	-0.41	0.25	**1.46**	**0.98**
STMDD59	Putative CCR4-associated factor	-0.09	-0.15	-0.05	0.10	**0.81**	0.12	-0.01	-0.09	-0.02	0.22
STMCV39	Similar to SRE1b	0.36	0.38	0.27	**0.81**	0.41	0.53	0.31	0.57	0.50	0.65
STMCS03	Homologue to STS14 protein precursor	0.18	0.77	0.38	**0.93**	0.36	0.40	0.04	0.02	0.24	0.21
STMCA80	Similar to *Nicotiana tabacum* wound induced mRNA	-0.14	-0.40	-0.35	**0.83**	0.25	-0.36	-0.69	-0.19	0.69	0.33
STMCV76	Homologue to Heat shock protein 83	-0.13	-0.43	0.03	**-0.81**	-0.20	-0.21	-0.26	0.00	-0.74	-0.18
STMEA19	Homologue to Heat shock cognate protein 80	-0.34	-0.50	-0.25	**-0.82**	-0.36	-0.47	0.07	-0.03	-0.28	-0.42
STMHS08	Homologue to Heat shock protein 81-1 (HSP81-1)	-0.25	-0.30	-0.28	**-0.85**	-0.40	-0.41	0.27	0.06	-0.38	-0.31
STMEQ11	Similar to DnaJ-like protein (putative molecular chaperone)	0.00	-0.33	-0.15	-0.72	**-0.83**	0.16	-0.20	-0.06	**-1.07**	**-1.04**
STMCG30	Homologue to cold-induced glucosyl transferase	-0.10	0.01	-0.39	**-0.91**	**-1.03**	-0.04	0.30	0.04	-0.41	**-1.30**
STMCR71	Weakly similar to dehydration-induced protein RD22 (BURP domain protein)	-0.01	-0.36	-0.09	**-0.93**	-0.70	0.03	-0.39	0.09	**-1.00**	-0.72
STMJO29	Homologue to dehydrin CI7 (Dhn2)	-0.47	0.68	0.27	**-0.83**	-0.61	-0.35	0.01	-0.28	0.18	**-1.35**
STMIV08*	Putative delta tonoplast intrinsic protein	-0.14	-0.55	-0.16	**-0.88**	-0.71	-0.48	-0.41	-0.18	**-1.23**	-0.77

[A] DEGs (|FC| ≥0.8; p <0.05) are indicated in bold and shaded in grey. DEGs that are down-regulated are boxed in.
[B] Some genes are represented by multiple clones on the TIGR 10-K (version 3) potato cDNA microarray. These genes are indicated with an asterisk. One representative clone shown. Redundant clones can be found in Table S1.
[C] *LapN* was annotated as an up-regulated DEG. This is in striking contrast to the previous studies monitoring the rare-class *LapN* RNAs by RNA blots or RNase protection studies or monitoring *LAP-N* protein levels (Chao et al. 2000; Tu et al. 2003). It is likely that the abundant wound-induced *LapA* transcript cross-hybridized to the *LapN* ESTs on the array, since the potato *LapN* EST has 95.6% and 88.9% identity with the tomato *LapN* and *LapA,* respectively.

Table 2.3 Differentially regulated genes after wounding in WT and *LapA-SI* lines represented by multiple clones on TIGR 10-K cDNA microarray.[A]

		WT					*LapA-SI*				
		0 hr	1 hr		8 hr		0 hr	1 hr		8 hr	
Clone number[B]	Description		L	S	L	S		L	S	L	S
STMEA11	LeXYL1 protein (β-d-xylosidase)	-0.26	**-1.15**	-0.60	-0.49	-0.63	-0.21	**-1.24**	-0.59	**-0.93**	**-0.91**
STMEA15		-0.20	**-1.12**	-0.60	-0.47	-0.48	-0.38	**-1.16**	-0.52	**-0.83**	-0.76
STMEI66*	Similar to plastidial delta-12 oleate desaturase	-0.11	-0.30	-0.09	**-1.98**	**-1.33**	0.12	-0.16	0.06	**-1.53**	**-1.60**
STMCU78		-0.17	-0.74	-0.38	**-2.16**	**-1.40**	-0.36	-0.15	0.01	**-1.58**	**-1.48**
STMCK79		-0.03	-0.28	0.05	**-1.86**	**-1.33**	0.09	-0.17	0.17	**-1.51**	**-1.56**
STMCM57		0.16	0.03	0.27	-0.76	-0.60	0.48	-0.19	0.03	-0.75	**-0.86**
STMGZ27*	Homologue to phospholipase A1	0.09	0.53	0.25	**1.28**	**0.94**	-0.24	0.22	0.27	**1.75**	**1.24**
STMGE39		0.14	0.20	0.33	**1.04**	0.67	0.11	0.16	0.26	**1.08**	**0.84**
STMCF50		0.22	**1.01**	0.51	**1.61**	**0.99**	0.65	0.13	0.24	**1.57**	**1.21**
STMCJ60*	S-adenosylmethionine synthetase 1	0.04	0.40	0.02	**1.28**	**0.80**	0.03	0.44	0.39	**1.10**	**1.26**
STMCD54		0.17	**0.85**	0.35	**1.68**	**1.05**	0.37	**0.80**	0.48	**1.61**	**1.38**
STMGS62		-0.06	0.21	0.03	**1.01**	0.73	-0.27	0.53	0.31	**0.94**	**1.14**
STMGZ79		-0.06	0.30	0.01	**1.07**	0.77	-0.22	0.56	0.34	**1.10**	**1.12**
STMCI86		0.08	0.57	0.16	**1.17**	**0.80**	0.13	0.39	0.31	0.74	**1.07**
STMCQ94		0.10	0.45	0.31	**1.40**	**0.96**	0.38	0.37	0.18	**0.85**	**1.16**
STMGU10	Homologue to gamma-aminobutyrate transaminase isozyme 2	0.07	-0.25	0.08	0.59	**0.90**	-0.34	0.07	0.40	**1.14**	**1.26**
STMCQ79		0.29	0.11	0.37	**0.93**	**0.96**	-0.02	0.00	0.59	**0.99**	**1.39**
STMCG71		0.05	0.02	0.04	0.50	**0.90**	-0.24	-0.22	0.18	**0.98**	**1.03**
STMGN32		0.02	-0.25	0.10	0.42	**0.83**	-0.43	-0.05	0.17	**0.73**	**1.06**
STMCB25		0.07	-0.22	0.25	0.45	**0.97**	-0.29	-0.19	0.32	0.74	**1.19**
STMHR62*	Similar to N-acetylornithine deacetylase-like protein	0.16	0.38	0.08	**1.98**	**1.84**	-0.38	0.68	0.49	**1.84**	**2.26**
STMGE43		0.13	0.41	0.07	**2.03**	**1.48**	0.03	0.36	0.32	**1.68**	**1.80**
STMCG42*	Similar to aminotransferase 2	-0.12	**-0.92**	-0.48	-0.24	-0.79	-0.23	-0.65	-0.48	-0.47	-0.47
STMJB23		-0.16	**-0.84**	-0.54	-0.30	-0.69	-0.25	-0.43	-0.39	-0.39	-0.39
STMJA37*	Aromatic amino acid decarboxylase 1A	0.15	0.76	0.04	0.14	**0.83**	0.00	**1.31**	0.09	0.13	0.39
STMEP88		0.19	**0.81**	0.43	0.44	0.63	0.45	**0.94**	0.07	0.07	0.23
STMGU61*	Similar to probable acetylornithine aminotransferase	0.12	**1.49**	0.16	**1.56**	**0.97**	-0.01	**1.13**	0.22	**0.83**	**0.83**
STMHX08		-0.01	**1.06**	0.10	0.73	0.63	-0.21	**0.83**	0.16	0.55	0.55
STMGC07*	Homologue to Cathepsin B-like cysteine proteinase	-0.01	**0.86**	0.21	**1.02**	0.49	0.35	0.37	0.02	**1.45**	**0.89**
STMJB45		0.00	**0.91**	0.06	**1.03**	0.77	-0.18	0.68	0.24	**2.00**	**1.24**

STMEI96		-0.02	0.41	-0.02	0.75	0.52	-0.21	0.37	0.12	**1.34**	0.74
STMEU11		-0.01	0.76	-0.14	**1.05**	0.64	-0.37	0.61	0.21	**2.05**	**1.20**
STMHO21		-0.05	0.15	0.00	0.46	0.36	-0.21	0.22	-0.03	**1.04**	0.53
STMDU15*	Homologue to isopentenyl diphosphate isomerase 1	0.28	0.26	0.26	**1.14**	0.56	0.29	0.23	0.32	0.65	**0.80**
STMDB11		0.21	0.35	0.35	**0.98**	0.41	0.25	0.19	0.37	0.65	0.75
STMCV10*	Similar to phytoene synthase 1	-0.11	**-0.91**	**-0.91**	**-0.93**	-0.73	-0.06	-0.61	-0.01	-0.70	-0.58
STMFB34		-0.15	**-0.85**	**-0.85**	**-0.99**	-0.61	-0.45	-0.26	-0.16	-0.54	-0.61
STMIY78*	Similar to 4-coumarate-CoA ligase-like protein	-0.36	**-0.97**	-0.86	**-2.06**	**-1.53**	-0.79	-0.36	-0.78	**-1.68**	**-2.09**
STMFB08		-0.05	0.72	-0.05	**-1.14**	**-1.24**	-0.01	0.55	-0.06	**-0.83**	**-1.98**
STMJE63*	Homologue to tyramine hydroxycinnamoyl transferase	-0.02	**1.13**	-0.10	0.53	-0.08	0.10	**0.92**	-0.24	0.57	-0.38
STMIP44		0.05	**0.89**	-0.20	0.40	-0.05	-0.05	0.74	-0.11	0.32	-0.42
STMEZ84		0.15	**1.61**	0.21	0.76	0.02	0.57	**0.96**	0.04	0.60	-0.49
STMIC76*	P-coumaroyl shikimate 3'-hydroxylase isoform 2	0.13	**0.87**	0.45	**1.23**	**1.11**	-0.05	**0.96**	0.64	**1.11**	**1.58**
STMIL92		0.14	0.77	0.53	**1.05**	**0.96**	-0.01	**0.83**	0.66	0.78	**1.45**
STMGP82*	Weakly similar to caffeoyl-CoA O-methyltransferase	0.29	0.64	0.25	**1.55**	**1.58**	0.28	0.58	0.15	**1.60**	**1.96**
STMGN18		0.15	0.17	-0.13	**0.82**	**0.87**	-0.24	0.51	0.44	**1.37**	**1.41**
STMCL06*	Homologue to 2-oxoglutarate-dependent dioxygenase (SPP2)	0.10	0.02	0.26	**1.12**	0.58	-0.19	-0.41	0.25	**1.46**	**0.98**
STMGI29		-0.06	-0.40	-0.11	0.41	0.30	-0.62	-0.52	0.01	**0.80**	0.73
STMCF39		0.03	-0.17	0.03	0.45	0.63	-0.35	-0.51	0.08	**0.99**	**1.09**
STMJA50*	Homologue to thioredoxin peroxidase	-0.12	**-0.80**	-0.34	**-0.82**	-0.26	-0.31	-0.26	-0.02	**-0.84**	-0.26
STMIX55		0.08	-0.63	-0.12	-0.66	-0.20	-0.08	-0.05	0.29	**-0.86**	-0.12
STMEW65*	Catalase isozyme 2	0.00	0.71	-0.12	**1.79**	0.47	0.24	0.09	-0.33	**1.36**	0.54
STMDR74		0.15	0.70	0.19	**1.18**	0.70	0.57	0.17	0.03	**1.03**	0.77
STMIN52*	S-adenosylmethionine decarboxylase proenzyme	0.24	0.55	0.38	**2.06**	**1.98**	0.02	0.65	0.62	**2.74**	**2.24**
STMIR64		0.20	0.47	0.39	**2.12**	**1.79**	-0.05	0.56	0.60	**2.57**	**2.19**
STMEO42		0.24	0.59	0.26	**1.88**	**1.49**	-0.07	0.57	0.46	**1.62**	**1.71**
STMCD62		0.30	0.72	0.28	**1.76**	**1.23**	0.05	0.70	0.43	**1.57**	**1.47**
STMCP75		0.45	**0.94**	0.39	**1.74**	**1.33**	0.11	0.67	0.44	**1.56**	**1.68**
STMIY61		0.01	-0.10	0.04	**1.68**	**1.04**	-0.34	0.26	0.02	**1.05**	**1.25**
STMIX62		0.22	0.50	0.59	**1.66**	**1.70**	0.02	0.54	0.57	**1.95**	**1.95**
STMCV17*	Spermidine synthase	0.41	**0.84**	0.56	**2.16**	**2.21**	0.32	0.68	0.64	**2.02**	**2.74**
STMCQ05		0.32	0.63	0.42	**1.49**	**1.41**	0.42	0.34	0.25	**1.22**	**1.73**
STMIW94*	Similar to IAA amidohydrolase-like	-0.04	**1.72**	0.25	0.09	0.07	0.02	**1.62**	0.59	0.39	0.33
STMIU23		-0.03	**0.93**	0.10	0.12	0.04	-0.14	**0.90**	-0.21	-0.03	0.08
STMCF04*	Similar to DWARF1/DIMINUTO1	0.20	0.33	0.26	**1.33**	**1.03**	0.10	0.18	0.45	**1.48**	**1.52**
STMCH16		0.23	0.19	0.31	**1.02**	**0.93**	0.27	0.03	0.48	**0.92**	**1.40**
STMCQ85*	Homologue to putative C-8 7 sterol isomerase	0.20	0.02	0.11	**1.25**	0.60	-0.20	0.09	0.34	**0.96**	**1.12**

STMCQ53		0.21	0.03	0.11	**0.99**	0.59	-0.17	0.10	0.38	**0.90**	**1.13**
STMDC82		0.27	0.17	0.27	**1.33**	0.61	0.03	0.07	0.33	**1.01**	**1.17**
STMJC16*	GAST1 protein precursor	-0.20	**-1.35**	-0.32	**-0.99**	0.07	-0.48	-0.45	-0.22	**-1.11**	-0.07
STMGD43		-0.04	**-1.23**	-0.17	-0.77	0.23	-0.37	-0.54	0.12	**-1.11**	0.05
STMGX36*	Lipoxygenase A	0.13	0.57	0.14	**2.53**	**0.91**	0.44	0.53	-0.03	**3.24**	**1.42**
STMGT19		0.09	0.35	-0.04	**2.60**	**1.03**	0.19	0.46	0.10	**3.23**	**1.47**
STMGL95		0.09	0.13	0.08	**1.00**	0.74	-0.03	0.23	0.12	0.77	**1.11**
STMFB93*	Pathogenesis-related protein (PR)1c	0.25	**1.27**	0.01	**1.01**	-0.48	**0.93**	0.03	-0.54	**0.94**	-0.76
STMFB44		0.08	**1.04**	-0.11	**0.82**	-0.33	0.55	-0.01	-0.66	**0.80**	-0.76
STMIS93*	Homologue to Endochitinase A precursor	-0.02	**1.31**	-0.40	**1.22**	-0.63	0.27	0.40	-0.46	**1.64**	-0.78
STMIX82		-0.09	**1.16**	-0.51	**1.06**	**-0.81**	0.21	0.24	-0.54	**1.45**	**-0.85**
STMEY55*	Similar to pathogenesis related protein 10	-0.14	**0.92**	-0.14	**1.19**	-0.39	0.32	0.24	-0.38	**1.46**	**-0.92**
STMCF73		-0.06	**1.14**	-0.11	**1.25**	-0.43	0.59	0.43	-0.40	**1.63**	**-0.94**
STMDW19		-0.06	**0.98**	-0.11	**1.17**	-0.43	0.25	0.27	-0.28	**1.69**	-0.73
STMFB60*	Pathogenesis-related leaf protein 6 precursor	-0.06	**1.97**	-0.13	**1.31**	-0.31	**1.13**	**0.82**	-0.38	**2.03**	-0.94
STMIN04		-0.12	**1.15**	-0.37	0.63	-0.30	0.52	0.72	-0.33	**1.48**	-0.60
STMIT44*	Homologue to osmotin-like protein	-0.04	**1.14**	-0.36	**1.42**	-0.28	0.35	0.64	-0.35	**1.58**	-0.44
STMCY79		0.13	**1.85**	-0.25	**1.95**	-0.43	**1.03**	0.48	-0.62	**2.05**	-0.94
STMIT70		-0.16	**1.01**	-0.51	**1.33**	-0.45	0.38	0.56	-0.47	**1.44**	-0.62
STMEO91*	Acidic 26 kDa endochitinase precursor	0.08	**1.57**	-0.07	**1.12**	-0.17	0.84	**0.96**	0.16	**2.46**	0.20
STMJD93		**0.04**	**0.91**	**-0.15**	**0.86**	**-0.12**	**0.27**	**0.63**	**0.33**	**1.92**	**0.12**
STMCP23*	Similar to endoplasmin homolog precursor (HSP90)	0.28	**0.84**	0.64	**1.35**	**1.40**	0.58	0.14	0.23	**1.30**	**1.62**
STMEG24		0.02	0.33	0.14	**1.07**	**1.08**	-0.09	0.12	0.39	**1.28**	**1.37**
STMIU61*	Homologue to proteinase inhibitor I	0.20	0.05	0.30	**3.28**	**3.45**	-0.41	0.28	0.27	**3.36**	**3.82**
STMCO50		0.45	0.57	0.56	**3.41**	**3.80**	0.20	0.18	0.42	**3.29**	**3.81**
STMCM05		0.46	0.50	0.82	**2.55**	**2.68**	0.54	0.33	0.33	**2.34**	**2.82**
STMDO41*	Polyphenol oxidase	0.50	**0.86**	0.32	**3.57**	**3.39**	0.47	**0.83**	0.44	**3.08**	**3.49**
STMDU79		0.52	**0.85**	0.36	**4.18**	**3.99**	0.32	**0.88**	0.54	**3.27**	**4.14**
STMGP96		0.38	0.61	0.37	**3.10**	**2.60**	0.28	0.46	0.19	**2.77**	**3.16**
STMEB67		0.50	0.45	0.19	**3.18**	**3.42**	0.16	0.56	0.26	**3.21**	**3.33**
STMEB19		0.59	0.18	0.29	**3.58**	**3.43**	0.16	0.71	0.40	**3.17**	**3.37**
STMIV08*	Putative delta tonoplast intrinsic protein	-0.14	-0.55	-0.16	**-0.88**	-0.71	-0.48	-0.41	-0.18	**-1.23**	-0.77
STMJO58		-0.12	-0.21	-0.11	-0.71	**-0.89**	-0.34	-0.25	-0.16	**-1.01**	**-0.86**
STMHT35*	Proteinase inhibitor II	0.59	0.20	-0.04	**2.75**	**3.14**	-0.13	0.38	-0.06	**3.27**	**3.70**
STMJA26		0.02	-0.55	-0.11	**0.84**	**1.38**	-0.32	-0.21	0.00	**1.05**	**1.58**

[A] DEGs (|FC| ≥0.8; p <0.05) are indicated in bold and highlighted in grey. Down-regulated DEGs are boxed in.

[B] Some genes were representated by multiple cDNA clones (spots) on the 10-K array. Only one representative clone for these genes appears in Tables 1-3. The identity of the redundant clones is provided here. Representative clones on other tables are marked with asterisks.

Table 2.4 Differentially expressed photosynthesis and tetrapyrole synthesis genes in response to wounding in WT and *LapA-SI* leaves.[A]

				WT					*LapA-SI*				
				0 hr	1hr		8hr		0 hr	1hr		8hr	
	Bin #	Clone ID	Description[B]		L	S	L	S		L	S	L	S
1 hr only	1	STMDM82	PRK, phosphoribulokinase / Uridine kinase family	0.37	**0.90**	0.50	0.35	0.01	0.52	0.50	0.30	0.15	0.02
	1	STMIX26	Oxygen-evolving enhancer protein 1, chloroplast precursor (OEE1)	-0.27	**-0.82**	-0.38	-0.77	-0.38	-0.34	-0.28	-0.18	**-1.13**	-0.39
	1	STMJB23	similar to aminotransferase 2	-0.16	**-0.84**	-0.54	-0.30	-0.69	-0.25	-0.43	-0.39	-0.39	-0.70
	1	STMCG42	similar to aminotransferase 2	-0.12	**-0.92**	-0.48	-0.24	-0.79	-0.23	-0.65	-0.48	-0.47	**-0.85**
	19	STMIS09	homologue to uroporphyrinogen decarboxylase, chloroplast precursor (URO-D)	-0.14	**-0.88**	-0.54	-0.28	-0.70	-0.30	-0.31	-0.37	-0.35	-0.68
	19	STMHU76	homologue to glutamate-1-semialdehyde 2,1-aminomutase, chloroplast precursor (GSA)	-0.16	**-0.92**	-0.34	-0.76	0.01	-0.31	-0.28	-0.04	-0.72	-0.05
1 hr & 8 hr	1	STMIU17	Fructose-1,6-bisphosphatase	-0.20	**-1.35**	-0.58	**-3.20**	**-2.07**	-0.41	-0.63	-0.08	**-3.24**	**-2.15**
	1	STMIY56	homologue to chlorophyll a-b binding protein CP24 10A	-0.22	**-1.53**	-0.50	**-2.87**	**-1.53**	-0.61	-0.68	0.03	**-2.55**	**-1.52**
	1	STMCD64	homologue to chlorophyll a-b binding protein CP24 10A	-0.06	**-0.95**	-0.32	**-2.46**	**-1.31**	-0.29	-0.59	0.08	**-2.61**	**-1.38**
	1	STMIY69	TOMCBPB chlorophyll a/b-binding protein Cab-3C	-0.20	**-1.25**	-0.38	**-2.66**	**-1.55**	-0.54	-0.51	0.07	**-2.73**	**-1.35**
	1	STMET04	Chlorophyll a/b-binding protein type III precursor	-0.14	**-0.92**	-0.25	**-2.20**	**-1.42**	-0.35	-0.68	-0.19	**-2.13**	**-1.43**
	1	STMIX78	homologue to photosystem II 22 kDa protein, chloroplast precursor (CP22)	-0.20	**-1.06**	-0.37	**-2.09**	**-1.13**	-0.48	-0.53	-0.26	**-1.74**	**-1.30**
	1	STMIU53	similar to proton gradient regulation 5	-0.52	**-1.06**	-0.31	**-1.99**	**-1.10**	-0.66	-0.43	-0.24	**-1.14**	**-1.41**
	1	STMEU31	similar to photosystem I reaction centre subunit N, chloroplast precursor (PSI-N)	-0.29	**-1.06**	-0.05	**-1.86**	**-1.00**	-0.56	**-0.81**	0.01	**-1.97**	**-1.21**
	1	STMIY09	similar to photosystem I reaction centre subunit N, chloroplast precursor (PSI-N)	-0.26	**-0.97**	-0.34	**-1.77**	**-1.01**	-0.54	-0.36	-0.12	**-1.86**	**-0.85**
	1	STMIR79	homologue to photosystem I reaction center subunit X psaK	-0.38	**-1.12**	-0.18	**-1.75**	-0.63	-0.76	**-1.01**	-0.37	**-1.79**	**-1.17**
	1	STMIX69	homologue to photosystem I reaction center subunit III	-0.07	**-0.85**	-0.04	**-1.22**	-0.21	-0.34	-0.21	0.15	**-1.30**	-0.20
	1	STMIX56	homologue to PSI-H precursor	-0.13	**-0.95**	-0.30	**-1.37**	-0.50	-0.52	-0.39	0.15	**-1.17**	-0.38

	1	STMIR58	similar to ultraviolet-B-repressible protein	-0.30	**-1.20**	-0.04	**-1.31**	-0.30	-0.44	-0.70	-0.48	**-0.84**	-0.54
	1	STMCI15	homologue to Glyceraldehyde-3-phosphate dehydrogenase B, chloroplast precursor	-0.10	**-0.95**	-0.30	**-1.26**	**-0.83**	-0.20	-0.18	-0.02	**-1.28**	-0.69
	1	STMIY23	similar to glycine decarboxylase complex H-protein	-0.26	**-1.05**	-0.34	**-1.10**	-0.53	-0.56	-0.56	-0.01	-0.76	-0.32
	1	STMHZ22	Photosystem II 10 kDa polypeptide, chloroplast precursor	-0.62	**-0.80**	0.00	**-1.06**	-0.08	-0.58	-0.61	-0.44	-0.42	-0.58
	1	STMIZ57	Photosystem II 10 kDa polypeptide, chloroplast precursor	-0.73	**-0.89**	-0.10	**-0.98**	-0.01	-0.72	-0.67	-0.37	-0.50	-0.51
	1	STMHQ84	Photosystem II 10 kDa polypeptide, chloroplast precursor	-0.65	**-0.82**	-0.10	**-0.94**	0.01	-0.59	-0.58	-0.48	-0.45	-0.48
	1	STMES83	similar to photosystem II core complex proteins psbY	-0.05	**-0.82**	0.03	**-0.91**	-0.20	-0.10	**-0.86**	0.12	-0.51	-0.15
	1	STMIX96	similar to ultraviolet-B-repressible protein	-0.42	**-0.86**	-0.07	**-0.85**	-0.31	-0.42	-0.50	-0.08	-0.47	-0.19
	19	STMDP32	homologue to NADPH:protochlorophyllide oxidoreductase	-0.09	**-1.37**	-0.11	**-2.65**	**-1.08**	-0.04	**-0.86**	0.09	**-2.84**	**-1.29**
	19	STMDH13	homologue to NADPH:protochlorophyllide oxidoreductase	-0.19	**-0.91**	-0.22	**-2.50**	**-1.34**	0.06	**-1.00**	-0.14	**-2.99**	**-1.61**
	19	STMJD64	homologue to leucine zipper-containing protein Copper response defect 1 (CRD1)	-0.08	**-0.95**	-0.37	**-2.24**	**-1.59**	-0.40	-0.37	0.05	**-2.20**	**-1.78**
	19	STMJE86	similar to LLS1 protein	0.02	**-1.02**	-0.30	**-2.04**	**-1.75**	0.26	**-0.84**	-0.04	**-2.04**	**-2.08**
	19	STMHO12	similar to LLS1 protein	-0.05	**-1.11**	-0.38	**-1.87**	**-1.57**	-0.01	-0.50	-0.06	**-1.81**	**-1.69**
	19	STMHL29	similar to AtGUN4	-0.23	**-1.50**	-0.38	**-1.78**	**-0.96**	-0.28	**-0.99**	0.01	**-1.52**	**-0.97**
	19	STMJB64	similar to uroporphyrinogen decarboxylase (chloroplast)	-0.14	**-1.05**	-0.39	**-1.43**	-0.65	-0.53	-0.50	-0.04	**-1.36**	-0.57
	19	STMHY74	homologue to Mg protoporphyrin IX chelatase	-0.24	**-0.93**	-0.37	**-1.73**	**-0.98**	-0.30	-0.53	-0.28	**-1.49**	**-1.22**
	19	STMJE39	homologue to magnesium chelatase, subunit ChlI	-0.19	**-1.03**	-0.45	**-0.83**	-0.38	-0.50	-0.48	-0.05	-0.76	-0.21
8 hr only	1	STMCF01	homologue chlorophyll a/b binding protein CP29	0.13	-0.46	0.07	**-1.11**	-0.78	0.18	-0.33	0.20	**-1.64**	-0.27
	1	STMCN09	Chlorophyll a-b binding protein 13, chloroplast precursor (LHCII type III CAB-13)	0.03	-0.09	0.10	**-1.85**	**-1.13**	0.13	-0.25	0.09	**-2.28**	**-1.20**
	1	STMCY82	Chlorophyll a-b binding protein 13, chloroplast precursor (LHCII type III CAB-13)	0.27	-0.13	0.23	**-2.01**	**-1.22**	0.41	-0.59	0.14	**-2.50**	**-1.18**
	1	STMCN64	Chlorophyll a-b binding protein 4, chloroplast precursor (LHCII type I CAB-4)	0.03	-0.21	-0.10	**-1.81**	**-1.19**	-0.04	-0.20	0.02	**-1.99**	**-1.37**

1	STMCP33	homologue to chlorophyll a-b binding protein 4, chloroplast precursor (LHCII type I CAB-4)	0.00	-0.26	-0.15	**-2.40**	**-1.51**	-0.14	-0.36	0.09	**-2.67**	**-1.65**
1	STMCQ90	Chlorophyll a/b-binding protein type I precursor (cab-6A)	-0.08	-0.61	-0.12	**-1.55**	-0.60	-0.43	-0.16	0.02	**-1.69**	-0.49
1	STMDP45	Chlorophyll a/b-binding protein type III precursor	-0.14	-0.59	0.07	**-1.91**	**-1.05**	-0.21	-0.39	-0.09	**-2.14**	**-1.48**
1	STMCJ09	Chlorophyll a/b-binding protein (cab-11)	-0.01	-0.25	0.11	**-1.62**	**-1.04**	0.13	-0.37	0.08	**-1.78**	-0.80
1	STMCK76	Chlorophyll a/b-binding protein (cab-11)	0.20	-0.15	0.09	**-1.71**	**-0.99**	0.16	-0.50	0.14	**-2.03**	-0.67
1	STMCN12	Chlorophyll a/b-binding protein (cab-11)	-0.04	-0.32	-0.10	**-1.65**	**-1.06**	-0.18	-0.56	0.12	**-1.98**	-0.55
1	STMCJ04	Chlorophyll a/b binding protein	-0.10	-0.13	-0.18	**-1.38**	-0.32	-0.03	-0.08	-0.14	**-1.76**	-0.50
1	STMCY88	Chlorophyll a-b binding protein 1B, chloroplast precursor (LHCII type I CAB-1B)	-0.17	-0.61	-0.25	**-2.54**	**-1.53**	-0.21	-0.47	0.07	**-2.65**	**-1.76**
1	STMCZ27	Chlorophyll a-b binding protein 1B, chloroplast precursor (LHCII type I CAB-1B)	0.00	-0.10	0.10	**-2.14**	**-1.25**	0.19	-0.24	0.08	**-2.38**	**-1.37**
1	STMIY91	Type I (26 kD) CP29 polypeptide	-0.14	-0.62	-0.32	**-1.38**	**-0.90**	-0.33	-0.17	0.01	**-1.93**	-0.77
1	STMCC39	Oxygen-evolving enhancer protein 2, chloroplast precursor (OEE2)	-0.32	-0.59	-0.34	**-0.89**	-0.29	-0.50	-0.34	-0.08	**-0.81**	-0.34
1	STMCD24	homologue to photosystem II 22 kDa protein, chloroplast precursor (CP22)	0.03	-0.36	-0.01	**-1.69**	**-1.05**	0.00	**-0.81**	-0.02	**-1.63**	**-1.29**
1	STMEZ80	similar to ultraviolet-B-repressible protein	-0.07	-0.71	0.24	**-0.99**	-0.36	-0.06	-0.70	0.14	-0.61	-0.52
1	STMCD42	similar to photosystem I reaction centre subunit N, chloroplast precursor (PSI-N)	-0.13	-0.61	0.26	**-1.97**	**-0.94**	-0.06	**-0.88**	0.18	**-2.04**	**-1.35**
1	STMCI32	similar to photosystem I reaction center subunit III	-0.04	-0.78	0.18	**-1.22**	-0.37	-0.12	-0.44	0.33	**-1.43**	-0.24
1	STMCQ21	similar to photosystem I reaction center subunit III	-0.04	-0.75	0.21	**-0.97**	-0.43	-0.11	-0.47	0.30	**-1.37**	-0.24
1	STMCX85	similar to photosystem I subunit O	-0.24	-0.53	0.13	**-1.32**	-0.67	0.03	**-0.81**	-0.14	**-1.81**	**-0.98**
1	STMHX87	homologue to photosystem I reaction centre subunit VI	-0.13	-0.51	-0.13	**-1.07**	-0.41	-0.28	-0.33	0.26	**-0.99**	-0.30
1	STMCG35	Ferredoxin--NADP reductase, leaf-type isozyme, chloroplast precursor (FNR)	-0.11	-0.39	-0.02	**-0.81**	-0.51	-0.02	-0.24	-0.14	**-0.84**	-0.50
1	STMEY02	similar to alanine-2-oxoglutarate aminotransferase 2	-0.20	-0.74	-0.24	**-0.83**	-0.78	-0.30	-0.28	-0.35	**-0.82**	**-0.94**
1	STMCL39	homologue to H-Protein	-0.21	-0.74	-0.04	**-1.20**	-0.44	-0.26	-0.61	-0.06	**-0.87**	-0.36
1	STMET51	homologue to hydroxypyruvate reductase	-0.24	-0.49	-0.33	**-0.91**	-0.55	-0.33	-0.46	-0.34	-0.65	**-0.86**
1	STMIY04	homologue to ribulose bisphosphate carboxylase small chain 1, chloroplast	-0.26	-0.56	-0.16	**-0.93**	-0.26	-0.53	-0.30	-0.10	-0.71	-0.16

	1	STMCM20	homologue to glyceraldehyde-3-phosphate dehydrogenase A, chloroplast precursor	0.08	-0.16	0.10	**-0.98**	-0.39	-0.11	-0.05	0.11	**-0.98**	-0.40
	1	STMCP14	homologue to glyceraldehyde-3-phosphate dehydrogenase A, chloroplast precursor	0.02	-0.22	0.02	**-0.81**	-0.59	-0.14	-0.19	-0.09	**-0.81**	-0.46
	1	STMDB44	homologue to RuBisCO activase, chloroplast precursor	-0.11	-0.10	0.05	**-1.36**	**-0.85**	-0.02	-0.08	-0.04	**-1.35**	**-1.39**
	1	STMES37	homologue to RuBisCO activase, chloroplast precursor	-0.09	-0.50	-0.26	**-1.91**	**-1.26**	0.05	-0.05	-0.17	**-1.65**	**-1.42**
	19	STMIJ77	similar to Glu-tRNA(Gln) amidotransferase subunit A	0.00	-0.77	0.01	**-0.97**	-0.36	-0.07	-0.42	0.04	**-1.05**	-0.29
	19	STMCN28	homologue to Leucine zipper-containing protein copper response defect 1 (CRD1)	0.07	-0.61	0.09	**-2.06**	**-1.26**	0.14	-0.36	0.26	**-1.93**	**-1.78**
LapA-SI only														
1 hr only	1	STMCM49	Photosystem II 10 kDa polypeptide, chloroplast precursor	-0.50	-0.52	0.32	-0.45	0.15	-0.22	**-0.90**	-0.25	-0.30	-0.48
8 hr only	1	STMCH36	ATP synthase C chain (Lipid-binding protein) (Subunit III)	0.07	0.31	0.13	0.37	0.24	0.23	0.40	0.11	**0.81**	0.26
	1	STMCB94	Oxygen-evolving enhancer protein 2, chloroplast precursor (OEE2)	-0.17	-0.75	0.04	-0.59	-0.19	-0.07	-0.57	-0.13	**-0.89**	-0.41
	1	STMCP64	Oxygen-evolving enhancer protein 1, chloroplast precursor (OEE1)	0.07	-0.30	-0.13	-0.73	0.03	0.03	-0.22	0.11	**-0.98**	0.13
	1	STMDR17	Oxygen-evolving enhancer protein 2, chloroplast precursor (OEE2)	0.00	-0.40	0.10	-0.72	-0.21	-0.10	-0.42	0.16	**-0.87**	0.02
	1	STMCK36	homologue to chlorophyll a-b binding protein 7, chloroplast precursor (LHCI type II CAB-7)	0.05	-0.10	-0.01	-0.54	-0.17	0.03	-0.12	0.10	**-0.90**	-0.08
	1	STMCO48	homologue to photosystem I subunit XI	0.17	-0.44	0.22	-0.77	-0.36	0.20	-0.65	0.24	**-1.19**	-0.38
	1	STMCQ33	homologue to photosystem I subunit XI	0.17	-0.28	0.13	-0.59	-0.32	0.08	-0.08	0.25	**-1.13**	-0.13
	1	STMCP03	homologue to photosystem I 20 kDa subunit (PSI-D), chloroplast precursor	0.15	-0.25	0.10	-0.65	-0.17	0.07	-0.48	0.15	**-0.99**	-0.05
	1	STMCZ35	homologue to photosystem I reaction center subunit IV A, chloroplast precursor (PSI-E A)	0.15	-0.24	0.35	-0.51	-0.32	0.32	-0.44	0.40	**-1.03**	-0.25
	1	STMCV30	similar to phosphoglycolate phosphatase precursor	0.14	-0.35	0.22	-0.69	-0.22	0.05	-0.26	0.13	**-0.92**	-0.28
	1	STMEQ70	homologue to (S)-2-hydroxy-acid oxidase, peroxisomal 2 (Glycolate oxidase 2) (GOX 2)	-0.18	-0.60	-0.49	-0.66	-0.75	-0.40	-0.19	-0.28	**-0.84**	-0.79

	1	STMCU21	homologue to glyceraldehyde-3-phosphate dehydrogenase A, chloroplast precursor	0.04	-0.23	-0.17	-0.65	-0.15	-0.02	-0.11	0.00	**-1.04**	-0.44
	1	STMJG96	similar to glyceraldehyde-3-phosphate dehydrogenase A, chloroplast precursor	0.04	-0.52	-0.18	-0.62	-0.56	0.00	-0.19	0.19	**-0.91**	-0.26
	1	STMEV18	homologue to glyceraldehyde-3-phosphate dehydrogenase B, chloroplast precursor	-0.11	-0.47	-0.21	-0.53	-0.66	-0.18	-0.26	-0.03	**-0.92**	-0.64
	1	STMCO53	Fructose-1,6-bisphosphatase	0.09	-0.14	0.34	-0.63	-0.29	0.67	-0.53	0.15	**-0.86**	-0.63
	1	STMIU18	Fructose-1,6-bisphosphatase	-0.15	-0.77	-0.31	-0.75	-0.53	-0.23	-0.41	-0.29	**-0.97**	-0.57
	1	STMCP81	homologue to phosphoribulokinase	-0.14	-0.79	-0.25	-0.73	-0.56	-0.12	-0.27	-0.05	**-1.07**	-0.49
	19	STMCE59	homologue to magnesium chelatase, subunit ChlI	-0.04	-0.69	-0.01	-0.60	-0.33	-0.13	-0.56	0.04	**-0.84**	-0.22
	19	STMCX44	homologue to S-adenosyl-L-methionine Mg-protoporphyrin IX methyltranserase	-0.05	-0.63	-0.13	-0.51	-0.34	-0.12	-0.69	-0.12	**-0.90**	-0.35
8 hr systemic only	1	STMEJ16	similar to ATP synthase protein I	-0.08	-0.76	-0.15	0.13	0.50	-0.51	-0.35	0.17	0.19	**0.82**
	1	STMEO76	similar to alanine-2-oxoglutarate aminotransferase 2	0.00	-0.24	-0.10	-0.60	-0.70	0.04	-0.13	0.00	-0.52	**-0.96**

[A] DEGs (|FC| ≥0.8; $p < 0.05$) are indicated in bold and shaded in grey. DEGs that are down regulated are boxed in.
[B] The DEGs in this table were not manually annoted and genes with duplicate spots or gene family member-specific spots were not determined.

Table 2.5 RNA and protein metabolism genes differentially regulated after wounding in WT and *LapA-SI* tomato leaves.[A]

				WT					*LapA-SI*				
				0 hr	1hr		8hr		0 hr	1hr		8hr	
	Bin #	Clone ID	Description[B]		L	S	L	S		L	S	L	S
RNA Metabolism													
1 hr only	27.1	STMGO76	similar to Poly(A) polymerase	0.03	**0.85**	0.10	0.58	0.45	-0.15	**0.99**	0.26	0.51	0.67
	27.2	STMDB40	similar to chloroplast sigma factor A (SIG1)	-0.07	**-0.93**	-0.22	-0.73	-0.50	0.09	-0.46	0.02	-0.53	-0.48
	27.3.22	STMHT30	similar to homeobox-leucine zipper protein 7 (HB-7)	-0.06	**0.80**	0.32	0.41	0.07	0.40	**0.96**	0.32	0.59	0.39
	27.3.27	STMFB46	similar to NAC domain protein NAC2	0.08	**1.05**	-0.07	0.39	-0.41	0.39	0.11	-0.23	0.38	-0.47
	27.3.29	STMJE94	weakly similar to Cycloidea protein, TCP family transcription factor	0.14	**1.06**	0.45	0.21	0.24	0.14	**1.21**	0.58	0.55	0.28
	27.3.3	STMFB78	Ethylene-responsive transcription factor ABR1	0.06	**1.17**	0.40	0.44	0.02	0.43	**0.80**	0.19	0.09	-0.03
	27.3.3	STMJN16	similar to Ethylene-responsive transcription factor RAP2-7	-0.02	**1.58**	0.19	0.46	0.46	-0.05	**1.58**	0.53	0.26	0.72
	27.3.30	STMCH76	similar to GT-2 factor (Fragment)	-0.07	**0.85**	0.18	0.24	-0.06	0.31	0.41	-0.11	0.19	-0.47
	27.3.32	STMFB09	homologue to AtWRKY75/StWRKY1 (potato SGN-U273670)	0.29	**0.88**	0.41	0.46	0.15	0.55	0.25	0.08	0.20	-0.01
	27.3.32	STMCN79	homologue to ATWRKY33	0.16	**1.10**	0.21	0.24	0.44	0.26	0.76	0.16	-0.17	-0.59
	27.3.32	STMIW03	similar to ATWRKY40	-0.01	**1.36**	-0.01	-0.30	-0.04	-0.29	**1.37**	0.17	-0.05	-0.02
	27.3.62	STMDH93	weakly similar to DDT domain-containing protein	0.12	**0.87**	0.34	0.34	0.05	**0.84**	-0.01	0.10	-0.23	-0.04
	27.3.67	STMEM46	weakly similar to transcription elongation factor-related	0.08	**0.97**	0.11	0.56	0.52	0.00	0.78	0.30	0.48	0.63
	27.3.8	STMCK07	similar to DNA binding protein	0.21	**1.15**	0.28	0.37	0.13	0.57	0.71	0.27	0.12	0.08
	27.3.8	STMDH55	similar to DNA binding protein	0.25	**1.26**	0.39	0.23	0.32	0.66	0.76	-0.05	0.31	0.07
	27.3.99	STMCZ22	similar to ARF GAP-like zinc finger-containing protein ZIGA3 (ZIGA3)	0.18	**0.84**	0.47	0.52	0.29	0.52	0.42	0.41	0.08	0.17
	27.3.99	STMCC31	homologue to MRNA binding protein precursor	-0.07	**-0.85**	-0.29	-0.74	-0.37	-0.43	-0.53	0.07	**-1.17**	-0.40
1 & 8 hr	27.2	STMCA66	similar to chloroplast sigma factor A (SIG1)	-0.18	**-1.06**	-0.36	**-0.94**	-0.75	-0.11	-0.63	0.03	**-0.95**	-0.62
	27.1.19	STMDH65	Ribonuclease P family protein	0.26	**0.83**	0.01	**1.61**	**1.06**	0.08	0.64	0.26	**1.28**	**1.37**
	27.3.23	STMCQ44	similar to Lil3 protein	-0.07	**-0.84**	-0.13	**-1.21**	-0.64	-0.28	-0.71	-0.11	**-1.15**	-0.62

	27.3.35	STMCU81	similar to BZIP transcription factor BZI-2	0.34	**1.18**	0.40	**0.96**	0.38	0.23	**0.82**	0.65	**1.43**	**0.87**
	27.3.6	STMCN84	weakly similar to MYC protein	0.23	**1.33**	0.62	**0.81**	0.27	0.58	**1.04**	0.20	0.02	0.24
	27.3.67	STMGA35	putative DNA Binding	0.30	**0.81**	0.68	**0.92**	0.17	**0.96**	-0.05	0.20	-0.08	-0.12
	27.3.7	STMDB34	similar to zinc finger (B-box type) family protein	-0.13	**-1.40**	-0.30	**-1.24**	**-1.00**	-0.11	**-1.20**	-0.20	**-1.33**	**-1.12**
	27.3.7	STMHT65	similar to CONSTANS-like protein CO1	-0.20	**-0.95**	-0.61	**-1.07**	**-1.18**	-0.20	-0.66	-0.48	**-1.16**	**-1.29**
	27.3.99	STMIY06	similar to Chloroplast RNA binding protein precursor	-0.21	**-0.86**	-0.43	**-1.05**	-0.46	-0.55	-0.57	-0.14	-0.66	-0.39
8 hr only	27.1	STMFB51	similar to RNA-binding gricine-rich protein-1 (RGP-1c)	0.00	0.29	0.09	**0.84**	**0.82**	0.00	-0.43	0.00	**1.05**	**0.87**
	27.1	STMDV82	similar to RNA-binding protein	0.03	0.44	0.29	**0.86**	0.36	0.14	0.09	0.20	0.58	0.64
	27.1	STMDZ02	similar to RNA-binding protein	0.08	0.36	0.27	**0.91**	0.40	0.11	0.06	0.13	0.71	0.76
	27.1	STMCX26	similar to RNA-binding protein	0.08	0.38	0.09	**1.02**	0.47	0.14	0.15	0.24	0.75	0.68
	27.1.1	STMGZ96	similar to arginine/serine-rich splicing factor RSP31	0.15	0.40	0.18	**0.86**	0.54	0.28	0.14	0.20	**0.80**	0.76
	27.3.14	STMGO41	similar to CCAAT-binding transcription factor (CBF-B/NF-YA)	-0.08	0.18	-0.13	**1.00**	0.37	-0.07	-0.02	-0.10	0.65	0.38
	27.3.23	STMHO25	similar to Lil3 protein	-0.08	-0.71	-0.15	**-0.87**	-0.49	-0.40	-0.43	0.00	**-0.98**	-0.44
	27.3.32	STMHA17	homologue to AtWRKY13	0.19	0.16	0.15	**1.36**	**1.51**	0.04	0.06	0.02	**1.44**	**1.68**
	27.3.35	STMEP60	similar to NPR1-interactor protein 1 TGA2	-0.12	0.77	-0.03	**0.86**	-0.28	0.22	0.28	-0.26	**1.10**	-0.72
	27.3.40	STMGW46	homologue to Nt-iaa28 deduced protein, AUX_IAA, AUX/IAA family	-0.32	-0.72	-0.36	**-1.03**	-0.29	-0.54	-0.39	-0.20	-0.61	-0.33
	27.3.40	STMDP46	homologue to Nt-iaa2.3 deduced protein, AUX_IAA, AUX/IAA family	-0.10	-0.23	0.12	**-0.89**	-0.40	-0.02	-0.12	0.00	-0.65	-0.35
	27.3.41	STMFB49	weakly similar to B3 DNA binding domain transcription factor	-0.05	-0.39	0.13	**-0.83**	-0.17	0.08	-0.65	0.01	**-1.18**	-0.34
	27.3.67	STMJH25	similar to remorin family protein	-0.20	-0.68	-0.38	**-1.10**	-0.59	-0.61	-0.30	-0.05	**-0.95**	-0.50
	27.3.69	STMJP20	similar to SET-domain-containing protein	-0.10	-0.63	-0.14	**-1.21**	**-0.80**	-0.19	-0.31	0.00	**-1.23**	-0.74
	27.3.8	STMDM43	similar to Ascorbate oxidase promoter-binding protein	-0.01	-0.30	-0.10	**-0.99**	**-1.27**	0.30	**-0.80**	0.09	**-1.21**	**-1.26**
	27.3.99	STMDH86	homologue to RCD1, Cell differentiation family	0.24	0.55	0.24	**0.84**	0.51	0.64	0.18	0.07	0.52	0.70
	27.3.99	STMHQ48	similar to transducin family protein	0.36	0.21	0.03	**1.86**	**2.03**	-0.03	0.11	0.11	**2.23**	**2.24**
	27.3.99	STMCK27	LeArcA1 protein, guanine nucleotide-binding family protein	0.35	0.40	0.47	**3.33**	**3.66**	0.12	0.26	0.31	**3.29**	**3.98**
8 hr Systemic Only	27.3.11	STMGT55	similar to zinc finger (C2H2 type) family protein	-0.02	-0.38	0.07	0.57	**0.88**	-0.53	-0.05	0.29	**0.86**	**1.11**
	27.3.22	STMEF08	homologue to Homeobox DNA-binding factor	0.05	-0.73	-0.30	0.50	**0.88**	-0.64	-0.68	-0.01	-0.23	**1.06**

	27.3.8	STMCY67	similar to Ascorbate oxidase promoter-binding protein	-0.01	-0.30	-0.10	-0.78	**-0.90**	0.28	-0.45	0.18	**-1.06**	**-0.92**
LapA-SI only													
0 hr	27.3.22	STMGF04	similar to Homeodomain protein GhHOX1	-0.01	0.42	0.56	0.65	0.07	**0.83**	-0.22	0.06	-0.10	-0.17
	27.3.22	STMGB83	similar to F6F9.25 protein (BEL1-like homeodomain 5 protein)	0.06	0.71	0.60	0.73	-0.01	**0.89**	-0.12	0.04	0.02	-0.30
	27.3.26	STMDH75	similar to MYB family transcription factor	0.21	0.50	0.32	0.41	0.02	**0.86**	-0.42	-0.10	-0.02	-0.14
	27.3.6	STMGB18	weakly similar to DNA-binding protein-like	0.13	0.42	0.52	0.74	0.21	**0.88**	-0.23	0.01	0.11	0.05
1 hr only	27.3.11	STMFB31	similar to Zinc finger protein PIF1	0.10	0.52	0.08	0.05	0.54	0.06	**0.91**	-0.10	-0.45	-0.02
	27.3.15	STMDQ49	homologue to transcription factor, CCAAT-binding, chain A	-0.29	-0.53	-0.17	-0.61	-0.23	-0.62	**-0.91**	-0.10	-0.37	-0.44
	27.3.27	STMIY82	homologue to Jasmonic acid 2	-0.18	0.66	-0.08	0.01	-0.26	-0.42	**0.88**	-0.08	0.75	-0.25
	27.3.32	STMJC11	homologue to WRKY-type transcription factor	0.05	0.26	-0.04	-0.23	-0.12	-0.09	**0.81**	-0.17	-0.10	-0.01
	27.3.32	STMJD75	similar to WRKY transcription factor 6	-0.06	0.76	-0.03	0.23	0.07	0.02	**0.98**	-0.30	0.34	0.01
	27.3.59	STMCI93	similar to methyl-CpG-binding domain-containing protein	-0.06	-0.34	-0.08	-0.06	0.07	0.17	**-0.81**	-0.18	-0.28	-0.16
	27.3.6	STMGI14	homologue to MYC transcription factor	0.07	0.64	0.16	0.38	0.65	0.04	**0.97**	0.30	0.33	0.54
	27.3.6	STMGY33	MYC transcription factor	0.04	0.42	-0.05	0.42	0.61	-0.14	**0.99**	0.27	0.25	0.63
	27.3.6	STMCU25	weakly similar to MYC protein	-0.12	0.77	0.26	-0.02	0.00	0.02	**1.02**	0.32	0.14	0.13
	27.3.64	STMGX55	similar to Avr9/Cf-9 rapidly elicited protein 74, RING finger containing	0.06	0.78	0.20	0.19	-0.04	0.26	**0.84**	0.03	0.27	-0.14
8 hr only	27.3.21	STMDT03	weakly similar to scarecrow-like transcription factor 11 (SCL11)	0.03	-0.47	-0.43	-0.56	-0.48	-0.07	-0.05	-0.58	**-1.14**	**-1.14**
	27.3.22	STMJL63	similar to Homeobox-leucine zipper protein	0.04	0.35	-0.40	0.56	0.36	0.31	0.74	0.21	**0.88**	0.65
	27.3.23	STMIN23	similar to heat shock transcription factor	0.11	0.48	0.07	0.16	0.14	0.29	0.15	0.02	**0.80**	-0.04
	27.3.25	STMCA87	homologue to Myb-like DNA-binding protein	-0.16	-0.79	-0.03	-0.75	-0.28	-0.24	-0.49	-0.01	**-1.02**	-0.18
	27.3.36	STMCE02	weakly similar to Piwi protein	0.20	0.10	0.26	-0.75	-0.38	0.46	-0.08	0.08	**-0.88**	**-0.83**
	27.3.44	STMES33	Adenosine A3 receptor, identical to MOM1 (mutation in a 'Morpheus molecule')	-0.12	-0.69	-0.06	-0.65	-0.36	-0.13	-0.50	0.11	**-1.15**	-0.24
	27.3.59	STMID61	weakly similar to PHD finger-like protein	-0.25	-0.54	-0.47	-0.61	-0.77	-0.49	-0.19	-0.18	**-0.91**	-0.76
	27.3.6	STMCO64	similar to basic helix-loop-helix (bHLH) family protein	0.09	-0.04	0.22	-0.29	-0.60	0.38	-0.37	0.11	**-0.89**	-0.65
	27.3.73	STMGN12	similar to TUDOR, RNA-binding protein	-0.01	0.33	0.06	0.75	0.48	-0.05	0.06	0.26	**0.81**	**0.82**
	27.3.99	STMDB23	MRNA binding protein precursor	0.02	-0.49	0.03	-0.47	-0.35	0.19	-0.58	0.11	**-0.86**	-0.43
	27.3.99	STMCR44	MRNA binding protein precursor	0.08	-0.57	-0.10	-0.58	-0.47	0.20	-0.58	-0.07	**-1.06**	-0.34
8 hr Systemic Only	27.3.12	STMGW14	similar to zinc finger (CCCH-type) family	0.07	-0.04	0.18	0.73	0.62	0.22	-0.14	0.38	0.60	**0.88**

			protein											
	27.3.62	STMCN34	similar to WD-40 repeat protein (MSI2)	0.14	0.18	0.15	0.32	0.69	0.00	0.70	0.46	0.36	**0.91**
Protein Metabolism														
1 hr only	29.2.2	STMDH15	homologue to 40S ribosomal protein	0.27	**0.85**	0.63	0.22	0.15	0.73	0.01	0.23	0.17	-0.01
	29.2.4	STMJD89	similar to Elongation factor TS	-0.30	**-0.83**	-0.20	-0.53	-0.01	-0.35	-0.58	-0.15	-0.18	-0.10
	29.4	STMDH09	homologue to Osmotic stress-activated protein kinase	0.24	**0.95**	0.61	0.34	0.16	0.78	0.04	0.16	0.07	0.03
	29.4	STMHS17	similar to Protein phosphatase 2C	0.07	**1.30**	0.23	0.44	0.02	-0.09	**1.30**	0.40	**0.81**	0.07
	29.5.1	STMDZ58	homologue to SBT2 protein (Subtilisin-like protease)	-0.10	**-1.11**	-0.30	-0.72	0.08	-0.33	-0.44	0.08	-0.53	0.22
	29.5.11.4.2	STMIQ20	similar to IBR, In Between Ring fingers	-0.07	**1.28**	0.42	-0.54	-0.20	-0.28	**1.16**	0.23	-0.14	-0.22
	29.5.11.4.3.2	STMEN90	similar to Kelch repeat-containing F-box family protein	0.03	**0.91**	-0.05	0.23	-0.15	-0.36	**0.84**	0.17	0.38	-0.39
	29.5.4	STMIY85	similar to aspartyl protease family protein	0.08	**1.09**	0.11	-0.07	-0.42	-0.14	**1.15**	0.11	0.07	**-0.84**
	29.6	STMGG82	homologue to Hsp70 protein	0.07	**0.81**	0.33	-0.12	-0.38	0.45	0.65	0.46	-0.08	-0.25
	29.6	STMCH67	homologue to Hsp70 protein	0.13	**1.47**	0.24	0.01	-0.19	0.13	**0.82**	0.47	0.07	-0.23
	29.7	STMGH29	similar to Galactosyltransferase	0.20	**0.82**	0.43	0.43	0.03	0.59	0.22	0.22	-0.03	-0.05
1 & 8 hr	29.2.1.1	STMCU43	weakly similar to 50S ribosomal protein L34, chloroplast precursor	0.00	**-0.88**	-0.08	**-1.11**	-0.58	-0.17	**-0.83**	0.20	**-1.41**	-0.76
	29.2.1.1	STMIW46	similar to ribosomal protein 30s	-0.18	**-0.96**	-0.17	**-0.90**	-0.09	-0.28	-0.57	-0.01	**-1.11**	-0.22
	29.2.1.1	STMHI26	similar to ribosomal protein S17	-0.17	**-0.95**	-0.33	**-0.81**	-0.22	-0.61	-0.64	0.05	-0.76	-0.18
	29.2.2	STMJA14	similar to 60S ribosomal protein L36	-0.29	**-1.10**	-0.46	**-2.55**	**-1.68**	-0.42	-0.55	-0.13	**-2.59**	**-1.65**
	29.2.2	STMCC66	Type I (26 kD) CP29 polypeptide, 60S ribosomal protein L7A (RPL7aB)	-0.33	**-1.21**	-0.50	**-1.93**	**-1.08**	-0.57	-0.50	-0.08	**-2.05**	**-0.96**
	29.3.4.3	STMCV50	similar to vacuolar sorting receptor	0.17	**0.83**	0.30	**1.43**	**1.04**	0.20	0.30	0.42	**1.57**	**1.43**
	29.4.1.56	STMCQ49	weakly similar to protein kinase family protein	0.17	**0.95**	0.67	**0.88**	**1.41**	-0.10	**1.21**	**0.80**	**1.02**	**1.72**
	29.5.11.1	STMGC93	Ubiquitin	0.16	**0.91**	0.57	**0.97**	0.10	0.46	0.41	0.32	0.59	-0.01
	29.5.11.4.2	STMJO85	weakly similar to Zinc finger (C3HC4) protein	-0.08	**-0.99**	-0.27	**-2.18**	**-1.52**	-0.07	-0.69	0.06	**-2.60**	**-1.41**
	29.5	STMCR61	Neutral leucine aminopeptidase preprotein precursor	0.48	**1.17**	0.07	**2.35**	**2.36**	-0.10	-0.48	-0.30	-0.49	-0.56
	29.5	STMGC30	Leucine aminopeptidase A	0.49	**1.00**	0.02	**2.80**	**2.67**	**-1.21**	-0.66	-0.62	**-0.97**	-0.79
	29.5.3	STMGC07	similar to Cathepsin B-like cysteine proteinase	-0.01	**0.86**	0.21	**1.02**	0.49	0.35	0.37	0.02	**1.45**	**0.89**
	29.5.3	STMJB45	homologue to Cathepsin B-like cysteine proteinase	0.00	**0.91**	0.06	**1.03**	0.77	-0.18	0.68	0.24	**2.00**	**1.24**

	29.5.5	STMCK62	weakly similar to serine carboxypeptidase S10 family protein	0.08	**0.95**	0.14	**1.07**	-0.07	0.66	0.52	0.04	**1.27**	0.16
1 hr Systemic & 8 hr	29.5.15	STMCM05	homologue to probable protease inhibitor P322	0.46	0.50	**0.82**	**2.55**	**2.68**	0.54	0.33	0.33	**2.34**	**2.82**
8 hr only	29.1	STMCC51	similar to Palmitoyl protein thioesterase	-0.30	-0.74	-0.36	**-1.07**	-0.31	-0.51	-0.24	-0.08	**-0.84**	-0.29
	29.2.1.1	STMCF33	similar to 30S ribosomal protein S1, chloroplast precursor (CS1)	-0.07	-0.50	-0.10	**-0.88**	-0.74	-0.12	-0.13	0.04	**-1.13**	-0.63
	29.2.1.2	STMCA58	similar to 50S ribosomal protein L21, mitochondrial (RPL21M)	0.10	-0.09	-0.09	**1.58**	**1.53**	-0.13	-0.04	0.09	**2.16**	**1.97**
	29.2.1.99	STMCJ03	similar to ribosomal protein S5 family protein	-0.01	-0.05	0.02	**-1.45**	-0.67	0.03	0.00	0.00	**-1.73**	-0.53
	29.2.2	STMHV38	homologue to 60S ribosomal protein L10 (EQM)	-0.16	-0.62	-0.30	**-1.57**	**-0.93**	-0.48	-0.41	-0.08	**-1.35**	**-1.00**
	29.3.4.3	STMCX65	homologue to Vacuolar processing enzyme-3	0.14	0.24	0.09	**0.86**	0.13	0.22	0.11	0.09	0.64	0.22
	29.4	STMDR26	homologue to Protein kinase, 41K	0.10	0.20	-0.14	**1.16**	0.67	-0.02	-0.22	-0.14	0.64	0.74
	29.4	STMEQ22	similar to Phototropin 2	-0.07	-0.50	-0.32	**-1.03**	**-0.85**	-0.06	-0.14	-0.06	**-0.92**	**-1.07**
	29.4	STMDP27	similar to SOS2-like protein kinase	-0.13	0.04	-0.26	**-0.97**	-0.58	-0.43	0.36	0.08	-0.35	-0.53
	29.4	STMCU58	similar to Phototropin 2	-0.09	-0.48	-0.27	**-0.88**	**-0.87**	0.17	-0.55	-0.11	**-0.95**	**-0.87**
	29.4.1.59	STMGF60	weakly similar to protein kinase family protein	0.10	0.66	0.57	**0.96**	0.32	**0.91**	-0.13	0.22	0.05	-0.42
	29.5.11.20	STMCU19	homologue to Proteasome subunit alpha type 6	0.13	0.32	0.14	**1.09**	0.62	-0.03	0.06	0.15	**1.18**	**0.86**
	29.5.11.4.2	STMHH47	similar to RING, Ring finger; E3 ubiquitin-protein ligase	0.24	-0.01	0.06	**1.47**	**1.66**	0.00	0.08	0.17	**1.18**	**1.76**
	29.5.11.4.3.2	STMGG22	weakly similar to kelch repeat-containing F-box family protein	0.21	0.74	0.56	**0.85**	0.43	0.66	0.28	0.27	0.27	0.40
	29.5.11.4.3.2	STMHK75	similar to Tubby-like protein 3;F-box family protein	0.10	-0.43	-0.59	**1.19**	**1.42**	-0.55	-0.18	-0.21	**1.02**	**1.73**
	29.5.11.4.3.2	STMHN37	weakly similar to Kelch repeat-containing F-box-like protein	0.39	0.32	0.01	**3.12**	**3.20**	-0.13	0.14	0.28	**3.54**	**3.33**
	29.5.11.5	STMEU27	similar to ubiquitin-specific protease 12 (UBP12)	-0.06	-0.52	-0.22	**-1.58**	**-1.27**	-0.03	-0.23	-0.04	**-1.64**	**-1.36**
	29.5.15	STMJA26	Proteinase inhibitor II	0.02	-0.55	-0.11	**0.84**	**1.38**	-0.32	-0.21	0.00	**1.05**	**1.58**
	29.5.15	STMCG57	Metallocarboxypeptidase inhibitor Iia	0.28	-0.07	0.43	**1.59**	**1.86**	0.39	-0.11	0.19	**1.15**	**1.94**
	29.5.15	STMHT35	Proteinase inhibitor II	0.59	0.20	-0.04	**2.75**	**3.14**	-0.13	0.38	-0.06	**3.27**	**3.70**
	29.5	STMDQ06	Neutral leucine aminopeptidase preprotein precursor	0.46	0.71	-0.33	**2.81**	**2.65**	**-1.77**	-0.50	-0.66	**-1.12**	-0.70
	29.5.15	STMCQ55	homologue to Aspartic protease inhibitor 8 precursor (pi8)	0.81	0.36	0.03	**3.18**	**3.41**	0.02	0.27	0.38	**2.47**	**4.02**
	29.5.15	STMIU61	Homologue to proteinase inhibitor I	0.20	0.05	0.30	**3.28**	**3.45**	-0.41	0.28	0.27	**3.36**	**3.82**

	29.5.15	STMCO50	Homologue to proteinase inhibitor I	0.45	0.57	0.56	**3.41**	**3.80**	0.20	0.18	0.42	**3.29**	**3.81**
	29.5.3	STMCJ34	similar to Cysteine protease precursor	0.16	0.32	-0.21	**0.83**	**0.89**	-0.12	-0.43	-0.16	**1.15**	0.78
	29.5.3	STMEU11	homologue to Cathepsin B-like cysteine proteinase	-0.01	0.76	-0.14	**1.05**	0.64	-0.37	0.61	0.21	**2.05**	**1.20**
	29.5.9	STMDF49	similar to AAA-type ATPase family protein, chaperone-like functions	-0.14	0.45	0.17	**-1.16**	**-0.94**	0.10	0.41	0.09	**-0.94**	**-1.27**
	29.5.9	STMGM71	similar to AAA-type ATPase family protein, chaperone-like functions	-0.06	0.09	0.06	**-0.85**	-0.74	-0.14	0.36	0.08	-0.76	**-0.87**
8 hr Systemic only	29.4	STMIT27	similar to Protein kinase	-0.27	-0.66	-0.49	-0.68	**-0.92**	-0.35	-0.44	-0.26	**-0.83**	**-0.94**
	29.5.15	STMIX49	homologue to Cystatin, cysteine protease inhibitor	0.07	0.07	0.21	0.51	**1.04**	-0.22	0.20	-0.06	0.45	0.65
LapA-SI only													
0 hr only	29.2.4	STMGT05	homologue to elongation factor 1-alpha	-0.34	-0.51	-0.51	-0.52	-0.49	**-0.82**	-0.05	-0.32	-0.10	-0.32
	29.5.11.4.2	STMCY59	similar to IBR, In Between Ring fingers	0.22	0.43	0.31	0.10	-0.15	**0.91**	0.42	0.17	-0.05	-0.45
	29.5.4	STMCR83	weakly similar to aspartyl protease family protein	0.42	0.61	0.55	0.49	0.24	**1.45**	0.36	0.34	-0.24	0.13
1 hr only	29.4	STMHO79	similar to protein phosphatase 2C	0.03	0.59	0.16	0.07	-0.12	0.00	**0.81**	0.21	0.35	-0.09
	29.4	STMDU54	homologue to MAP kinase phosphatase	-0.09	0.41	-0.40	0.08	-0.29	-0.32	**0.86**	-0.02	0.05	-0.46
	29.5.11.4.3.2	STMCA77	similar to F-box family protein	-0.25	0.63	0.26	0.26	0.14	0.09	**0.81**	-0.11	0.20	0.60
	29.5.11.4.3.2	STMGU08	similar to Avr9/Cf-9 rapidly elicited protein 189	0.03	0.67	0.02	-0.10	-0.03	-0.01	**0.98**	0.02	0.06	-0.23
	29.5.11.4.3.2	STMFB18	similar to Kelch repeat-containing F-box family protein	-0.24	-0.63	-0.18	-0.25	-0.25	-0.10	**-0.90**	-0.29	-0.41	-0.77
	29.6	STMCM21	similar to probable FKBP-type peptidyl-prolyl cis-trans isomerase 2, chloroplast precursor	0.05	-0.43	-0.22	0.39	0.17	0.30	**-0.84**	-0.34	-0.13	0.03
1 & 8 hr	29.2.1.1	STMCB75	similar to Ribosomal protein L1	-0.13	-0.47	-0.10	-0.53	-0.20	-0.64	**-1.01**	-0.41	**-1.27**	**-1.15**
	29.2.1.1	STMCX09	similar to Ribosomal protein L1	0.05	-0.30	0.03	-0.41	-0.16	-0.57	**-0.91**	-0.21	**-1.08**	**-0.97**
	29.5.11.4.3.2	STMFB16	similar to Kelch repeat-containing F-box family protein	-0.18	-0.40	-0.05	-0.06	-0.32	0.28	**-0.99**	-0.31	-0.50	**-0.81**
	29.5.5	STMJP52	similar to serin carboxypeptidase-like protein	0.10	0.64	0.13	0.52	0.78	-0.04	**0.91**	0.56	**0.96**	**1.06**
8 hr only	29.2.1.99	STMCG60	similar to plastid ribosomal protein L19,	-0.09	-0.55	0.03	-0.55	-0.22	-0.10	-0.63	0.05	**-1.02**	-0.33
	29.2.1.99	STMCZ29	homologue to 50S ribosomal protein L27, chloroplast precursor (CL27)	0.14	-0.36	0.06	-0.51	-0.21	-0.06	-0.58	0.10	**-0.83**	-0.15
	29.2.2	STMER23	weakly similar to 60S ribosomal protein L13-1 (Cold induced protein C24A)	-0.06	-0.09	-0.01	-0.45	-0.65	-0.19	-0.34	-0.19	**-0.84**	**-0.83**
	29.2.3	STMEW29	similar to eukaryotic translation initiation factor 3 subunit 3	0.02	-0.13	0.06	-0.74	-0.53	0.15	-0.12	-0.01	**-0.93**	-0.58

	29.4	STMIG40	similar to protein phosphatase type 2C	-0.05	-0.21	0.03	-0.73	-0.16	-0.16	-0.17	0.01	**-0.85**	-0.43
	29.4	STMIM14	similar to protein kinase family protein / Ire1 homolog-2 (IRE1-2)	-0.20	-0.48	-0.06	-0.57	-0.23	-0.17	-0.22	0.00	**-0.82**	-0.21
	29.5	STMIX54	similar to ATP-dependent protease	-0.04	0.23	-0.10	0.30	0.02	0.10	0.64	0.32	**1.20**	0.25
	29.5.11.4.2	STMJF07	similar to RING, Ring finger; E3 ubiquitin-protein ligase	0.03	0.23	-0.01	0.60	0.06	0.08	0.26	0.13	**0.84**	0.20
	29.5.3	STMHO21	similar to Cathepsin B-like cysteine proteinase	-0.05	0.15	0.00	0.46	0.36	-0.21	0.22	-0.03	**1.04**	0.53
	29.5.3	STMEI96	similar to Cathepsin B-like cysteine proteinase	-0.02	0.41	-0.02	0.75	0.52	-0.21	0.37	0.12	**1.34**	0.74
	29.5.3	STMCI03	weakly similar to OTU-like cysteine protease	0.23	-0.22	0.25	-0.48	-0.13	0.37	-0.53	0.28	**-0.87**	-0.24
	29.6	STMCM38	similar to Peptidyl-prolyl cis-trans isomerase, chloroplast precursor	-0.07	-0.67	-0.05	-0.61	-0.20	0.12	-0.71	0.04	**-1.13**	-0.42
8 hr Systemic only	29.3.4.3	STMGI03	similar to vacuolar sorting receptor	0.05	0.28	0.11	0.56	0.63	-0.03	0.17	0.12	0.67	**0.96**
	29.4	STMCR96	similar to Serine/threonine kinase	0.03	0.28	0.21	-0.05	-0.28	0.31	-0.33	-0.24	-0.41	**-0.90**
	29.4	STMCZ26	similar to SOS2-like protein kinase	0.03	0.77	0.32	-0.55	-0.49	0.24	0.56	0.17	-0.27	**-0.94**
	29.5.1	STMJP90	homologue to Subtilisin-like protease	0.00	-0.34	-0.17	0.72	0.66	0.02	-0.03	0.13	0.54	**0.86**
	29.5.11.4.2	STMII86	similar to zinc finger (C3HC4-type RING finger) family protein	-0.22	-0.32	-0.55	-0.32	-0.47	-0.22	-0.67	-0.59	-0.63	**-0.84**
	29.5.11.4.2	STMIA79	similar to zinc finger (C3HC4-type RING finger) family protein	-0.29	0.56	0.10	-0.35	-0.35	-0.51	0.07	-0.17	0.22	**-0.91**

[A] DEGs (|FC| ≥0.8; p <0.05) are indicated in bold and shaded in grey. DEGs that are down-regulated are boxed in.
[B] The DEGs in this table were not manually annoted and genes with duplicate spots or gene family member-specific spots were not determined.

Table 2.6 General metabolism genes differentially expressed in WT and *LapA-SI* leaves after wounding.[A]

BIN Name	BIN #	Clone ID[B]	Annotation	WT 0 hr	WT 1hr L	WT 1hr S	WT 8hr L	WT 8hr S	*LapA-SI* 0 hr	*LapA-SI* 1hr L	*LapA-SI* 1hr S	*LapA-SI* 8hr L	*LapA-SI* 8hr S
Cell Wall	10.6.3	STMEA11*	LeXYL1 protein (β-d-xylosidase)	-0.26	**-1.15**	-0.60	-0.49	-0.63	-0.21	**-1.24**	-0.59	**-0.93**	**-0.91**
	10.6.3	STMIQ06	LeXYL2 protein (β-d-xylosidase)	-0.15	**-0.96**	-0.52	-0.34	-0.33	-0.35	**-0.84**	-0.55	-0.75	-0.67
	10.7	STMEP06	Similar to BR-regulated protein BRU1 (Xyloglucan Endotransglycosylase; XTH16)	0.02	**-0.88**	-0.49	**-1.80**	**-0.93**	-0.50	**-0.81**	-0.30	**-1.27**	**-1.24**
	10.7	STMEI79	Probable xyloglucan endotransglucosylase/hydrolase 1 (LeXTH1)	-0.09	-0.33	-0.12	**-1.06**	-0.59	-0.38	-0.69	-0.11	**-1.33**	**-1.07**
	10.7	STMCV68	Xyloglucan endotransglucosylase-hydrolase (XTH7)	-0.14	-0.72	-0.27	**-0.89**	-0.46	0.07	-0.69	-0.36	**-1.14**	**-1.21**
	10.7	STMCX13	Homologue to xyloglucan endotransglucosylase-hydrolase (XTH5)	-0.16	-0.13	-0.57	-0.59	**-1.11**	0.05	-0.79	-0.59	-0.53	**-1.40**
	10.6.3	STMGL07	Homologue to pectate lyase	-0.34	**-1.06**	-0.62	**-0.91**	-0.51	-0.78	-0.62	-0.50	**-0.83**	-0.71
	10.5.2	STMGL17	Homologue to proline-rich protein	0.04	**-0.80**	-0.62	**-1.28**	-0.50	-0.49	-0.50	-0.10	**-0.85**	-0.53
	10.5.2	STMJI39	Homologue to proline-rich protein	0.08	-0.40	-0.64	**-0.86**	-0.41	-0.32	-0.38	-0.13	-0.67	-0.46
Lipid Metabolism	11.9.2	STMEU40	Similar to Lipase-like protein	0.01	**0.83**	0.11	**1.46**	**1.08**	-0.04	0.46	0.11	**1.07**	**1.15**
	11.2	STMGH41	Homologue to FAD2 (FAD2.1)	0.28	**0.88**	0.25	0.59	0.18	**1.15**	0.72	0.22	0.67	0.08
	11.2	STMES31	Homologue to FAD2 (FAD2.2)	0.02	-0.51	-0.07	**-1.33**	**-0.95**	0.12	-0.35	0.13	**-1.65**	**-1.07**
	11.2	STMCP71	Delta 9 desaturase (homologue to FAD5)	0.01	-0.32	0.10	**-1.42**	**-1.34**	0.35	-0.12	0.35	**-1.41**	**-1.82**
	11.2	STMEI66*	Similar to plastidial delta-12 oleate desaturase; FAD6	-0.11	-0.30	-0.09	**-1.98**	**-1.33**	0.12	-0.16	0.06	**-1.53**	**-1.60**
Amino Acid Synthesis	13.1.1	STMCG42*	Similar to alanine:glyoxylate aminotransferase	-0.12	**-0.92**	-0.48	-0.24	-0.79	-0.23	-0.65	-0.48	-0.47	-0.47
	13	STMEY02	Similar to putative alanine aminotransferase	-0.20	-0.74	-0.24	**-0.83**	-0.78	-0.30	-0.28	-0.35	**-0.82**	**-0.94**
	13	STMCE75	Similar to anthranilate synthase alpha subunit (AS1)	0.04	0.27	-0.03	**0.83**	0.23	0.27	-0.22	-0.26	0.26	0.20
	13.1.3.4	STMCJ60*	S-adenosylmethionine synthetase 1	0.04	0.40	0.02	**1.28**	**0.80**	0.03	0.44	0.39	**1.10**	**1.26**
	13.1.1	STMCJ20	Gamma-aminobutyrate transaminase isozyme 1	0.19	**1.04**	0.35	0.63	0.35	0.11	0.64	0.45	**0.81**	0.50
	13.1.1	STMGU10	Homologue to gamma-aminobutyrate transaminase isozyme 2	0.07	-0.25	0.08	0.59	**0.90**	-0.34	0.07	0.40	**1.14**	**1.26**
	13.1.1	STMGU61*	Similar to probable acetylornithine	0.12	**1.49**	0.16	**1.56**	**0.97**	-0.01	**1.13**	0.22	**0.83**	**0.83**

			aminotransferase												
Isoprenoids	16.1	STMDU15*	Homologue to isopentenyl diphosphate isomerase 1	0.28	0.26	0.26	**1.14**	0.56	0.29	0.23	0.32	0.65	**0.80**
	16.1	STMEF65	Farnesyl pyrophosphate synthase	-0.19	**-0.90**	-0.90	-0.72	-0.21	-0.44	-0.41	-0.33	**-0.82**	-0.18
	16.1	STMCA79	Sesquiterpene synthase 1	-0.18	-0.63	-0.63	**-0.82**	0.05	-0.14	-0.33	0.12	-0.42	-0.04
	16.1	STMCV10*	Similar to phytoene synthase 1	-0.11	**-0.91**	-0.91	**-0.93**	-0.73	-0.06	-0.61	-0.01	-0.70	-0.58
	16.1	STMDH72	Homologue to β-carotene hydroxylase	-0.07	-0.40	-0.40	**-1.22**	**-0.94**	0.04	-0.26	-0.11	**-1.02**	**-1.38**
Phenylpropanoids	16.2	STMJE63*	Homologue to tyramine hydroxycinnamoyl transferase	-0.02	**1.13**	-0.10	0.53	-0.08	0.10	**0.92**	-0.24	0.57	-0.38
	16.2	STMGC82	Weakly similar to 4-coumarate-CoA ligase-like	0.06	**1.27**	0.54	0.64	0.17	0.43	0.66	0.31	0.32	0.04
	16	STMCR27	Chorismate synthase 1	0.09	**0.82**	0.37	0.76	0.32	0.17	0.17	0.14	0.29	0.29
	16.2	STMDR02	Similar to 4-coumarate-CoA ligase-like	0.13	**2.92**	0.84	**0.85**	0.39	0.28	**2.44**	**1.05**	0.65	0.56
	16.2	STMIY78*	Similar to 4-coumarate-CoA ligase-like protein	-0.36	**-0.97**	-0.86	**-2.06**	**-1.53**	-0.79	-0.36	-0.78	**-1.68**	**-2.09**
	16.2	STMCS41	Homologue to cinnamic acid 4-hydroxylase	0.05	**1.45**	0.67	**0.88**	**1.04**	-0.07	**0.91**	0.68	**1.55**	**1.45**
	16.2	STMIC76*	p-coumaroyl shikimate 3'-hydroxylase isoform 2	0.13	**0.87**	0.45	**1.23**	**1.11**	-0.05	**0.96**	0.64	**1.11**	**1.58**
	16.2	STMES07	Homologue to hydroxycinnamoyl transferase	0.20	**0.93**	0.45	**1.40**	**1.47**	-0.10	0.65	0.52	**1.31**	**1.79**
	16.8	STMGP82*	Weakly similar to caffeoyl-CoA O-methyltransferase	0.29	0.64	0.25	**1.55**	**1.58**	0.28	0.58	0.15	**1.60**	**1.96**
	16	STMGC95	Weakly similar to 3-dehydroquinate dehydratase	0.12	0.59	0.59	**1.12**	**0.86**	0.78	-0.13	0.11	0.33	0.69
	16.2	STMDZ37	Similar to cinnamoyl-CoA reductase-like protein	0.04	0.52	0.18	**1.18**	-0.03	0.78	-0.13	0.11	0.33	0.69
	16.2	STMCC79	Similar to Caffeic acid 3-O-methyltransferase	0.00	-0.48	0.04	**-0.87**	-0.19	-0.12	0.24	0.29	-0.31	0.16
Flavanoids	16.8	STMHP28	Homologue to chalcone synthase 2	-0.14	**-1.09**	-0.23	**-1.11**	-0.74	-0.09	-0.44	-0.20	**-1.11**	**-0.81**
	16.8	STMCQ37	Weakly similar to leucoanthocyanidin dioxygenase-like protein	0.05	**1.71**	0.51	**2.53**	**1.36**	0.18	**1.43**	0.54	**2.53**	**1.89**
	16.8	STMIU56	Similar to chalcone-flavonone isomerase	-0.21	-0.46	-0.03	**-0.90**	-0.34	-0.25	-0.28	0.09	-0.57	-0.55
	16.8	STMCK87	Similar to malonyltransferase	-0.02	0.13	0.26	-0.62	**-0.85**	0.37	-0.58	0.18	**-0.87**	**-1.12**
Redox	21	STMCQ83	Superoxide dismutase [Cu-Zn] 1 (cytosolic)	0.13	**0.81**	0.59	0.27	-0.03	0.20	0.53	0.46	0.62	0.02
	21	STMJG34	Superoxide dismutase [Cu-Zn] 2 (plastid)	-0.18	-0.69	-0.20	**-1.10**	-0.29	-0.44	-0.26	0.07	-0.42	-0.08
	21	STMDC64	Homologue to putative glutathione peroxidase	0.02	**0.94**	0.39	0.17	-0.16	0.16	0.17	0.05	0.30	-0.50
	21	STMJA50*	Homologue to thioredoxin peroxidase	-0.12	**-0.80**	-0.34	**-0.82**	-0.26	-0.31	-0.26	-0.02	**-0.84**	-0.26
	21	STMCV49	Similar to chloroplast thioredoxin	0.08	-0.22	0.21	**-1.34**	-0.70	0.26	-0.53	0.27	**-1.10**	**-0.87**
	21	STMIS65	Probable glutathione S-transferase	-0.35	-0.43	-0.33	**-1.09**	-0.62	-0.46	-0.12	-0.30	-0.76	**-0.86**
	21	STMEW65*	Catalase isozyme 2 (peroxisome)	0.00	0.71	-0.12	**1.79**	0.47	0.24	0.09	-0.33	**1.36**	0.54
	10.7	STMGH65	Cytosolic ascorbate peroxidase	0.29	0.67	0.23	**1.52**	**0.96**	0.22	0.40	0.39	**1.30**	**1.15**

	21	STMDP29	Homologue to disulfide isomerase-like protein (ER/wall/vac)	0.08	0.31	0.02	**0.97**	0.49	-0.18	0.13	0.28	**1.44**	**0.97**
	21	STMDF17	Similar to protein disulfide-isomerase precursor (multiple loc)	0.15	0.49	0.20	**1.24**	**1.21**	0.33	0.22	0.35	**1.17**	**1.62**
Polyamines	22	STMCF12	Arginine decarboxylase	0.32	**1.01**	0.26	0.77	-0.01	0.70	0.24	0.02	0.06	-0.37
	29.5	STMHR62*	Similar to N-acetylornithine deacetylase-like protein	0.16	0.38	0.08	**1.98**	**1.84**	-0.38	0.68	0.49	**1.84**	**2.26**
	22	STMHW26	Similar to agmatine iminohydrolase	-0.28	**-0.91**	-0.20	**-0.97**	-0.34	-0.42	-0.41	-0.01	**-1.09**	-0.26
	22	STMCV17*	Spermidine synthase	0.41	**0.84**	0.56	**2.16**	**2.21**	0.32	0.68	0.64	**2.02**	**2.74**
	22.1.2	STMIN52*	S-adenosylmethionine decarboxylase proenzyme	0.24	0.55	0.38	**2.06**	**1.98**	0.02	0.65	0.62	**2.74**	**2.24**
	13	STMDV29	Ornithine decarboxylase	0.34	0.41	0.47	0.67	**1.11**	0.05	0.74	0.60	0.50	**1.45**

[A] DEGs (|FC| ≥0.8; $p < 0.05$) are indicated in bold and shaded in grey. DEGs that are down regulated are boxed in.
[B] Some genes were representated by multiple cDNA clones (spots) on the 10-K array. Only one representative clone for these genes appears in Tables 1-3. The identity of the redundant clones is provided here.

Table 2.7 Summary of hormone metabolism and response genes differentially expressed in response to wounding in WT and *LapA-SI* leaves.[A]

			WT					*LapA-SI*				
			0 hr	1 hr		8 hr		0 hr	1 hr		8 hr	
BIN	Clone ID[B]	Description		L	S	L	S		L	S	L	S
17.1 Abscisic Acid												
	STMGC08	9-*cis*-epoxy-carotenoid dioxygenase 2	0.11	**0.94**	0.34	0.45	0.42	0.38	**0.82**	0.08	0.49	0.42
17.2 Auxin												
	STMIW94*	Similar to IAA amidohydrolase-like	-0.04	**1.72**	0.25	0.09	0.07	0.02	**1.62**	0.59	0.39	0.33
	STMCM67	Similar to IAA-amino acid hydrolase 3	0.19	**1.85**	0.67	**2.78**	1.24	0.22	**1.25**	0.57	**2.41**	**1.73**
	STMGN14	Similar to IAA-amino acid hydrolase 6	0.13	**0.91**	0.33	**0.81**	**0.87**	-0.04	**0.88**	0.62	**0.97**	**1.10**
	STMDP46	Homologue to Nt-iaa2.3 deduced protein	-0.10	-0.23	0.12	**-0.89**	-0.40	-0.02	-0.12	0.00	-0.65	-0.35
	STMGW46	Homologue to Nt-iaa28 deduced protein	-0.32	-0.72	-0.36	**-1.03**	-0.29	-0.54	-0.39	-0.20	-0.61	-0.33
	STMJA37*	Aromatic amino acid decarboxylase 1A	0.15	0.76	0.04	0.14	**0.83**	0.00	**1.31**	0.09	0.13	0.39
17.3 Brassinosteroid												
	STMGL44	Similar to squalene monooxygenase 1	-0.07	-0.48	-0.36	**-0.92**	-0.66	-0.12	-0.05	0.00	**-0.80**	-0.69
	STMCQ85*	Homologue to putative C-8 7 sterol isomerase	0.20	0.02	0.11	**1.25**	0.60	-0.20	0.09	0.34	**0.96**	**1.12**
	STMGG42	DWARF1/DIMINUTO	0.12	0.45	0.30	**1.27**	**0.82**	0.19	0.13	0.41	**0.86**	**1.24**
	STMCF04*	Similar to DWARF1/DIMINUTO1	0.20	0.33	0.26	**1.33**	**1.03**	0.10	0.18	0.45	**1.48**	**1.52**
	STMGY73	Steroid 5 alpha reductase (DET2)	0.17	**0.80**	0.41	0.63	0.70	-0.15	**0.85**	0.56	0.66	1.03
17.5 Ethylene												
	STMER30	Homologue to aminocyclopropane-1-carboxylate oxidase	-0.15	0.57	-0.21	**0.93**	0.11	-0.29	0.44	-0.07	**1.23**	0.26
	STMIK39	Homologue to ethylene response factor 5 (ER5)	-0.08	-0.64	-0.25	0.09	**0.91**	-0.08	-0.50	-0.41	-0.27	0.13
17.6 Gibberellic Acid												
	STMCY85	Similar to ent-kaurenoic acid oxidase	0.22	**0.90**	0.44	**1.26**	**0.96**	0.40	0.47	0.60	**1.39**	**1.49**
	STMJC16*	GAST1 protein precursor	-0.20	**-1.35**	-0.32	**-0.99**	0.07	-0.48	-0.45	-0.22	**-1.11**	-0.07
17.8 Salicylic Acid												
	STMEP60	Similar to NPR1-interactor protein TGA2	-0.12	0.77	-0.03	**0.86**	-0.28	0.22	0.28	-0.26	**1.10**	-0.72

[A] DEGs (|FC| ≥0.8; p <0.05) are indicated in bold and shaded in grey. DEGs that are down-regulated are boxed in.
[B] Some genes are represented by multiple clones on the TIGR 10-K (version 3) potato cDNA microarray. These genes are indicated with an asterisk. One representative clone shown. Redundant clones can be found in Table S1.

Table 2.8 Genotype DEGs at 1 and 8 hr after wounding.[A]

				WT					*LapA-SI*				
				0 hr	1 hr		8 hr		0 hr	1 hr		8 hr	
	BIN #	Clone ID[B]	Annoation		L	S	L	S		L	S	L	S
1-hr Local													
	34	STMDJ91	Similar to vacuolar proton pump	0.04	**0.17**	0.00	0.28	0.16	-0.81	**-1.19**	-0.72	-0.63	-0.66
	34	STMFB95	Homologue to chloride channel protein	0.11	**0.11**	0.12	0.15	0.03	-0.70	**-1.03**	-0.44	-0.50	-0.76
8-hr Local													
	20	STMGZ34	Heat shock cognate protein 80	-0.11	-0.14	-0.01	**-0.72**	-0.37	-0.05	0.12	0.07	**-0.05**	-0.30
	20	STMIB24	Similar to DnaJ-like protein	-0.22	-0.32	-0.41	**0.29**	-0.35	-0.02	-0.83	-0.47	**-0.63**	-0.91
	29	STMIX54	Similar to ATP-dependent Clp proteolytic subunit (ClpP)	-0.04	0.23	-0.10	**0.30**	0.02	0.10	0.64	0.32	**1.20**	0.25
	29	STMIX83	Similar to 60S ribosomal protein L6	-0.23	-0.48	-0.13	**-0.79**	-0.28	-0.62	-0.22	0.01	**0.04**	0.00
	20/29	STMEU11	Homologue to cathepsin B cysteine proteinase	-0.01	0.76	-0.14	**1.05**	0.64	-0.37	0.61	0.21	**2.05**	1.20
	10.2	STMCZ18	Endo-1,4-beta-glucanase	0.17	0.19	0.23	**0.23**	0.02	0.49	0.00	0.24	**-0.39**	-0.08
	34	STMCC78[B]	Similar to ABC transporter F family protein 5	-0.39	-0.61	-0.60	**-1.86**	-1.55	-0.53	-0.16	-0.19	**-0.69**	-1.59
	25	STMEA95	Weakly similar to carboxylesterase	-0.16	0.19	-0.12	**0.29**	-0.10	0.17	0.40	0.05	**1.30**	0.51
	26	STMHX28	Similar to UDP-glycosyltransferase	-0.20	-0.18	-0.36	**-0.31**	-0.32	0.06	-0.06	-0.36	**0.64**	-0.06
8-hr Systemic													
	10.7	STMHG34	Xyloglycan endo-transglycosylase	0.06	-0.01	-0.38	-0.19	**0.48**	0.12	0.02	-0.45	-0.59	**-0.61**
	35	STMGV86	Similar to hypothetical protein VITISV_011279	0.05	0.09	-0.16	0.05	**0.04**	0.20	-0.75	-0.37	-0.82	**-0.99**
8-hr Local & Systemic													
	29	STMCB75	Similar to 50S ribosomal protein L1	-0.13	-0.47	-0.10	**-0.53**	**-0.20**	-0.64	-1.01	-0.41	**-1.27**	**-1.15**

[A] At each experimental point, the|FC| ≥0.8 ($p<0.05$) of each gene was determined. gDEGs were identified by comparing gene signals in the WT and *LapA-SI* lines. Those signals that were significantly different between the genotypes (gDEGs) at each timepoint are indicated in bold. Down-regulated gDEGs are boxed in. gDEGs that have RNAs at lower levels in *LapA-SI* relative to WT are in light grey. gDEGs that have RNAs at higher levels in LapA-SI relative to WT are in dark grey.
[B] Two cDNAs (STMCC78 and STMDP40) representing the ABC transporter F family protien 5 gene were on the TIGR 10-K (version 3) microarray. Signals from STMCC78 are shown.

Table 2.9 Top 100 differentially expressed genes at 1 hr after injury of *LapA-SI* relative to WT leaves.[A]

Bin #	Bin Name[B]	Clone name	Name	FC	p-val
29	Protein (stress)C	STMGC30	Leucine aminopeptidase 2, chloroplast precursor	**-1.66**	0.00
10.5	Cell wall	STMEP76	Cell wall protein precursor	**-1.40**	0.22
20	Stress	STMCY79	Osmotin-like protein OSML13 precursor	**-1.37**	0.38
10.6	Cell wall (Stress)	STMEF73	Putative polygalacturonase	**-1.37**	0.22
34	Transport	STMDJ91	Vacuolar ATP synthase subunit D	**-1.36**	0.00
20	Stress	STMFB93	Basic PR-1 protein precursor	**-1.25**	0.31
20	Stress	STMFB60	Pathogenesis related protein isoform b1 precursor	**-1.15**	0.55
16	Secondary metabolism	STMET49	Cell wall protein precursor	**-1.15**	0.39
34	Transport	STMFB95	Similar to Chloride channel Stclc1	**-1.13**	0.04
35	Not assigned	STMHG96	Octicosapeptide/Phox/Bem1p family protein	**-1.11**	0.39
10.5	Cell wall	STMIX33	Xanthine dehydrogenase-like protein	**-1.08**	0.40
35	Not assigned	STMCV94	Nucleoporin-like protein	**-1.07**	0.36
20	Stress	STMFB44	Prb-1b	**-1.06**	0.29
26	Stress[D]	STMEH13	Glucan endo-1,3-beta-glucosidase, basic isoform 2 precursor	**-1.03**	0.49
35	Not assigned	STMCE46	Similar to unknown protein	**-1.00**	0.22
20/33	Stress(Development)	STMGA34	Ethylene-responsive late embryogenesis-like protein (ER5)	**-0.99**	0.31
35	Not assigned	STMGC87	None	**-0.98**	0.39
30	Signaling	STMDJ33	Mitogen-activated protein kinase	**-0.98**	0.39
20	Stress	STMCS25	Similar to Early nodulin 12B precursor	**-0.96**	0.37
20	Stress	STMCP16	Proteinase inhibitor type-2 TR8 precursor	**-0.95**	0.45
35	Not assigned	STMFA10	Similar to Twin LOV protein 1	**-0.95**	0.33
27/33	RNA/Development	STMFB46	Putative NAC domain protein NAC2	**-0.93**	0.24
35	Not assigned	STMEQ20	Expressed protein	**-0.92**	0.36
20	Stress	STMIX82	Endochitinase 2 precursor	**-0.92**	0.39
27	RNA	STMDH75	MYBR29-like	**-0.92**	0.38
33	Development	STMGF23	Putative calcium binding protein	**-0.92**	0.27
20	Stress	STMIS93	Homologue to Endochitinase A precursor	**-0.90**	0.40
29	Protein(Stress)	STMDH09	Similar to osmotic stress-activated protein kinase	**-0.90**	0.45
35	Not assigned	STMCZ47	None	**-0.89**	0.51
26	Misc	STMCK41	Similar to BYJ15	**-0.89**	0.06
35	Not assigned	STMGA81	None	**-0.89**	0.40
27	RNA	STMDH93	DDT domain-containing protein	**-0.88**	0.50
11.9	Lipid metabolism	STMCF50	Similar to EEF53 protein	**-0.87**	0.36
35	Not assigned	STMGB47	Putative xylose isomerase	**-0.87**	0.27
27	RNA	STMGA35	Hypothetical protein	**-0.86**	0.47
35	Not assigned	STMGE86	Similar to Protein SET DOMAIN GROUP 41	**-0.86**	0.39
15	Metal handling	STMCO76	Metallothionein	**-0.85**	0.48
35	Not assigned	STMCP59	Domain of Unknown Function (DUF926)	**-0.85**	0.72
35	Not assigned	STMGV86	Similar to unknown protein	**-0.84**	0.16
29	Protein	STMDH15	P40-like protein	**-0.84**	0.39
27	RNA	STMGB83	BEL1-related homeotic protein 30	**-0.83**	0.30

35	Not assigned	STMCS73	None	**-0.83**	0.54
35	Not assigned	STMGA75	Hypothetical protein	**-0.82**	0.50
13	Amino acid metabolism	STMGA53	Similar to Ornithine carbamoyltransferase, chloroplast precursor	**-0.82**	0.50
26	Misc	STMFB75	Short chain dehydrogenase	**-0.82**	0.18
31	Cell	STMCR57	Cyclophilin ROC7-like	**-0.81**	0.47
35	Not assigned	STMCN87	None	**-0.81**	0.38
29	Protein	STMGF60	U-box domain, putative	**-0.80**	0.36
29/30	Protein/Signaling	STMGA95	SNF1-related protein kinase	**-0.79**	0.36
34	Transport	STMGH95	Similar to KEA6; potassium:hydrogen antiporter	**-0.78**	0.38
35	Not assigned	STMCK09	Putative Cdc2-related protein kinase CRK2	**-0.78**	0.42
35	Not assigned	STMGA83	Probable gibberellin receptor GID1L2	**-0.78**	0.47
35	Not assigned	STMGH27	Putative tyrosine-specific transport protein	**-0.78**	0.57
35	Not assigned	STMCM08	None	**-0.78**	0.47
21	Redox	STMDC64	Probable phospholipid hydroperoxide glutathione peroxidase	**-0.77**	0.22
35	Not assigned	STMGC45	None	**-0.77**	0.43
13/22	Polyamine metabolism	STMCF12	Arginine decarboxylase	**-0.77**	0.50
35	Not assigned	STMCH05	None	**-0.76**	0.55
29	Protein	STMCK53	None	**-0.75**	0.55
29	Protein	STMCJ34	Cysteine protease	**-0.74**	0.39
27	RNA	STMCM32	DNA-binding protein	**-0.74**	0.37
27	RNA	STMCF95	Similar to Phytochrome Interacting Factor 3	**-0.74**	0.49
17.2/27	Hormone metabolism/RNA	STMGB57	Similar to Nt-iaa28 deduced protein	**-0.74**	0.38
35	Not assigned	STMCV46	Kinesin related protein	**-0.74**	0.53
20	Stress	STMFB59	Class IV chitinase	**-0.74**	0.38
28	DNA	STMGB89	Putative RNA helicase	**-0.74**	0.48
28	DNA	STMGG87	Similar to DNA mismatch repair protein	**-0.74**	0.50
29	Protein	STMEZ18	Chloroplast protease precursor	**-0.74**	0.40
35	Not assigned	STMGC88	None	**-0.73**	0.56
35	Not assigned	STMGG63	None	**-0.73**	0.55
20	Stress	STMCS03	Similar to STS14 protein precursor	**-0.73**	0.48
35	Not assigned	STMHG92	None	**-0.73**	0.40
31	Cell	STMGA77	Similar to kinesin-related protein (MKRP1)	**-0.73**	0.54
35	Not assigned	STMCR11	Probable signal peptidase complex subunit 2	**-0.73**	0.47
4	Glycolysis	STMIR25	Similar to Glyceraldehyde-3-phosphate dehydrogenase	**-0.72**	0.43
13	Amino acid metabolism	STMGC95	3-dehydroquinate dehydratase / shikimate dehydrogenase	**-0.72**	0.58
29	Protein	STMGH33	N-acetylglucosaminyltransferase	**-0.72**	0.29
16	Secondary metabolism	STMGA58	Similar to caffeic acid O-methyltransferase	**-0.72**	0.54
35	Not assigned	STMJJ90	Putative purine nucleotide binding protein	**0.72**	0.35
16	Secondary metabolism	STMCC79	Similar to Catechol O-methyltransferase	**0.72**	0.07
8	TCA / org. transformation	STMIW27	Similar to Carbonic anhydrase	**0.73**	0.39
2/34	Major CHO metabolism/Transport	STMEJ57	Similar to Glucose-6-phosphate/phosphate translocator 2	**0.73**	0.35
16	Secondary metabolism	STMEH59	Similar to 10-hydroxygeraniol oxidoreductase	**0.73**	0.06

30	Signaling	STMEU56	Similar to Lectin-like protein kinase	**0.74**	0.33
1	Photosynthesis	STMIY69	Light harvesting chlorophyll a /b binding protein	**0.74**	0.37
35	Not assigned	STMGJ82	Similar to NTRB (NADPH-dependent thioredoxin reductase B)	**0.75**	0.22
26	Misc	STMGM02	Similar to Secretory peroxidase	**0.76**	0.01
1	Photosynthesis	STMCI15	Similar to Glyceraldehyde-3-phosphate dehydrogenase B,	**0.77**	0.15
35	Not assigned	STMJN07	Esterase-related	**0.77**	0.08
31	Cell	STMCA54	Kinesin, putative	**0.80**	0.20
34	Transport	STMHE26	Similar to CAX	**0.81**	0.15
35	Not assigned	STMJB37	None	**0.81**	0.24
29	Protein	STMEF10	Similar to Putative casein kinase II catalytic (Alpha) subunit	**0.83**	0.08
26	Misc	STMCK85	Putative GDSL-motif lipase/acylhydrolase	**0.84**	0.24
1	Photosynthesis	STMIY56	Chlorophyll a-b binding protein CP24 10A, chloroplast precursor	**0.85**	0.15
35	Not assigned	STMGO63	Similar to En/Spm-like transposon protein	**0.86**	0.24
35	Not assigned	STMEN59	Similar to En/Spm-like transposon protein	**0.88**	0.37
17.6	Hormone metabolism	STMJC16	GAST1 protein precursor	**0.90**	0.24
5	Fermentation	STMJA78	Similar to Aldehyde dehydrogenase 1 precursor	**1.03**	0.06
35	Not assigned	STMEL61	Similar to Protodermal Factor 1	**1.04**	0.10

[A] The top 100 cDNA spots with largest fold changes (|FC|=0.72-1.66) at 1 hr after wounding in *LapA-SI* leaves were identified. The significance of signal changes in LapA-SI relative to WT plants at 1 hr after wounding is indicated (p value). Due to the redundancy in cDNA spots (see footnotes C-D), the top 103 spots were examined.

[B] MapMan often placed genes in multiple BINs; therefore multiple designations are indicated. It should be noted that there was no extensive manual annotation of the cDNAs that appear in this table. Therefore, spots with similar names may encode for RNAs from the same gene.

[C] Three *Lap* spots were indentified in the top 100 spots. *LapA* (STMDQ06) and two spots called out as LapN (STMGC30, STMCR61). LapN was annotated as an up-regulated DEG. This is in striking contrast to the previous studies monitoring the rare-class *LapN* RNAs by RNA blots or RNase protection studies or monitoring LAP-N protein levels (Chao et al. 2000; Tu et al. 2003). It is likely that the abundant wound-induced *LapA* transcript cross-hybridized to the *LapN* ESTs on the array, since the potato *LapN* EST has 95.6% and 88.9% identity with the tomato *LapN* and *LapA*, respectively. For this reason only the *LapA* spots are shown.

[D] *GluB2* was placed in the Misc Bin by MapMan. This is a pathogen induced cDNA and was recategorized to stress. Two spots for GluB were in the top 100 (STMEB78, STEMEH13). Only the STEMEH13 data is shown.

Table 2.10 Top 100 differentially expressed genes at 8 hr after injury of *LapA-SI* relative to WT leaves.[A]

BIN #	BIN title[B]	Clone name	Name	FC	p-val
29	Protein (stress)[C]	STMDQ06	Neutral leucine aminopeptidase preprotein precursor	**-3.93**	0.00
20	Stress	STMCP56	Catechol oxidase B, chloroplast precursor	**-1.30**	0.25
20	Stress[D]	STMCI55	Polyphenol oxidase (similar to tomato PPO-B)	**-1.11**	0.26
35	Transport	STMCV94	Nucleoporin-like protein	**-1.09**	0.24
35	Not Assigned	STMEZ06	None	**-1.04**	0.21
35	Not Assigned	STMGF46	Similar to Os06g0298500	**-1.03**	0.23
33	Development	STMCY35	Hypothetical protein T11I11.190	**-1.02**	0.14
35	Not Assigned	STMCZ24	None	**-1.02**	0.28
28	DNA	STMGG77	Exonuclease-like protein	**-1.02**	0.42
27	RNA	STMGA35	Hypothetical protein	**-1.00**	0.31
35	Not Assigned	STMGA93	None	**-0.97**	0.23
35	Not Assigned	STMDH05	Hypothetical protein	**-0.96**	0.50
31	Cell	STMGG29	Hypothetical protein	**-0.94**	0.21
20	Stress	STMIB24	DnaJ-like protein	**-0.92**	0.02
35	Not Assigned	STMCP59	Domain of Unknown Function (DUF926)	**-0.92**	0.75
35	Not Assigned	STMCK71	T27I1.4 protein	**-0.92**	0.07
20	Stress	STMCV60	Cysteine protease inhibitor 1 precursor	**-0.91**	0.07
20	Stress	STMDU79	Polyphenol oxidase (similar to tomato PPO-D)	**-0.91**	0.39
31	Cell	STMGA77	Similar to kinesin-related protein (MKRP1)	**-0.91**	0.36
34	Transport	STMDJ91	Vacuolar ATP synthase subunit D	**-0.91**	0.13
35	Not Assigned	STMGB70	None	**-0.90**	0.45
29	Protein	STMGF60	U-box domain, putative	**-0.90**	0.18
35	Not Assigned	STMGA66	None	**-0.90**	0.09
13	Amino acid metabolism	STMIT78	3-methylcrotonyl-CoA carboxylase alpha chain	**-0.90**	0.43
35	Not Assigned	STMCX60	None	**-0.90**	0.23
35	Not Assigned	STMGB22	Integral membrane family protein	**-0.89**	0.35
20	Stress	STMCV86[E]	Jasmonate ZIM-domain protein 3	**-0.88**	0.07
35	Not Assigned	STMGV86	Similar to unknown protein	**-0.87**	0.10
35	Not Assigned	STMDZ77	Hcr9-OR3A	**-0.86**	0.10
31	Cell	STMCZ94	HOBBIT protein	**-0.86**	0.41
35	Not Assigned	STMCH84	None	**-0.85**	0.35
35	Not Assigned	STMGG94	Similar to LPD1 (Lipoamide dehydrogenase 1)	**-0.85**	0.45
35	Not Assigned	STMGA05	Similar to glycotransferase	**-0.85**	0.45
35	Not Assigned	STMCZ47	None	**-0.84**	0.52
35	Not Assigned	STMCE11	None	**-0.84**	0.44
27	RNA	STMCF95	Similar to Phytochrome Interacting Factor 3	**-0.83**	0.35
33	Development	STMGD80	Similar to latex allergen from Hevea brasiliensis	**-0.83**	0.23
20	Stress	STMGB30	Dehydration-responsive protein RD22 precursor	**-0.81**	0.30
35	Not Assigned	STMGB11	Polyunsaturated fatty acid synthase subunit A	**-0.80**	0.52
29	Protein	STMGD52	Kelch repeat-containing F-box family protein	**-0.79**	0.32
26	Misc	STMGH28	GDSL-motif lipase, putative	**-0.79**	0.18

16	Secondary metabolism	STMDZ37	Putative cinnamoyl-CoA reductase-like protein	**-0.79**	0.60
35	Not Assigned	STMCK05	WD-40 repeat family protein (nucleoporin SHE-1-like)	**-0.79**	0.36
35	Not Assigned	STMCR90	WD-40 repeat family protein (Transducin family protein)	**-0.79**	0.33
27	RNA	STMCN84	Transcription factor MYC7E	**-0.79**	0.49
13	Amino acid metabolism	STMGC95	3-dehydroquinate dehydratase / shikimate dehydrogenase isoform 2	**-0.79**	0.54
26	Misc	STMGG16	Short-chain dehydrogenase/reductase (SDR) family protein	**-0.78**	0.17
35	Not Assigned	STMGH27	Putative tyrosine-specific transport protein	**-0.78**	0.58
20	Stress	STMCR35	Proteinase inhibitor I	**-0.78**	0.58
35	Not Assigned	STMGC87	None	**-0.78**	0.50
29	Protein	STMCV88	Retrotransposon protein, putative, unclassified	**-0.77**	0.23
13	Amino acid metabolism	STMGA53	Ornithine carbamoyltransferase, chloroplast precursor	**-0.77**	0.52
35	Not Assigned	STMGD70	None	**-0.76**	0.23
26	Misc	STMER56	Invertase/pectin methylesterase inhibitor family protein	**-0.76**	0.07
29	Protein	STMCH04	F-box family protein	**-0.76**	0.51
35	Not Assigned	STMCX88	Similar to nodulin-like protein	**-0.76**	0.43
35	Not Assigned	STMGG48	Similar to protein kinase	**-0.76**	0.45
27/33	RNA/Development	STMGF04	Homeodomain protein GhHOX1	**-0.75**	0.48
26	Misc	STMCH88	Flavin containing monooxygenase 3-like	**-0.75**	0.54
29	Protein	STMCB75	50S Ribosomal protein L1	**-0.75**	0.00
35	Not Assigned	STMGH51	None	**-0.73**	0.43
35	Not Assigned	STMGB84	Probable carbohydrate esterase	**-0.73**	0.41
29	Protein	STMCR83	Putative aspartyl protease	**-0.73**	0.39
27	RNA	STMEF08	Homeobox	**-0.73**	0.19
33	Development	STMCM36	Cupin family protein	**-0.73**	0.31
29	Protein	STMCK53	None	**-0.73**	0.55
28	DNA	STMGB89	Putative RNA helicase	**-0.73**	0.43
13	Amino acid metabolism	STMGU61	Similar to alanine--glyoxylate aminotransferase	**-0.73**	0.23
17.5	Ethylene	STMGD11	ERF/AP2 transcription factor family (ATERF-9)	**-0.73**	0.50
35	Not Assigned	STMFA05	ABC1 family protein	**-0.73**	0.53
28	DNA	STMCR64	Putative phosphoesterase	**-0.72**	0.34
20	Stress	STMDV12	Disease resistance-responsive family protein	**-0.72**	0.28
4	Glycolysis	STMGF18	Phosphoenolpyruvate carboxylase kinase 2	**-0.72**	0.51
34	Transport	STMGD94	Similar to ABC transporter 10	**-0.72**	0.43
15	Metal Handeling	STMGS26	Methallothioneine-like protein	**0.72**	0.41
27/33	Development/Stress	STMIY82	Jasmonic acid 2	**0.74**	0.36
35	Not Assigned	STMHI64	None	**0.74**	0.41
27/33	RNA/Development	STMCV89	No apical meristem (NAM) family protein	**0.75**	0.64
29	Protein	STMIR76[I]	60S Ribosomal protein L36	**0.76**	0.18
35	Not Assigned	STMEQ18	Putative phytosulfokine peptide precursor	**0.77**	0.33
34	Transport	STMJJ19	Peptide transporter-like protein	**0.78**	0.80
35	Not Assigned	STMHQ27	None	**0.79**	0.21
10.5	Cell Wall	STMGX29	Extensin precusor	**0.80**	0.41

20	Stress	STMEK46	Hsp90-2-like	**0.81**	0.14
11.6	Cell Wall	STMIP64	Lipid transfer protein LTP1	**0.82**	0.42
29	Protein	STMIX83	60S ribosomal protein L6	**0.83**	0.03
29	Protein	STMJC30	Phytophthora-inhibited protease 1	**0.84**	0.28
1	Photosynthesis	STMIU53	PGR5-like	**0.84**	0.26
20	Stress	STMIN04	Pathogenesis-related protein 1b precursor	**0.85**	0.51
20	Stress	STMIU06	Pathogenesis-related protein P2 precursor	**0.89**	0.28
29	Protein	STMIX54	Clp protease proteolytic subunit (ClpP)	**0.90**	0.00
20	Stress	STMGE38	TAS14 (Le4) dehydrin	**0.92**	0.43
26	Misc	STMHX28	Cytokinin-O-glucosyltransferase 3	**0.95**	0.01
26	Misc	STMID59	Cytochrome P450	**0.95**	0.10
29	Protein	STMJB45[F]	Cathepsin B-like cysteine proteinase	**0.97**	0.02
20	Stress	STMBB21	Glucan endo-1,3-beta-glucosidase A precursor	**0.98**	0.33
35	Not Assigned	STMEA95	Weakly similar to carboxylesterase	**1.01**	0.02
20	Stress	STMJO29	Cold-stress inducible protein	**1.01**	0.18
34	Transport	STMCC78[H]	ABC transporter protein F family protein 5-like	**1.17**	0.00
20	Stress	STMEO91[G]	Class II chitinase	**1.34**	0.19

[A] The top 100 cDNA spots with largest fold changes (|FC|=0.73-3.93) at 8 hr after wounding in *LapA-SI* leaves were identified. The significance of signal changes in *LapA-SI* relative to WT plants at 8 hr after wounding is indicated (*p* value). Due to the redundancy in cDNA spots (see footnotes D-H), the top 107 spots were examined.

[B] MapMan often placed genes in multiple BINs; therefore multiple designations are indicated. It should be noted that there was no extensive manual annotation of the cDNAs that appear in this table. Therefore, spots with similar names may encode for RNAs from the same gene.

[C] *LapN* was annotated as an up-regulated DEG. This is in striking contrast to the previous studies monitoring the rare-class *LapN* RNAs by RNA blots or RNase protection studies or monitoring LAP-N protein levels (Chao et al. 2000; Tu et al. 2003). It is likely that the abundant wound-induced *LapA* transcript cross-hybridized to the *LapN* ESTs on the array, since the potato *LapN* EST has 95.6% and 88.9% identity with the tomato *LapN* and *LapA*, respectively.

[D] *PPO-B* was represented by two cDNAs (STMDB46,STMCI55) in the top 100 spots with largest fold changes.Only the STMCI55 data is shown.

[E] Jasmonate Zim-domain three was represented by two cDNAs (STMCV86, STMCZ36) in the top 100 spots with largest fold changes.Only the STMCV86 data is shown.

[F] Capthepsin B was represented by two cDNAs (STMJB45, STMEU11) in the top 100 spots with largest fold changes.Only the STMJB45 data is shown.

[G] Class II chitinase (acidic) was represented by two cDNAs (STMJD93, STME091) in the top 100 spots with largest fold changes.Only the STME091 data is shown.

[H]ABC transporter protein F family protein 5-like was represented by two cDNAs (STMCC78 ,STMDP40) in the top 100 spots with largest fold changes. Only the STMCC78 data is shown.

[I]60S Ribosomal protein L36 was represented by two cDNAs (STMIR76,STMJA94) in the top 100 spots with largest fold changes. Only the STMIR76 data is shown.

Table 2.11 Differentially expressed genes in *LapA-SI* leaves before wounding[A].

				LapA-SI					WT				
				0 hr	1hr		8hr		0 hr	1hr		8hr	
BIN #	BIN Title	Clone ID[B]	Annotation[C]		L	S	L	S		L	S	L	S
20	Stress	STMFB93	**Basic PR-1 protein**	**0.93**	0.03	-0.54	**0.94**	-0.76	0.25	**1.27**	0.01	**1.01**	-0.48
20	Stress	STMGE38	**TAS14 (Le4) - dehydrin**	**1.31**	0.71	0.09	**2.16**	-0.16	0.18	**1.26**	0.48	**1.24**	0.12
27	RNA	STMGB83	**BEL1-related homeotic protein 30**	**0.89**	-0.12	0.04	0.02	-0.30	0.06	0.71	0.60	0.73	-0.01
27	RNA	STMDH75	**MYBR29-like**	**0.86**	-0.42	-0.10	-0.02	-0.14	0.21	0.50	0.32	0.41	0.02
27	RNA	STMCS81	**SlWRKY42**	**1.02**	0.35	0.26	0.24	0.17	0.39	0.52	0.58	0.45	0.31
17.6	GA	STMGA83	**Similar to gibberellin receptor GID1**	**0.97**	0.29	0.11	0.39	-0.18	0.10	**1.07**	0.54	**0.97**	0.12
34	Transport	STMDJ91	**Similar to vacuolar ATP synthase**	**-0.81**	**-1.19**	-0.72	-0.63	-0.66	0.04	0.17	0.00	0.28	0.16
26	Misc	STMGC17	Cytochrome P450 monooxygenase	**0.82**	-0.21	0.32	0.09	0.14	0.14	0.19	0.41	0.26	0.12
29	Protein	STMGT05	Elongation factor 1-alpha	**-0.82**	-0.05	-0.32	-0.10	-0.32	-0.34	-0.51	-0.51	-0.52	-0.49
17.3	BR	STMGB76	Homolog to 24-sterol C-methyltransferase	**0.81**	-0.13	-0.04	-0.09	-0.18	-0.02	0.31	0.52	0.37	0.11
34	Transport	STMGL05	Homolog to Bet1-like SNARE 1-1	**-0.91**	-0.40	-0.47	-0.32	-0.78	-0.56	-0.49	-0.69	-0.32	-0.52
11	Lipid Metabolism	STMCH75	Homolog to diacylglycerol kinase 1	**0.89**	0.00	0.22	0.07	-0.12	0.20	0.45	0.16	0.27	-0.02
26	Misc	STMCP17[D]	Homolog to secretory peroxidase	**0.86**	0.40	0.49	0.23	0.04	0.25	0.47	0.43	0.08	-0.09
31	Cell	STMGA77	Kinesin related protein	**0.84**	-0.15	0.09	-0.05	-0.07	0.23	0.57	0.58	**0.86**	0.27
29	Protein	STMCV94	Nucleoporin-like protein	**0.99**	-0.19	-0.07	-0.07	-0.19	0.22	**0.89**	0.57	**1.02**	0.31
11	Lipid Metabolism	STMGA93	Polyunsaturated fatty acid synthase subunit A	**1.17**	-0.06	0.16	-0.20	-0.01	0.30	0.38	0.40	0.77	0.12
29	Protein	STMCR83	Putative aspartyl protease	**1.45**	0.36	0.34	-0.24	0.13	0.42	0.61	0.55	0.49	0.24
35	Not Assigned	STMCX75	Putative membrane protein	**0.83**	0.23	0.47	-0.22	0.39	0.31	0.52	0.39	0.29	0.05
13	Amino Acid Metabolism	STMCP40	Putative urease accessory protein D	**0.81**	0.04	0.15	0.01	0.18	0.32	0.55	0.50	0.61	0.34
16	Secondary Metabolism	STMCY27	Similar to anthocyanin acyltransferase	**0.86**	**1.02**	**1.25**	**1.35**	**2.17**	0.50	**1.40**	**1.28**	**1.67**	**2.04**
29	Protein	STMCK09	Similar to cdc2-related protein kinase	**0.82**	-0.12	0.00	-0.14	-0.16	0.24	0.67	0.57	0.46	0.17
27	RNA	STMGB18	Similar to DNA binding protein	**0.88**	-0.23	0.01	0.11	0.05	0.13	0.42	0.52	0.74	0.21
26	Misc	STMGC47	Similar to geraniol 10-hydroxylase	**0.80**	-0.11	-0.02	-0.08	-0.01	0.07	0.51	0.40	0.64	0.16
35	Not Assigned	STMGA05	Similar to glycotransferase	**0.84**	0.15	0.43	0.05	0.21	0.36	0.73	0.71	**0.89**	0.22
27	RNA	STMGG63[E]	Similar to Homeodomain protein GhHOX1	**0.86**	-0.13	0.03	0.05	-0.16	0.15	0.60	0.54	0.65	0.07
30	Signaling	STMGA09	Similar to leucine-rich repeat transmembrane protein kinase	**1.10**	-0.37	0.22	-0.08	-0.18	0.27	0.23	0.36	0.52	-0.18
11	Lipid Metabolism	STMGH41	Similar to omega-6 fatty acid desaturase	**1.15**	0.72	0.22	0.67	0.08	0.28	**0.88**	0.25	0.59	0.18

13	Amino Acid	STMGA53	Similar to ornithine carbamoyltransferase	**0.82**	-0.08	0.25	0.02	0.09	0.34	0.74	0.58	0.78	0.33
35	Not Assigned	STMGC87	Similar to probable N-glycosylase/DNA lyase	**0.85**	-0.11	0.00	-0.12	-0.05	0.29	**0.87**	0.54	0.66	0.14
29	Protein	STMCY59	Similar to protein binding / zinc ion binding protein	**0.91**	0.42	0.17	-0.05	-0.45	0.22	0.43	0.31	0.10	-0.15
29	Protein	STMGG48	Similar to protein kinase	**0.81**	-0.17	-0.04	-0.02	0.16	0.09	0.40	0.56	0.74	0.25
26	Misc	STMGG16	Similar to short-chain dehydrogenase/reductase family protein	**0.86**	-0.02	0.00	0.08	-0.20	0.19	0.59	0.34	**0.86**	0.32
29	Protein	STMGF60	Similar to U-box domain	**0.91**	-0.13	0.22	0.05	-0.42	0.10	0.66	0.57	**0.96**	0.32
35	Not Assigned	STMCV87	Weakly similar to acyltransferase	**0.92**	0.23	0.10	0.04	-0.15	0.17	0.74	0.64	0.75	0.12
35	Not Assigned	STMCH48	Similar to uncharacterized protein	**0.92**	0.02	0.06	-0.05	0.12	0.24	0.64	0.51	0.63	0.15
35	Not Assigned	STMCJ58	Similar to uncharacterized protein	**0.96**	**1.03**	0.13	**1.20**	**1.18**	0.29	**0.82**	-0.02	**1.18**	0.50
35	Not Assigned	STMCX39	Similar to uncharacterized protein	**0.85**	0.19	0.02	0.10	0.05	0.22	0.52	0.36	0.43	0.06
35	Not Assigned	STMCY35	Similar to uncharacterized protein	**0.81**	0.10	0.14	0.21	0.31	0.32	0.78	0.39	**1.24**	0.51
35	Not Assigned	STMDH05	Similar to uncharacterized protein	**1.06**	-0.13	0.05	0.04	-0.13	0.11	0.53	0.68	**1.00**	0.29
35	Not Assigned	STMDH23	Similar to uncharacterized protein	**0.85**	0.19	0.34	-0.07	0.26	0.34	0.38	0.37	0.61	0.29
35	Not Assigned	STMDH93	Similar to uncharacterized protein	**0.84**	-0.01	0.10	-0.23	-0.04	0.12	**0.87**	0.34	0.34	0.05
35	Not Assigned	STMDJ60	Similar to uncharacterized protein	**0.81**	0.43	0.51	-0.02	0.44	0.24	0.52	0.34	0.20	0.04
35	Not Assigned	STMGA35	Similar to uncharacterized protein	**0.96**	-0.05	0.20	-0.08	-0.12	0.30	**0.81**	0.68	**0.92**	0.17
35	Not Assigned	STMGA52	Similar to uncharacterized protein	**1.14**	-0.07	0.15	-0.04	-0.02	0.23	0.39	0.54	0.65	0.21
35	Not Assigned	STMGA70	Similar to uncharacterized protein	**0.82**	0.24	0.34	-0.06	0.22	0.31	0.44	0.25	0.36	0.11
35	Not Assigned	STMGA81	Similar to uncharacterized protein	**0.83**	-0.10	0.06	-0.04	-0.26	0.24	0.79	0.45	0.47	0.09
35	Not Assigned	STMGB11	Similar to uncharacterized protein	**0.87**	-0.11	-0.01	-0.13	-0.15	0.04	0.55	0.56	0.67	-0.06
35	Not Assigned	STMGB70	Similar to uncharacterized protein	**0.88**	-0.16	-0.04	-0.11	-0.08	0.05	0.49	0.61	0.79	0.14
35	Not Assigned	STMGG29	Similar to uncharacterized protein	**0.85**	-0.24	0.01	-0.25	-0.23	0.10	0.45	0.54	0.69	0.28

[A] DEGs are indicated in bold and are shaded in grey. DEGs are defined as those genes with |FC| ≥0.8, p< 0.05 relative to the WT 0 hr contol. Suppressed DEGs are boxed in.
[B] Genes which are represented by multiple clones on the TIGR 10K (version 3) potato cDNA microarray are indicated with an asterisk. All clones shown in table.
[C] The top 7 gDEGs (in bold) were tested by RT-PCR or qPCR.
[D] The 0-hr DEG encoding a secretory peroxidase was represented by two cDNAs (STMCP17, STMDB17) . Only the STMCP17 data is shown and is representative of both array spots.
[E] The 0-hr DEG encoding a homeodomain protein similar to GhHOX1 was represented by two cDNAs (STMGF04, STMGG63). Only the STMGG63 data is shown and is representative of both array spots.

Table 2.12 Putative 0-hr gDEG homologues, gene family members and primers for RT-PCR.

Potato EST	Potato EST	Homolog Search	Estimated Gene Family Size	Tomato gene	Tomato locus	BLASTN E-value
PR-1 (basic)[A]	STMFB93	BLASTN	5	***PR-1c***	SGN-U579345	0
		BLASTN		***PR-1a1***	SGN-U577839	2.00E-92
		BLASTN		***PR-1a2***	SGN-U578064	3.00E-23
				PR-1b	SGN-U579545	1.00E^-22
				PR-1a	SGN-U578815	1.00E^-22
GID1[A]	STMGA83	BLASTN	5[C]	***GID***	SGN-U581883	0
				GID-like2	SGN-U581650	8.00E-36
				GID-like3	SGN-U581885	8.00E-33
				GID-like1	SGN-U578033	4.90E-01
				GID-like4	SGN-U582131	2.60E-01
Bel1[A]	STMGB83	BLASTN	≥ 7	***BEL-like***	SGN-U569649	4.00E-47
				Bel-like3	SGN-U579380	8.00E-33
WRKY[A]	STMCS81	BLASTN	~81	***SlWRKY42***	SGN-U601704	3.00E-21
MYB[A]	STMDH75	BLASTN	100s	***MYBR29-like***	SGN-U570156	0
Vaculolar ATPase subunit D[A]	STMDJ91	BLASTN	1	***vATPD***	SGN-U578627	0
Dehydrins (Dhn)[B]	STMGE38	BLASTN	4	***TAS14 (Dhn1, Le4)***	SGN-U581493	8.00E-29
	STMJO29	BLASTN		***pDhn2 (Dhn2)[D]***	SGN-U581375	1.00E-166
		BLASTX		***pDhn3(Dhn3)[D]***	SGN-U576909	NA
		BLASTX		***pDhn4(Dhn4)[D]***	SGN-U581470	NA
ER5[A] (LEA)	STMGA34	BLASTN	2	***ER5***	SGN-U577990	0
	STMIQ26	BLASTN		***ER5-like (LEA-like)[E]***	SGN-U577361	0
Control				***eIF4***	SGN-U581466	NA

[A] Genes were identified based on BLASTN search of Sol Genomics Network (SGN) using the corresponding potato EST.
[B] Genes were identified based on BLASTN search of SGN using potato EST or Arabidopsis Dhn2, Dhn3*, and* Dhn4 genes (Hundertmark et al., 2008).
[C] *GID1* family members were identified based on BLASTN search of Sol Genomics Network (SGN) using potato EST as well as GID1 hits.
[D] Tomato *Dhn2-4* were named here for the first time. *Dhn4* RNAs were not detected in leaves and therefore was not further studied (data not shown).
[E] STMIQ26 is annotated as a LEA-like protein. BLASTN search indicated that the tomato LEA-like cDNA has high sequence identity (2E-39) with tomato's ER5. For this reason, it is referred to as ER5-like.

Table 2.13 Primers and their conditions used for RT-PCR or qPCR analysis

	Gene	Unigene	Primers (5'-3')	Ta(°C)	Amplicon Size (bp)
RT-PCR	*PR-1c*	SGN-U579345	TGTGGACACTATACTCAGGT	57	380
			ATACACACATCCAATAAAGC		
	PR-1a1	SGN-U577839	CCTCCATTTTCGTTGCTTGT	57	369
			CCGACTTACGCCATACCACT		
	PR-1a2	SGN-U578064	GCTCAAAATTCACCCCAAGA	55	391
			CCCAATTGCCTACAGGATCA		
	GID	SGN-U581883	CAGGGCATGAGGTGAATCTGTTGT	57	315
			ACATTTCTCAGGCCAAGACTCGGT		
	GID-like2	SGN-U581650	GGGTCCCTTTTTGTTTTGCT	56	309
			TTTTACCCCAATCAGCCTCA		
	GID-like3	SGN-U581885	TCCATGCAGTTTCACTCTCG	56	344
			GGACGACGAAGCATGTTGTA		
	BEL-like	SGN-U569649	AATTAGCGGGTCACTCTTGACGGT	55	318
			AGTCAATACCCGAACCACCACCTT		
	Bel-like3	SGN-U579380	TGGAGCAGCAAAACCATACA	57	335
			TTAAGCCAGTTTGCCTTGCT		
	SlWRKY42	SGN-U601704	TGGGCTTGAAGGTCCATGTGAAGA	50	390
			ATGTGTAGTGGTGCAGGAGGGAAT		
	MYBR29-like	SGN-U570156	TCCCATCCACTTCCCGAAGAACAA	57	368
			TCGGAGCAGTTTCACCCTTGGTAA		
	vATPD	SGN-U578627	CATCTAAGTAGCTTGGAAAA	55	315
			GTGATCGAACTTTCAGAGTA		
	TAS14 (Dhn1, Le4)	SGN-U581493	GGCACAATACGGCAATCAAGACCA	57	317
			ACTCACCTTCATGTTGTCCAGGCA		
	Dhn2	SGN-U581375	AGCTCCAGTGATGAGGAGGA	50	361
			CCTCTTCAGCCTTTGAGTGG		
	Dhn3	SGN-U576909	CAAGGCCAATTGCACCTAAT	60	380
			CAGTGCATTCCAGGGATCTT		
	Dhn4	SGN-U581470	CTGCAGCAGTTGGTGAAA	60	339
			GTTGCTCCGGTTTTGTTGTT		
	ER5	SGN-U577990	GATGTGCCAGTGAAGGTACCTC	57	458
			GTGATATGTTCGAATATGGTATCC		
	ER5-like (LEA-like)	SGN-U577361	CTACTGTGGCAAGGGGTGTT	57	349
			TGGTGTTGGCTTTTGATTGA		
	eIF4	SGN-U581466	GGCAGCAGAAGGTTCTCA	58	300
			TCAATCTGTTGTGCAAGCTC		
qPCR	*Pr1c*	SGN-U579345	TCACAATGCAGCTCGTAGAC	60	157
			GCAGCTAGGTTTTCACCGTA		
	PR1A2	SGN-U578064	TCATTCTGGTTCAGGGGAGA	55	92
			AGTTTGGCTTTTCCGACACC		
	TAS14	SGN-U581493	TGGGAGGAGAAAGAAGGGTT	54	149
			AAGGTGTTCAATGCATCCCA		
	Dhn2	SGN-U581375	TGAAGAAGTGGAACCCAAGG	57	80
			CCTCCTCATCACTGGAGCTA		

Dhn3	SGN-U576909	GGACAACAACTTCGTCATTCT GCCCTTTTTCTTCTTCCTCCT	54	87
ER5	SGN-U577990	ACCGGAGGCTGACATCACGGA CCTGCCGGAGCATTTGAGAGT	60	145

CHAPTER 3: Leucine Aminopeptidases as the Major Plant Cys-Gly Dipeptidase and the Potential Role of Leucine Aminopeptidase A in Retrograde Signaling During the Tomato Wound Response

ABSTRACT

Plant responses to wounding involve a complex network of signaling molecules that regulate expression of nuclear defense genes. Many of these signaling compounds are highly abundant or begin their synthesis within the plastid. Recently, the tomato plastid-localized leucine aminopeptidase A (LAP-A) has been shown to modulate nuclear late wound-response gene expression. It was therefore hypothesized that LAP-A may act through a known plastid-localized defense signal. In this chapter, levels of key phytohormones were monitored in WT, *LapA* silenced (*LapA-SI*), and *LapA* overexpression lines during wounding. Preliminary data suggests that salicylic acid (SA) levels may be elevated in the *LapA-SI* line corresponding to increases in SA-responsive RNAs (*Pathogenesis-Related 1b* and *Thionin)*. In addition, a new function for plant LAPs as Cys-Gly dipeptidases was characterized and the relevance of this function to glutathione and reactive oxygen species (ROS) metabolism is discussed. Together, data in this chapter suggest that LAP-A may act through SA and/or ROS metabolism and signaling in order to modulate wound responses in tomato. Modulation of SA or ROS may be due in part to LAP-A's role as the major Cys-Gly dipeptidase in tomato.

INTRODUCTION

Recently, studies have focused on the regulatory role of plastids in defense signaling (Bonaventure and Baldwin 2010; Padmanabhan and Dinesh-Kumar 2010). This interest is in part due to the fact that plastids are the site of many defense-response molecules including those involved in primary defense signaling [jasmonic acid (JA) and salicylic acid (SA)] and modulators of defense [abscisic acid (ABA), gibberellic acid (GA), cytokinins (CK), and H_2O_2] (Baier and Dietz 2005; Uppalapati et al. 2007; Vranova et al. 2012; Wasternack 2007).

JA and JA-Ile signaling is the primary pathway for wound responses in tomato (Li et al. 2005; Sun et al. 2011). While JA-regulated defenses are often the dominant response to damage, this signaling pathway is integrated into a complex and dynamic defense phytohormone signaling network. The most well-studied relationship in phytohormone cross-talk is the mutual antagonism between JA and SA (Robert-Seilaniantz et al. 2011; Thaler et al. 2012). While there are exceptions, SA broadly is involved in defense responses to biotrophic pathogens, while JA is involved in defense signaling against necotrophic pathogens and chewing insects (Glazebrook 2005). In most studies, JA and SA mutually repress each other's responses; however, the effect of this antagonism is dependent on the timing and concentration of each elicitor (Koornneef et al. 2008; Thaler et al. 2002). Moreover, JA and SA can also act additively or synergistically depending on the timing, intensity and duration of each signal (Mur et al. 2006). For example, SA can enhance or suppress JA-responsive *Thionin* (*Thi*), while JA can enhance or suppress SA-responsive *Pathogenesis Related protein 1* (*PR-1*) depending on the concentration of modulating elicitor. The exact mechanism of cross-talk has yet to be elucidated though many potential key players have been identified such as glutaredoxin GRX480, , Mitogen-activated Protein Kinase 4 (MPK4), Nonexpresser of Pathogenesis-Related gene 1 (NPR1), and transcription factors such as MYC2, ET-response factor 1 (ERF1), NACs (petunia NAM and Arabidopsis ATAF1, ATAF2, and CUC2), and various WRKYs and JAZs (Koornneef and Pieterse 2008; Thaler et al. 2012; Zheng et al. 2012).

The plastid-derived isoprenoid hormones, ABA, GA, and CK, have also been implicated in modulating the wound responses in tomato. ABA accumulates in response to wounding and the bioactive peptide systemin in tomato and is essential for a robust wound response and defense against chewing insects (Birkenmeier and Ryan 1998; Peña-Cortés et al. 1995; Peña-Cortés et al. 1996; Peña-Cortés et al. 1989; Peña-Cortés et al. 1991). GA in Arabidopsis and potato negatively regulates JA responses (Navarro et al. 2008; Peña-Cortés et al. 1989). Little is known about GA signaling in tomato; however, one study has shown that GA positively regulates JA- and wound-responsive class I chitinase (*Chi9*) and β-1,3-glucanase (*GluB*) (Wu and Bradford 2003). A few studies in plants have shown that CK can modulate defense signaling; however, no clear pattern of regulation has emerged (Erb et al. 2012). To date, no studies have investigated the role of CKs in wound responses in tomato; however, one study has implicated a tomato cytokinin-response factor (*SlCRF1/Pti6*) in defense against biotrophic pathogens, suggesting that there may be some cross-talk (Gu et al. 2002; Shi et al. 2012; Zhou et al. 1997).

In plants, the primary sources of wound- and pathogen-induced reactive oxygen species (ROS) are plasma membrane-localized respiratory bust oxidase homologues (Rboh) and pH-dependent peroxidases (Suzuki et al. 2011; Torres et al. 2006). In tomato, H_2O_2, most likely originating from *Rboh*, is required for a robust expression of late-wound response genes, but H_2O_2 does not modulate early wound responses (Orozco-Cárdenas and Ryan 1999; Orozco-Cárdenas et al. 2001; Sagi et al. 2004). While apoplastic ROS has been the focus of much research in wound responses, recent studies have implicated plastid-generated ROS in plant defenses (Baier and Dietz 2005; Maruta et al. 2012; Padmanabhan and Dinesh-Kumar 2010). This work has focused on pathogen defense where chloroplastic ROS has been shown to be up-regulated after infection (Caplan et al. 2009; Liu et al. 2007b). In addition, in both Arabidopsis and tobacco, plastid ROS is required for a robust hypersensitive response (Liu et al. 2007b; Zurbriggen et al. 2009). Finally, chloroplast-generated ROS also induces unique expression patterns, which include the up-regulation of many nuclear-encoded SA- and JA-response genes such as PR1- and several WRKYs (Danon et al. 2005; Gadjev et al. 2006). Relatively little is

known about chloroplastic ROS in wounding; however, one study has implicated chloroplastic ROS in lipid oxidation in JA synthesis and signaling (Przybyla et al. 2008).

Plants have a complex network of enzymes and small antioxidant compounds to maintain ROS homeostasis (Mittler et al. 2004). The most well-studied antioxidant network is the ascorbate (Asc)–glutathione (GSH) cycle (Foyer and Noctor 2011). Briefly, Asc scavenges ROS via Asc peroxidase (APX) to generate monodehydroascorbate (MDHA). MDHA can either be regenerated to Asc by MDHA reductase (MDHAR) or is rapidly converted to dehydroascorbate (DHA) and requires DHA reductase (DHAR) to regenerate Asc. DHAR utilizes GSH to reduce DHA, forming GSSG, which in turn is reduced back to GSH by GSH reductase (GR).

In addition to its role in ROS metabolism, GSH has been implicated plant development, cell cycle regulation, xenobiotic and heavy metal detoxification, sulfur assimilation, and resistance to a wide range of abiotic and biotic stresses (Noctor et al. 2012). In particular, several studies in Arabidopsis have shown that GSH is essential for pathogen and insect defense due its role as a precursor for the phytoalexin camalexin and toxic glucosinolates, respectively (Ball et al. 2004; Maughan et al. 2010; Mhamdi et al. 2010; Parisy et al. 2007; Schlaeppi et al. 2008); these defenses are unique to the order Capparales (Halkier and Gershenzon 2006; Su et al. 2011). On the other hand, GSH has been implicated in SA defenses either through helping to reduce NPR1 and induce SA responses or by acting with cytosolic NPR1 through an unknown pathway to suppress JA-regulated defenses (Koornneef et al. 2008; Noctor et al. 2012; Spoel et al. 2003).

GSH is a tripeptide composed of Glu, Cys, and Gly in which the Glu attaches to the Cys at the γ–carboxyl group of Glu (differentiating it from a typical peptide bond). GSH is synthesized in two ATP-dependent steps within the plastid and/or cytosol. The plastid-localized γ-EC synthase (γ-ECS; GSH1) forms the γ-Glu-Cys bond and either a plastid or cytosolic GSH synthase (GSH-S; GSH2) joins the Gly to the γ-Glu-Cys dipeptide (Meister 1988; Mullineaux and Rausch 2005; Noctor et al. 2002; Rennenberg 1982; Wachter et al. 2005). Increases in GSH after stress have been correlated with transcript accumulation of *GSH1* and *GSH2* and post-translational activation of γ–ECS (Hell and Bergmann 1990; Hothorn et al. 2006; Noctor et al. 2002; Queval et al. 2009;

Sung et al. 2009; Xiang and Oliver 1998). GSH increases have also been correlated with transcription accumulation of Cys biosynthesis genes including all three *Adenosine 5'-Phosphosulphate Reductases* (*APR*) and the plastid-localized *Serine Acetyltransferase* (*SAT*) (Noctor et al. 2012; Queval et al. 2009).

While GSH biosynthesis in plants is fairly well-established in plants, less is known about GSH catabolism. At least four classes of enzymes have been identified that could be involved in initial GSH turnover (Figure 3.1; Noctor et al. 2012). These include: i) carboxypeptidases whose activity has been demonstrated in barley to remove the Gly from the C-terminus of GSH (Wolf et al. 1996); ii) phytochelatin synthase, which metabolizes GSH to generate phytochelatin, which may have a role during heavy metal stress (Blum et al. 2007) iii) γ–glutamyl cyclotransferase (GGC), which has been proposed to be the major GSH degradation enzyme in Arabidopsis (Ohkama-Ohtsu et al. 2008); and iv) γ–glutamyl transpeptidase (GGT), which has been shown to have active homologues in several species including Arabidopsis, maize, barley, tobacco, and tomato (Ferretti et al. 2009; Martin and Slovin 2000; Masi et al. 2007; Ohkama-Ohtsu et al. 2007a; Ohkama-Ohtsu et al. 2007b; Storozhenko et al. 2002).

In Arabidopsis, cytosolic GGC is proposed to convert the γ-Glu of GSH to 5-oxoproline (5-OP) and release a Cys-Gly dipeptide (Ohkama-Ohtsu et al. 2008). 5-OP is then metabolized by 5-OPase to release free Glu, while the Cys-Gly is processed by a yet unidentified plant dipeptidase. While GGC activity has been detected in Arabidopsis and tobacco (*Nicotiana tabacum*; Ohkama-Ohtsu et al. 2008; Steinkamp and Rennenberg 1985; Steinkamp et al. 1987), no obvious homologues of the human GGC have been identified in plants (Oakley et al. 2008).

GGT produces a γ–glutamyl peptide and also releases Cys-Gly (Noctor et al. 2012). The γ–glutamyl peptide is then further processed by GGC and 5-OPase. GGT activity has been primarily detected in the apoplast, while one Arabidopsis GGT (GGT3) is vacuolar localized. While in Arabidopsis, GGC is the predominant enzyme involved in initial GSH breakdown and turnover, no studies have been performed in other species to determine which pathway is preferred. For

instance, another pathway must be utilized to breakdown GSH in tobacco since its GGC homologue was unable to utilize GSH directly (Steinkamp et al. 1987).

Of all the potential steps involved in GSH degradation in plants, only Cys-Gly hydrolysis has not been studied. However, Cys-Gly hydrolysis has been studied in other kingdoms. Cys-Gly dipeptidase activity is important not only important for GSH catabolism to release free Cys and potential recycle GSH (Baudouin-Cornu et al. 2012; De Donatis et al. 2010), but also for removal of excess Cys-Gly, which is a damaging oxidant (del Bello et al. 1999; Del Corso et al. 2002; Dominici et al. 1999; Enoiu et al. 2007). Various aminopeptidases have been implicated as Cys-Gly dipeptidases in other kingdoms; however, in rat liver, bovine lens, and the bacterium *Treponema denticola*, a M17 leucine aminopeptidase (LAP) is the major or only Cys-Gly dipeptidase (Cappiello et al. 2004; Chu et al. 2008; Jösch et al. 2003). A LAP homologue in *Escherichia coli* has also shown Cys-Gly dipeptidase activity; however, *other E. coli* peptidases have Cys-Gly dipeptidase activity as well (Suzuki et al. 2001).

In plants, there are two distinct classes of LAP proteins: the relatively neutral LAP-N and the acidic LAP-A (Chao et al. 2000; Gu et al. 1996). LAP-A has been shown to be localized to the stroma in mesophyll plastids and LAP-N is also predicted to be localized in this subcellular compartment (Narváez-Vásquez et al. 2008; Tu et al. 2003). LAP-N and LAP-A have different stabilities, peptidase substrate preferences, chaperone activities, and distinctly different expression patterns within the plant; *LapN* is constitutively expressed in all plants, while *LapA* is induced in response to stress and is only present in a subset of Solanaceous plants (Chao et al. 1999; Chao et al. 2000; Scranton et al. 2012; Tu et al. 2003). LAPs are important in insect deterrence in *Solanum lycopersicum* (tomato) and *Solanum nigrum* (nightshade) (Fowler et al. 2009; Hartl et al. 2008). The tomato LAP-A positively modulates the expression of core JA-dependent late wound responses including *Ser proteinase inhibitors* (*PinI* and *PinII*) and *polyphenol oxidase* (*PPO*; Fowler et al. 2009). Recently, the tomato LAP-A was also shown to negatively modulate the expression of *Pathogenesis-Related* genes (*PR-1c* and *PR-1a2*) and late-wound dehydrins (*TAS14* and *Dhn3*; Chapter 2). In addition, work described in Chapter 2

demonstrated that LAP-A is also important for a robust early wound response. However, no known mechanism for LAP-A's action has been elucidated. Due to LAP-A's localization in the plastid, in this Chapter, experiments were performed using WT, *LapA*-silenced (*LapA-SI*), and *LapA*-overexpression (*LapA-OX*) plants to determine if LAP-A acts through a known plastid-localized defense signal. Levels of key phytohormone were monitored in WT and *LapA* mis-expression lines before and after wounding. Preliminary data suggests that SA levels may be elevated in the *LapA-SI* lines corresponding to increases in SA-responsive RNAs (*Pathogenesis-Related 1b* and *Thionin*). In addition, a new function for plant LAPs as Cys-Gly dipeptidases was characterized and its relevance to glutathione and reactive oxygen species (ROS) metabolism is discussed. Together, data in this chapter suggests that LAP-A may act through SA and/or ROS metabolism and signaling in order to modulate wound responses in tomato. Modulation of SA or ROS may be due in part to LAP-A's role as the major Cys-Gly dipeptidase in tomato.

RESULTS

LAP-A Does Not Regulate Key Phytohormones After Wounding

Several major phytohormones that are known to regulate or modulate wound signaling initiate their synthesis within the plastid, including JA (Wasternack et al. 2006), SA (Uppalapati et al. 2007), ABA, GA, and CK (Vranova et al. 2012). Since LAP-A is localized to the plastid, it was hypothesized that LAP-A may affect the synthesis of one or more of these key phytohormones in order to affect nuclear gene expression during wounding. Therefore, LC-MS analysis was performed on WT, *LapA-SI*, and *LapA-OX* leaf tissue 0 to 24 hr after wounding to quantify key phytohormone levels. Since little is known about the extent of the role of CK on tomato wound signaling and since CK is not as readily ionizable, studies were focused on JA, JA-Ile, SA, ABA, and GA. Methods were optimized to measure major GA forms in tomato: GA1, GA3, GA4, and GA7 (Table 3.1; Sponsel 2010). Unfortunately, GA levels were below the level of detection in the tomato leaf tissues (data not shown). This is not surprising since GA levels range within 10^{-9} to 10^{-11} g/g fresh weight (Sponsel 2010).

JA and JA-Ile levels increased significantly, peaking between 0.5 and 1 hr after wounding, consistent with previous studies (Figure 3.2;Doares et al. 1995b; Suza et al. 2010). Jasmonate levels were comparable with Suza et al. (2010). Also consistent with previous reports, JA-Ile levels were significantly lower than JA levels, less than half of the total JA at 0.5 hr. There was large biological variation in the two biological replicates and neither JA nor JA-Ile levels were significantly different between the genotypes. This is consistent with the fact that *LapA-SI* lines are compromised in their response to JA and LAP-A was proposed to act downstream of JA and JA-Ile biosynthesis (Fowler et al. 2009).

ABA levels did increase in response to wounding in WT plants, but this increase was not statistically significant [Figure 3.2; ANOVA, Tukey post-hoc test ($p<0.05$)]. This is in contrast to previous studies that showed significant increases in ABA levels in response to mechanical wounding (Peña-Cortés et al. 1995; Peña-Cortés et al. 1996). The data reported here is more consistent with Birkenmeier and Ryan (1998). Their studies showed significant but more modest increases in ABA levels in response to wounding at the whole leaf level. Differences in wound induction in this current study may be due in part to the fact basal levels of ABA in our tomato plants were up to 10-fold higher than previous studies (Birkenmeier and Ryan 1998; Peña-Cortés et al. 1995; Peña-Cortés et al. 1996). Differences in basal levels may be due to genotype or age differences, or control plants in this study may have undergone abiotic stress prior to the experiment. Moreover, ABA levels were not significantly different between the genotypes. ABA levels were slightly higher in the *LapA-SI* at all timepoints compared to WT, but were not significant due to high biological variation.

To ensure that lack of significant differences in ABA levels was not due to increased catabolism of ABA, the major ABA breakdown products dihydrophaseic acid (DPA) and phaseic acid (PA) and conjugate ABA-glucose ester (ABA-GE) were also measured. Unlike previous studies in tomato, appreciable DPA levels were not detected (Andrade et al. 2008). However, while ABA-GE and PA did accumulate, their levels did not increase significantly in response to wounding and were not significantly different between the genotypes (Figure 3.2). Unlike previous

study in tomato, PA was more abundant that ABA-GE (Andrade et al. 2008), however studies in Arabidopsis have seen PA levels that are more abundant than ABA-GE (Huang et al. 2007; Okamoto et al. 2009). Differences in metabolite levels may be due differences in genotypes, growing conditions, or age of the plants.

There was a small increase in SA levels in response to wounding, although this change was not significant [Figure 3.2; ANOVA, Tukey post-hoc test ($p<0.05$)]. This is consistent with the fact that SA levels do not increase after to wounding in tobacco (Malamy et al. 1990; Schmelz et al. 2003) and SA is a negative regulator of wound responses (Doares et al. 1995a; Peña-Cortés et al. 1993). Basal SA levels were approximately half of those seen in other studies (Schmelz et al. 2003; Schmelz et al. 2009; Uppalapati et al. 2007). However, this may be due to differences in the method of analysis (GC vs LC MS) or differences in genotypes. Again no significant differences were detected between the genotypes. However, there was a trend showing SA levels were slightly elevated in *LapA-SI* lines, particularly at 0, 0.5, 1, and 24 hr after wounding. Lack of significant differences may be due to high variability between biological replicates (Figure 3.3). A reciprocal decrease in SA levels in the *LapA-OX* lines was not detected.

LAP-A Modulates the Expression of Defense-Response Genes Before and After Wounding

While the increases in SA levels in the *LapA-SI* line were not significant, they may point to a change in the SA-JA relationship in the *LapA-SI* lines, which may influence LAP-A's effect on JA-dependent late wound responses. Therefore, to determine if SA responses were affected in the *LapA* mis-expression lines, transcript levels of the SA-responsive basic *Pathogenesis-Related 1b* (*PR-1b*) (Chao et al. 1999; Uppalapati et al. 2007; Vidya et al. 1999) were measured in WT, *LapA-SI*, and *LapA-OX* lines 0, 1, 12, and 24 hr after wounding. *LapA* RNA levels are shown for comparison.

PR-1b transcripts increased by 12 and 24 hr after wounding in WT (Figure 3.4). There was high variability between biological replicates; therefore both of the independent experiments are shown. This is consistent with previous studies that have shown that *PR-1b* RNAs accumulate in response to spider mite feeding, wounding, and MeJA treatment in tomato (AbuQamar et al.

2009; Chao et al. 1999; Kant et al. 2004; Puthoff et al. 2010; Chapter 2). Moreover, *PR-1b* levels were increased in the *LapA-SI* lines, particularly at 24 hr. However, the timing and level of *PR-1b* RNA increase was variable between the two biological replicates. These data are consistent with the microarray study (Chapter 2), which showed that *PR-1b* was up-regulated in the *LapA-SI* 8 hr after wounding, again this was not significant (Chapter 2, Table 2.1). A reciprocal decrease in *PR-1b* transcripts was not seen in the in the *LapA-OX* lines (Figure 3.4), consistent with SA measurement trends (Figure 3.2).

Recently, a *PR-1* homologue (At2g19990; *PR-1*) was shown to be synergistically regulated by SA and JA in Arabidopsis (Mur et al. 2006). This Arabidopsis *PR-1* is most amino acid similarity to the tomato *PR-1c* (BLASTX 6E-51) and also has high similarity to the tomato *PR-1b* (BLASTX 4E-41).The regulation of the Arabidopsis *PR-1* is consistent with previous studies that have shown that the tomato *PR-1b* RNAs increase in response to SA, MeJA, and wounding (AbuQamar et al. 2009; Chao et al. 1999; Uppalapati et al. 2007; Vidya et al. 1999). These data are also consistent with increases in JA and JA-Ile levels after wounding, as well as an elevated SA trend (Figure 3.2) and *PR-1b* levels (Figure 3.4) in the *LapA-SI* lines after wounding.

To determine if the *LapA-SI* line may alter both JA and SA signaling, the expression of defense-related *Thionin* (*Thi*) was measured. *Thi* is induced by JA in Arabidopsis and barley and by wounding in Arabidopsis and tomato (Andresen et al. 1992; Sagi et al. 2004; Vignutelli et al. 1998). In addition, *Thi* is synergistically regulated by SA and JA in Arabidopsis (Mur et al. 2006). In WT tomato, *Thi* was not strongly induced after wounding in WT plants (Figure 3.4); this is in contrast to previous studies (Sagi et al. 2004). However, *Thi* RNA levels were consistently elevated in the *LapA-SI* lines. Again, the timing and increase in *Thi* RNA levels in the *LapA-SI* lines was highly variable between biological replicates. In general, reciprocal lower levels of *Thi* RNAs were detected in the *LapA-OX* line relative to WT and *LapA-SI*. In addition, *Thi* and *PR-1* RNA levels have similar patterns of expression in the *LapA* mis-expression lines. If *PR-1b* and *Thi* are similarly regulated by SA-JA synergy in tomato as in Arabidopsis, these data would suggest that SA signaling is altered in the *LapA* mis-expression lines.

Plant LAPs Have Cys-Gly Dipeptidase Activity

GSH is another small molecule that is synthesized and highly abundant in the plastid (Noctor et al. 2012). GSH levels increase in response to stress and GSH has been shown to be involved in defense. GSH acts either through its role in ROS catabolism and redox homeostasis or through an unknown role in NPR1-mediated regulation of SA and JA antagonism. In addition, GSH has also been shown to induce *PR-1* expression in Arabidopsis (Gomez et al. 2004; Senda and Ogawa 2004). Interestingly, LAPs in other species hydrolyze the GSH metabolite Cys-Gly, which, at least in cows, has been shown to be important in GSH metabolism (Cappiello et al. 2004; Chu et al. 2008; De Donatis et al. 2010; Jösch et al. 2003; Suzuki et al. 2001). In addition, Cys-Gly itself is a ROS (del Bello et al. 1999; Del Corso et al. 2002; Dominici et al. 1999; Enoiu et al. 2007), which may influence the ROS signaling that modulates wound responses in tomato (Orozco-Cárdenas et al. 2001).

Given GSH's role in defense and *PR-1* expression, experiments were performed to determine if LAP-A is also Cys-Gly dipeptidase. The rate of hydrolysis of Cys-Gly was determined for purified His_6-LAP-A over a range of substrate concentrations (Figure 3.5A). His_6-LAP-A had a K_m of 1.8 mM and a V_{max} of 144.93 µmol/min/mg protein. These values are comparable to His_6-LAP-A's activity towards its most preferred dipeptide substrate, Leu-Gly, which had a K_m of 3.2 mM and V_{max} of 241 µmol/min/mg protein (Gu and Walling 2000). The Cys-Gly dipeptidase activity was abolished in the presence of the general LAP inhibitor bestatin (20 nM) and in the LAP-A mutant K354E, which lacks peptidase activity (Figure 3.5B; Gu et al. 1999; Gu and Walling 2002).

Since the tomato His_6-LAP-A hydrolyzed Cys-Gly, the neutral LAPs of tomato and Arabidopsis were tested for Cys-Gly dipeptidase activity. The tomato His_6-LAP-N and Arabidopsis His_6-LAP1 and His_6-LAP2 all displayed Cys-Gly dipeptidase activity (Figure 3.5B). Moreover, the neutral LAPs had more activity towards Cys-Gly than His_6-LAP-A in the presence of 5 mM substrate. His_6-LAP-N's activity of 228 µmol/min/mg protein was ~2.5-fold higher than His_6-LAP-A. Together this data demonstrates that plant LAPs are also Cys-Gly dipeptidases.

Since plant LAPs hydrolyzed Cys-Gly *in vitro*, assays were performed to determine if LAPs function as Cys-Gly dipeptidases *in vivo*. Cys-Gly dipeptidase activity was measured from total soluble protein extracts from control and wounded leaves of WT, *LapA-SI* and *LapA-OX* lines. In healthy, unwounded WT levels of soluble Cys-Gly activity were 166 µmol/min/g protein (Figure 3.5C). In unwounded *LapA-OX* plants, Cys-Gly dipeptidase activity was 1900 µmol/min/g protein; this was ~11-fold higher than in WT leaves. Cys-Gly dipeptidase activity was almost completely abolished in the *LapA-SI* lines. In WT leaves at 24 hr after wounding, Cys-Gly dipeptidase activity increased ~4-fold; this correlated with a ~20-fold increase *LapA* transcript levels (Figure 3.4; Figure 3.5C). Similarly, Cys-Gly dipeptidase activity was higher in the *LapA-OX* line and was barely detected in the *LapA-SI* line (Figure 3.5C). In addition, Cys-Gly dipeptidase activity was shown to increase ~8-fold with 24 hr MeJA treatment (Figure 3.5D). Moreover, addition of bestatin eliminated Cys-Gly dipeptidase activity in both the control WT as well as the MeJA-induced dipeptidase activity. It should be noted that *in vivo* Cys-Gly measurement results are representative of only one biological replicate and therefore will be repeated in future work. Together these data demonstrated that the soluble Cys-Gly activity directly correlates with *LapA* transcript levels and LAP activity suggesting that LAP-A is the major or only Cys-Gly dipeptidase in tomato.

Putative GSH Metabolism Genes in Tomato

Since there are several proposed models for GSH metabolism in plants (Noctor et al. 2012) and since GSH metabolic genes have not been characterized in tomato, BLAST search analysis was performed to verify which GSH metabolism genes are present in tomato (Table 3.2). Since the tomato LAPs are localized to the plastid, the location of putative GSH metabolism proteins were predicted to determine if LAP could act in concert with any of these enzymes. Like Arabidopsis, tomato has three APR proteins and a SAT2 protein localized to the plastid to synthesize Cys (Martin et al. 2005; Noji et al. 1998). In addition, tomato has homologues of GSH1 and GSH2, which, like Arabidopsis, are predicted to reside in the plastid. The Arabidopsis GSH2 has a splice variant without the plastid transit sequence (Wachter et al. 2005), but searches of

tomato Unigene database (http://solgenomics.net/) did not provide evidence for a similar transcript in tomato. However, potential truncated transcripts may have been incorrectly aligned with transcripts containing the transit sequence. While carboxypeptidase and GGC activities have been discovered in plants (Ohkama-Ohtsu et al. 2008; Wolf et al. 1996), the enzymes responsible for this activity have yet to be identified. Finally, phytochelatin synthase, GGT, and 5-OPase genes were also identified in tomato. While phytochelatin synthase and 5-OPase are predicted to be cytosolic, GGT is predicted be localized to the plasma membrane, which is consistent with a previous report demonstrating apoplast-localized GGT activity in tomato fruit (Martin and Slovin 2000).

Optimization of GSH and H_2O_2 Measurement Assays

Extraction methods were optimized based on methods in Queval and Noctor (2007). Extraction methods for thiols is typically done with HCl, while extraction of H_2O_2 for luminol assays is typically done with $HClO_4$ (Queval and Noctor 2007; Queval et al. 2008). Since these extractions are similar, thiol and H_2O_2 measurements were taken from the same HCl extractions since this is the more common method for thiol extraction and HCl extraction has been used previously for H_2O_2 measurements (Queval and Noctor 2007; Queval et al. 2008). After initial acid extraction, the extracts were too acidic for the chemical and enzymatic assays to measure thiols and H_2O_2. Therefore, extracts need to be neutralized before further analysis. It was determined that unknown compound(s) within the tomato extract had pH-dependent coloration. Acidic solutions (0.2 M HCl) had a pink hue that faded to clear at a pH between 5-6 (Figure 3.6B). When an extract had a pH above 6, a yellow hue was observed and its intensity increased with increasing pH. Therefore, the color of the extract could be used as a pH indicator during the neutralization process.

GSH is readily oxidized in alkaline or even neutral conditions (Noctor et al. 2011). Therefore, to accurately measure endogenous redox states of GSH, it was important to determine the effect of pH on the oxidative state of GSH in tomato extracts. The redox status of samples with various pHs was determined. Oxidiative state of GSH [% oxidized glutathione (GSSG)] was directly

proportional to the pH of the extract (Figure 3.6C). Healthy tissue should have no more than 10% GSSG (Noctor et al. 2011). When pH was kept below 5, GSSG levels were below 10%, while above pH of 5, GSSG levels increased up to 43% (Figure 3.6C). In addition, preliminary studies showed that pH affected the amount of H_2O_2 detected. When extracts had a pH greater than 5, the total amount of H_2O_2 detected was reduced by more than half (Figure 3.6D). Therefore, extracts were kept below a pH of 5 throughout the analysis to maximize the detection of H_2O_2 and GSH and reduce experimental oxidation of GSH. Experiments to measure thiols and H_2O_2 on wounded *LapA* mis-expression lines were not performed due to repeated growth chamber failures (June-October 2012) that induced primed stress responses within the plants prior to experimentation.

Since LAP's Cys-Gly dipeptidase activity could affect GSH metabolism, assays were optimized to test if GSH or its metabolites Cys-Gly, Cys, and γ-Glu-Cys were altered in WT, *LapA-SI* and *LapA-OX* lines before or after wounding (Figure 3.6A). GSH also has the potential to affect the redox status of the cell, in particular the redox-sensitive SA signaling component NPR1 (Noctor et al. 2012). GSH's reductive power is affected by its total levels and by its own redox status. Finally, GSH metabolism affects H_2O_2 metabolism, which can contribute to wound signaling (Noctor et al. 2012; Orozco-Cárdenas et al. 2001). Therefore, assays were also optimized to determine if the GSH redox status or H_2O_2 levels were also altered in the same wounded tissues (Figure 3.6A).

Total GSH levels and oxidized GSH levels can be monitored by measuring the amount of glutathione reductase (GR)-dependent 5,5'-dithiobis(2-nitrobenzoate) (DTNB) reduction (Queval and Noctor 2007). The rate of DNTB reduction is proportional to the total amount of GSH. However, the time of when GR activity is constant is limited; therefore, it was important to determine if assay measurements using a 96-well plate could capture the linear rate of activity. GR activity was constant for at least 6 minutes, and the rate of DTNB reduction was directly proportional to the total GSH (Figure 3.7A-B). GSH levels measured in tomato leaves were within this linear range (Figure 3.7B).

Queval et al. (2008) demonstrated that the levels of H_2O_2 measured with *in vitro* assays are directly dependent on the amount of tissue assayed. They showed dramatic increases in amounts of H_2O_2 detected in samples that when less than 20 mg tissue/ml extraction buffer was used. It is unclear what the source of this discrepancy is in Arabidopsis and if similar limitations applied to tomato extracts (Queval et al. 2008). Furthermore, this assay had not been performed with a HCl extraction. Therefore, to maximize sensitivity and decrease risk of technical variation, 30 mg/ml HCl buffer was used to extract H_2O_2 from tomato leaves. Preliminary data shows that H_2O_2 concentrations were ~2000 nmol/g FW (Figure 3.8D), which is similar to previously published studies that used a $HClO_4$ extraction method and 30 mg/ml of Arabidopsis leaves (Queval et al. 2008).

HPLC is used to measure different thiol compounds simultaneously. Bromobimane is typically attached these compounds to allow fluorescent detection and help retain small thiols on a reverse-phase column (Kosower and Kosower 1995; Noctor et al. 2011). During experimentation to optimize thiol separation, it was discovered that excess bromobimane reduced the sensitivity of the mass spectrophotometer over many samples (Figure 3.8A). Therefore, excess bromobimane was removed with dichloromethane extraction (Figure 3.8B). In addition, a solvent delay strategy was used to remove excess bimane and other contaminants in the extracts by diverting the proportion of the extract that did not contain thiols of interest from the mass spectrophotometer (Figure 3.8D). In addition, Cys and Glu-Cys are present at low levels in the cell (Noctor et al. 2011). Therefore, a low limit of detection was required. Preliminary studies demonstrated that pmol amounts of GSH, Cys-Gly, γ-Glu-Cys, and Cys could be detected. This indicated that they system had the sensitivity to detect nmols/g FW of these thiols, which is within the range present within tomato leaves (Figure 3.7C; data not shown).

LAP-A Differentially Modulates Late-Wound Response Gene Family Members

In tomato, H_2O_2 signaling modulates late-wound response genes such as *PinI*, *PinII*, *PPO*, an aspartic proteinase inhibitor (*CDI*), and a metallocarboxypeptidase inhibitor (*CP*I; Orozco-Cárdenas et al. 2001). ROS and ROS signaling have also been implicated in retrograde signaling

(Galvez-Valdivieso and Mullineaux 2010). Finally, given LAP-A's potential role to modulate the antioxidant GSH metabolism through its Cys-Gly dipeptidase activity, studies were performed to determine if LAP-A modulated ROS signaling after wounding.

Since H_2O_2 and LAP-A positively modulate the tomato late wound response, experiments were designed to complement the *LapA-SI* lines by co-treating plants with H_2O_2 before and during wounding. However, in testing control experiments for *PinI*, *PinII*, and *PPO-F* expression by qRT-PCR, unanticipated patterns of expression were discovered in the *LapA-SI* and *LapA-OX* lines after wounding. Previous studies monitored early (*Allene Oxide Synthase* and *Lipoxygenase-D*) and late (*PinI*, *PinII*, and *PPO-F*) wound response genes in WT, *LapA-SI* and *LapA-OX* plants (Fowler et al. 2009). Late wound-response gene RNA levels measured by RNA blot and qRT-PCR were lower in the *LapA-SI* lines and higher in the *LapA-OX* line in response to wounding.

Surprisingly, when *PinI*, *PinII*, and *PPO-F* transcripts were measured by qPCR they accumulated to higher levels in the *LapA-SI* lines relative to WT, particularly at 12 hr after wounding (Figure 3.9). Reciprocally, these three transcripts were suppressed in the *LapA-OX* lines. This is in direct contrast to RNA blot and qRT-PCR analyses previously described (Fowler et al. 2009). In the RNA blot analyses, full-length cDNA clones were used to measure mRNA accumulation. Therefore, the labeled cDNA probe could have cross-hybridized with one or more *PinI*, *PinII* or *PPO* gene family members with at least 60% identity. In tomato, *PinI*, *PinII* and *PPO* belong to multi-gene families with seven, five, and seven members, respectively, with >60% nucleotide sequence identity (Figure 3.10; Newman et al. 1993). Therefore, previous RNA blot signals could reflect the cumulative expression of several different gene family members.

Upon further examination, it was discovered that previously utilized qRT-PCR primers for *PinI* and *PPO* had high enough nucleotide identity to prime at least two different family members each (Figure 3.11; Primer set 1; Fowler et al. 2009). In addition, *PinI* and *PinII* primers used in the current study could also prime two genes each (Figure 3.11; Primer set 2). Therefore, discrepancies between current qRT-PCR analysis and previous RNA blot and qRT-PCR analysis could reflect differential modulation of different gene family members in the late-wound response.

Further studies will be required to determine how each of the *PinI*, *PinII* and *PPO* family members is regulated in order to assess the studies of the *LapA* mis-expression lines.

LAP-A May Modulate ROS-Dependent Transcripts During Wounding

Endogenous H_2O_2 contents are difficult to quantify and methods are often too insensitive to detect changes in response to stress (Queval et al. 2008; Queval et al. 2009). Instead, transcriptional markers have proven to be more sensitive, reliable and quantitative measures of oxidative stress (at least in Arabidopsis). However, no systematic study has been performed to determine H_2O_2-dependent transcripts in tomato. Therefore, potential tomato ROS-dependent transcripts were identified through a literature search of ROS-responsive genes in Arabidopsis and tomato (Table 3.3). These genes included those involved in Cys, GSH, and ROS metabolism. In addition to *Thi*, an asparaginase (*Asp*) was identified as being Rboh-dependent after wounding in tomato (Sagi et al. 2004). *Asp* has been predicted to act as an anti-nutritional enzyme in defense by degrading Asn within the insect midgut (Liu et al. 2007a).

Preliminary screens of the 14 potential ROS-responsive genes were tested for induction in response to H_2O_2 treatment of tomato plants. WT tomato shoots were treated via their transpiration stream with glucose and glucose oxidase for 10 hr. Four genes either did not respond (*APR1*) or were repressed [*Asp*, *glutathione reductase* (*GRX*), and *SAT2*] in response to H_2O_2 treatment (Figure 3.12). The remaining genes were further tested for ROS-dependency by treating WT tomato plants with glucose and glucose oxidase with and without catalase, which catabolizes H_2O_2, for 10 hr (Figure 3.12-13). Six of these genes had diverse and unanticipated responses to H_2O_2. *Ascorbate peroxidase 3* (*APX3*) did not accumulate after H_2O_2 treatment and did not respond to catalase treatment. S*uperoxide dismutase 2* (*SOD2*) did not respond to H_2O_2 treatment, but was increased by catalase treatment, suggesting repression by H_2O_2. *APX7*, and *γ–glutamyl transferase* (*GGT*) decreased in response to H_2O_2; this was slightly alleviated by catalase treatment, again suggesting repression by H_2O_2. *SAT1* and *SAT3* had unusual expression patterns that were inconsistent with regulation by H_2O_2. For example, *SAT1* RNAs

accumulated after H_2O_2 treatment and were further induced by catalase treatment, while *SAT3* was down-regulated by H_2O_2 and even further suppressed by catalase treatment.

Four genes displayed a pattern typical of ROS-responsive genes including two genes involved in Cys biosynthesis (*APR2*, *APR3*), one involved in ROS catabolism (*monodehydroascorbate reductase 1*; *MDHAR1*) and one involved in defense (*Thi*). These genes were both induced in response to H_2O_2 and this induction was decreased or absent in the presence of catalase (Figure 3.13). These confirmed H_2O_2-dependent genes were tested for transcript accumulation in WT, *LapA-SI* and *LapA-OX* lines 0, 1, 12, and 24 hr after wounding. *Thi* had already been tested and showed both wound induction and LAP-A modulated expression (Figure 3.4). *APR2*, *APR3*, and *MDHAR1* all showed low levels of RNA throughout the time course and in each genotype (Figure 3.14). *MDHAR1* RNAs increased slightly (~1.5 fold) at 12 hr after wounding, but more replicates are needed to show significant differences. Neither, *APR3* nor *MDHAR1* showed any significant trends between genotypes. Only *APR2* RNAs showed significant increases (~3 fold) at 24 hr after wounding. At this timepoint, *APR2* transcript levels may have been lower in the *LapA-OX* lines, but further biological replicates are needed to demonstrate significant differences. If confirmed, reduced *APR2* expression in the *LapA-OX* line would be consistent with decreased *Thi* levels in *LapA-OX* and increased *Thi* expression in the *LapA-SI* line. Together this data suggests that H_2O_2 signaling may also be altered in *LapA* mis-expression lines.

DISCUSSION

In this Chapter, experiments were performed to explore whether LAP-A acts through known hormone signals in order to affect wound signaling in tomato leaves. Initial measurements of the phytohomornes JA, JA-Ile, ABA, and SA revealed that there is a wide biological variability within tomato. Data shown was representative of three to four biological replicates from pools of three four-week old plants. Data from other labs often rely on pseudoreplicates, in which biological replicates are grown at the same time and pooled into different replicates for analysis. Therefore, our data may represent more true biological variation. In this study, most of the hormones were

not different between genotypes. In particular, JA and JA-Ile were not significantly different between the genotypes, consistent with previous studies that demonstrated that LAP-A acts downstream of JA biosynthesis and perception (Fowler et al. 2009).

However, this preliminary work did suggest the *LapA-SI* had increased SA levels, particularly at 24 hr after wounding; however this trend was not consistent in all the biological samples (Figure 3.3). Furthermore, it is important to note that a reciprocal trend was not seen in the *LapA-OX* line. This suggests that LAP-A modulation of SA metabolism is complex or that perhaps the LAP-N, which is only mis-expressed in the *LapA-SI* line, also influences SA levels. Supporting the idea that LAP-A influences SA levels, the SA-responsive *PR-1b* and *Thi* had variable, but higher expression levels in *LapA-SI* compared to WT before and after wounding. Moreover, the expression of *PR-1b* and *Thi* appeared to be correlated with each other in the different biological replicates, suggesting that they were co-regulated. However, the expression of these genes was either the same as WT or lower in *LapA-OX*, consistent with SA levels in this line. Together these data suggested that SA signaling may be up-regulated in the *LapA-SI line* and either down or not regulated in the *LapA-OX* line. Measurements of more biological replicates are needed for both SA levels and *PR-1b* and *Thi* expression to confirm these trends.

SA is known to be a negative regulator of JA-dependent wound responses in tomato. Several studies in tomato have shown that SA or aspirin repress the accumulation of *PinI*, *PinII*, and *PPO* RNAs after wounding or treatment with wound signaling molecules (Doares et al. 1995a; Doares et al. 1995b; Peña-Cortés et al. 1993; Peña-Cortés et al. 1995; Thaler et al. 2002). The extent of this antagonism and whether it occurs up- or down-stream of JA biosynthesis and perception is most likely due to the difference in timing, duration and concentration of treatment with each elicitor. LAP-A only partially modulates *PinI*, *PinII*, and *PPO* (Fowler et al. 2009), consistent with an incomplete antagonism with SA in which perhaps both JA signaling and SA signaling are present to varying degrees. In addition, *PR-1* is known to be co-regulated by JA and SA in Arabidopsis and the tomato *PR-1b* is known to be up-regulated in response to SA, MeJA, and wounding (AbuQamar et al. 2009; Chao et al. 1999; Mur et al. 2006; Uppalapati et al. 2007; Vidya

et al. 1999). *Thi* is also known to be synergistically regulated by SA and JA in Arabidopsis (Mur et al. 2006); however while *Thi* is known to be regulated by wounding in tomato, its responsiveness to JA and SA remain to be determined in tomato (Sagi et al. 2004). Given that JA-dependent *PinI*, *PinII*, and *PPO* are down-regulated in the *LapA-SI* line (Fowler et al. 2009), up-regulation of *PR-1b* and *Thi* are more likely due to an up-regulation by SA or a combination of JA and SA signaling in this line. Alternatively, down-regulation of JA signaling may de-repress SA signaling in the *LapA-SI* line. Future studies will need to determine if *PR-1b* and *Thi* can be synergistically regulated by SA and JA. In addition, to discriminate between SA and JA signaling, markers such as *PR-3* could be tested for their expression, since *PR-3* is only induced by SA and not JA in tomato (Doares et al. 1995a).

PR-1 is also highly induced in response to ethylene (ET) treatment in tomato and ET is essential for a robust JA or wound-induced *PinII* induction (O'Donnell 1996). However, the highly ET-inducible gene *ET-responsive 5* (*ER5*) was not differentially regulated in either of the *LapA* mis-expression lines (Chapter 2). In fact, *ER5* may be slightly down-regulated in *LapA-SI* 1 hr after wounding. Therefore, it is likely that ET signaling is not responsible for the LAP-A-dependent mis-expression of *PR-1b* and *Thi*. This could be further confirmed using the defense marker *PR-4*, which has been shown to be induced by ET but not by JA or SA signaling in tomato (Chao et al. 1999).

GSH is a signal molecule that is highly abundant in the plastid and is known to regulate defense signaling in plants, including inducing the expression of genes such as *PR-1* (Gomez et al. 2004; Senda and Ogawa 2004). While GSH biosynthesis is fairly well-established in plants, the GSH catabolic pathway(s) remain elusive. At least four enzymes have been implicated in initial GSH breakdown, the two most well-characterized of which result in the release of Cys-Gly (Noctor et al. 2012). Cys-Gly is a potential source of essential reduced sulfur as well as a potentially damaging ROS (Baudouin-Cornu et al. 2012; De Donatis et al. 2010; del Bello et al. 1999; Del Corso et al. 2002; Dominici et al. 1999; Enoiu et al. 2007).

Chemical and genetic data in this Chapter indicated that LAPs are the major Cys-Gly dipeptidase in tomato and possibly in Arabidopsis. While LAP-N is able to hydrolyze Cys-Gly, *LapN* is a rare class transcript and constitutive levels of LAP-N protein are low (Chao et al. 2000; Tu et al. 2003). Therefore, it is not clear how much LAP-N contributes to overall Cys-Gly catabolism. Future studies with *LapA* and *LapN* RNAi lines will determine which LAP is the major Cys-Gly dipeptidase in tomato. Given that the Arabidopsis LAPs also hydrolyze Cys-Gly efficiently *in vitro*, studies with available knock-out *lap1*, *lap2*, and *lap3* single and double mutants (*lapl1/2* and *lap1/3*) should to be performed to determine if LAP acts the major Cys-Gly dipeptidase in Arabidopsis.

LAP's placement within the plastid is an ideal location to recycle Cys for GSH biosynthesis similar to bovine and yeast models (Baudouin-Cornu et al. 2012; Cappiello et al. 2004; De Donatis et al. 2010). However, enzymes that catalyze the hydrolysis of the Glu γ bond have different subcellular locations making the tomato GSH cycle complex (Figure 3.1). For example, the tomato GGT is predicted to be localized in the apoplast, making transit of the reactive Cys-Gly to the plastid unlikely unless transporters efficiently import Cys-Gly into the cytosol and that plastid. Alternatively, GSH could be acted upon by an unknown GGC-like activity to release Cys-Gly. GGC is the proposed major GSH catabolic enzyme in Arabidopsis and its activity has been detected in *N. tabacum* (Ohkama-Ohtsu et al. 2008; Steinkamp and Rennenberg 1985; Steinkamp et al. 1987). In Arabidopsis, GGC is believed to be localized to the cytosol, making Cys-Gly transport to the plastid more feasible (Ohkama-Ohtsu et al. 2008). Since the identity of the gene encoding the plant GGC enzyme is unknown, tomato should be tested for the presence and location of GGC activity. Interestingly, the Arabidopsis LAP1 is predicted to be localized to the cytosol (Bartling and Weiler 1992), making it a more ideal candidate to catabolize cytosolic Cys-Gly.

In other kingdoms, aminopeptidases from multiple families that hydrolyze Cys-Gly have been identified. In addition to M17 aminopeptidases (LAPs), the M20A Dug1p and members of the M1 and M19 peptidase families have all been shown to hydrolyze Cys-Gly in at least one species

(Dringen et al. 2001; Grau et al. 1979; Kaur et al. 2009; Mcintyre and Curthoys 1982; Rankin et al. 1980; Robinson et al. 1953). However, plants lack a Dug1p homologue and the closest homologue to the M19 peptidases in plants is LAP (data not shown). Plants do have M1 aminopeptidases. In tomato, as in Arabidopsis, there are three M1 peptidases (Table 3.2): meiotic prophase aminopeptidase 1-like (MPA1-like), aminopeptidase M1-like (APM1-like), and TAF2-like 2 (TAF2L2)/leukotriene A4 hydrolase-like (LTA4HL) (Peer 2011; Walling 2006). In Arabidopsis, MPA1 has been shown to be localized to the cytosol and the apoplast (Kaffarnik et al. 2009), while AMP1 has been shown to be localized to the secretory pathway and the plasma membrane (Peer et al. 2009). TAF2L2 is localized to the cytosol and the nucleus, but also has the potential to interact with AMP1 potentially in the secretory pathway or plasma membrane (Hosein et al. 2010). Therefore, these enzymes are poised to digest Cys-Gly that may be generated by apoplastic GGT activity in tomato. Given that AMP1 and potentially TAF2L2 are membrane associated, future work should rule out any potential Cys-Gly dipeptidase activity by membrane-bound or membrane-associated proteins.

Given LAP-A's potential role in GSH metabolism and LAP-A's similar regulation of late-wound response genes as H_2O_2, it was hypothesized that LAP-A may act through H_2O_2 to regulate gene expression. Therefore, experiments were designed to complement the *LapA-SI* wound-response phenotype with H_2O_2. However, the control experiments used newly designed gene-specific qPCR primers for *PinI*, *PinII*, and *PPO-F* demonstrated that LAP-A modulates at least one member of each of these gene families in a manner that was not anticipated. The *PinI*, *PinII*, and *PPO-F* members studied in this Chapter were negatively modulated by LAP-A compared to the positive modulation seen in Fowler et al. (2009). This discrepancy is most likely due to the fact that previous RNA blot and qRT-PCR studies measured multiple and/or unique gene family members from the current study. For the *Pin* gene families, primers in the previous and current study were designed before complete gene sequences of all the family members were available. Therefore, family member-specific primers for each member will have to be design and analyzed in the future. While PPO family members are known to be differentially regulated (Thipyapong et

al. 1997; Thipyapong and Steffens 1997), differential regulation of individual *PinI* and *PinII* family members has never been examined. Therefore, it will be interesting to determine how each family member is regulated by wounding and potentially modulated by LAP-A in the future.

Since H_2O_2 complementation studies could not be performed due to the unexpected complexity of LAP-A regulation of the late wound-response gene families, assays were designed to ROS-responsive gene expression in the *LapA* mis-expression lines as an indirect measurement of altered oxidative stress. Putative ROS-responsive genes were identified and their expression tested in response to H_2O_2 or catalase treatments in tomato. Many of the genes predicted to H_2O_2–responsive based on their expression in Arabidopsis or tomato fruit were either not induced or actually down-regulated at 10 hr after H_2O_2 treatment (Table 3.3). These data suggest a divergence in ROS gene regulation between tomato leaves and Arabidopsis or tomato fruit. The experiments presented here cannot exclude that the genes may be up-regulated at earlier or later times after ROS stress. In addition, this study does not rule out the possibility of regulation at other levels of gene expression. Many ROS metabolism genes are regulated in part at the post-translational level. Examples include an APR (Bick et al. 2001), catalase (Shao et al. 2008; Volk and Feierabend 1989), and γ–ECS (Hell and Bergmann 1990; Hothorn et al. 2006; Noctor et al. 2002).

Only four genes behaved in a ROS-dependent manner: *APR2*, *APR3*, *MDHAR1*, and *Thi*. Of these, only *APR2* and *Thi* showed significant wound induction in WT leaves. In addition, *APR2* may have been down-regulated in *LapA-OX* line 24 hr after wounding. H_2O_2 and ROS have been shown to induce *PR-1* expression in Arabidopsis and modulate wound-induced *Thi* expression in tomato (Baier and Dietz 2005; Sagi et al. 2004). Therefore, lower *APR2* RNAs in *LapA-OX* leaves was consistent with lower *Thi* transcript levels in the *LapA-OX* line and higher *PR-1b* and *Thi* in *LapA-SI*. Together, these data suggest that there is higher oxidative stress in the *LapA-SI* line and lower oxidative stress in the *LapA-OX* line. However, this data is inconsistent with previous studies that demonstrated that H_2O_2 was a positive regulator of late-wound responses (Orozco-Cárdenas et al. 2001). If ROS metabolism is altered in the *LapA* mis-expression lines, this

discrepancy may be due to the type, location, and amount of ROS that is produced in the *LapA* lines, since ROS signaling is unique for each ROS species and is unique to the timing and location of the signal (Gadjev et al. 2006; Mittler et al. 2011; Møller and Sweetlove 2010). Discrepancies may also be due to ROS interaction with other signaling pathways that may also be altered in the *LapA* mis-expression lines, such as altered SA or JA signaling.

Disentangling GSH, SA and H_2O_2 signaling may be tricky. First, GSH is a well-established antioxidant that has the potential to affect ROS accumulation (Foyer and Noctor 2011). Second, ROS signaling is known to be important for defense gene expression and ROS itself can induce defense genes (Baier and Dietz 2005; Danon et al. 2005; Levine et al. 1994; Suzuki et al. 2011). In addition, H_2O_2 can increase SA levels, while SA can increase stress-induced ROS accumulation presumably due to its down-regulation of ROS scavenging enzymes (Klessig et al. 2000; Leon et al. 1995; Shirasu et al. 1997). Finally, GSH and SA signaling are also entangled since GSH has been implicated in SA defenses, either through helping to reduce NPR1 and induce SA responses or by acting with cytosolic NPR1 through an unknown pathway to suppress JA defense (Koornneef et al. 2008; Noctor et al. 2012; Spoel et al. 2003). Finally, GSH is also known to induce *PR-1* gene expression (Gomez et al. 2004; Senda and Ogawa 2004). Therefore, in the future careful examination of the timing and level of SA, GSH, and H_2O_2 synthesis and signaling is required to determine which, if any, of these signals is the cause of differential gene regulation in the *LapA* mis-expression lines.

EXPERIMENTAL PROCEDURES

Plant Materials and Growth Conditions

Solanum lycopersicum L. UC82 (wild-type, WT), *LapA-SI*, and *LapA-OX* were previously described (Fowler et al. 2009). Plants were grown according to Chao et al. (1999) in a growth chamber with an 18-hr (28°C)/6-hr (24°C) light (300 µE)/dark cycle. However, growth chambers were prone to overheating and intermittently shutting off, resulting in tomato plants being exposed to highly variable abiotic stress. Therefore, consistent, healthy biological replicates for thiol and H_2O_2 measurement studies were not collected.

Wound, MeJA and H_2O_2 Treatments

Three- to four-week-old plants were used in the wounding and H_2O_2 treatment studies. Plants were wounded by crushing each leaflet with a pair of needle-nosed pliers. Wounded leaves were collected at designated times by freezing in liquid N_2. Plants were treated with exogenous MeJA by incubating excised shoots in flasks with 0.005% ethanol (control) or 1000 µM MeJA and 0.005% ethanol for 24 hr as previously descibed (Fowler et al. 2009). Briefly, H_2O_2 treatments were performed as described by Orozco-Cárdenas et al. (2001). Previous studies only treated for 2 hr followed by water treatment (Orozco-Cárdenas et al. 2001); however preliminary studies that monitored *PinI* RNAs demonstrated that H_2O_2 signaling was not as persistent in our tomato lines (Figure 3.15). The treatment time was chosen to correspond to the peak wound response time for genes that are regulated by H_2O_2 so that genes could be tested for complementation after wounding with H_2O_2 (Fowler et al. 2009; Orozco-Cárdenas et al. 2001). Therefore, plants in this study were treated continuously with H_2O_2 for 10 hr. Briefly, plants were excised at the base of the stem with a razor blade. Excised plants were placed in 10 mM phosphate buffer (pH 6.0) alone or with 50 µM glucose (Glu) and 2.5 U/ml glucose oxidase (GO; Sigma, St. Louis, MO). As a negative control, plants were also place in a phosphate buffer solution with 50 µM Glu, 2.5 U/ml GO, and 2.5 U/ml catalase (Cat, Sigma). For each treatment, the leaves of three four-week-old plants at each time point were pooled together for analysis. Four biological replicates were used for LC-MS analysis of phytohormones while two biological replicates were used for gene expression analysis studies.

LC-MS Analysis of Phytohormones

LC-MS analysis was performed according to Chung et al. (2008) with minor modifications. Briefly, ~500 mg of wounded tomato leaves were ground in liquid N_2 with a mortar and pestle. Dihydro-JA and ^{13}C-JA-Ile (generously provided by Dr. Gregg Howe; 66.6 pmols each), d2-GA4 (Australian National University, Canberra, Australia; 40 p mols), d4-SA (C/D/N isotopes, Pointe-Claire, Canada; 160 p mols), and d4-ABA (800 p mols) and 400 p mols each of d5-ABA GE, d3-DPA,

and d3-PA (National Research Council of Canada, Saskatoon, Canada), were used as internal standards. Homogenized tissue was resuspended in 2.5 ml of ethyl acetate and centrifuged for 10 min at 4°C at 12,000 g. The pellet was reextracted with 1 ml ethyl acetate. Combined extractions were evaporated with a stream of N_2 gas at 55°C. Dehydrated extracts were resuspended in 0.3 ml 70% methanol/water (v/v) and cleared by centrifugation at 13,000xg for 30 min at 4°C. Cleared extract (5 µl) was separated on UPLC BEH C18 column (1.7 µM, 2.1 x 50 mm) connected to an Acquity ultraperformance liquid chromatography system (Waters), which was linked to a Quattro Premier XE tandem quadrupole mass spectrometer (Waters). A gradient mobile phase was applied as previously described (Chung et al. 2008). Multiple-reaction monitoring (MRM) transition mass to charge ratios and spectrometer conditions are provided in Table 3.1. Cone voltages (CV) and collision energies (CE) were optimized and retention times were determined using labeled and unlabeled standards. MRM and conditions for JA and JA-Ile and their labeled standards were previously described (Chung et al. 2008). LC-MS optimization and experiments were performed at Michigan State University Mass Spectrometry Core with support from Dr. Gregg Howe, Dr. Abe Koo, and Dr. Dan Jones.

Total Protein Extraction from Tomato Leaves

One gram of leaf tissue was homogenized in 3 ml cold Buffer A [50 mM Tris-HCl, pH 6.8, 15% (v/v) glycerol, 1% (v/v) β–mercaptoethanol and 150 mg insoluble polyvinylpyrrolidone (PVP)] in a pre-chilled dounce homogenizer. Homogenized tissue was cleared by centrifuged at 10,000g for 20 min at 4°C. The Bradford method was used to determine protein concentrations using IgG as a standard (Bio-Rad Protein Assay Kit I, Bio-Rad, Hercules, CA).

Over-expression and Purification of LAP Proteins from E. coli

The *E. coli* vectors that express the His_6-LAP-A, His_6-LAP-N, the His_6-LAP-A mutant (K354E), His_6-LAP1, and His_6-LAP2 were previously described (Gu and Walling 2002; Scranton et al. 2012; Tu et al. 2003). His_6-LAP fusion proteins were expressed in and purified from *E. coli* according to Scranton et al. (2012). Briefly, overnight cultures were diluted 1:20 to a final volume of 0.5 L. Cultures were grown at 37°C (wild-type and mutant His_6-LAP-A) or 30°C (His_6-LAP1,

His_6-LAP2, and His_6-LAP-Ns) to an OD_{600}=0.6. After induction with 0.4 mM IPTG, cultures were grown for an additional 6-18 hr at 37°C (wild-type and mutant His_6-LAP-A), 30°C (His_6-LAP1, His_6-LAP2), or 22°C (His_6-LAP-N). Cells were resuspended in 50 ml pre-chilled Buffer B (50 mM $NaPO_4$, pH 8.0, 300 mM NaCl) with 75 mM lysozyme and incubated on ice for 30 min. Cells were lysed by sonicatation and the lysate was cleared by centrifugation at 10,000 g for 30 min at 4°C. His_6-LAP proteins were purified using Ni/nitrilotriacetic acid resin columns (Qiagen, Valencia, CA) as previously described (Gu and Walling 2000). LAP-A wild-type and mutant proteins and LAP1 and LAP2 proteins were stored at -20°C in 25 mM sodium phosphate (pH 8.0), 250 mM NaCl, 125 mM imidazole, and 50% glycerol. LAP-N protein was used on the day it was purified. Total protein concentrations were determined as described above.

Cys-Gly Dipeptidase Activity Assay

Cys-Gly dipeptidase activity was determined according to Cappiello et al. (2004) with minor modifications. Purified His_6-LAP (50 ng) or cleared leaf extract (20 µg) was added to a mixture with 0.25 - 5 mM Cys-Gly, 5 mM dithiothreitol (DTT), 0.2 $MnCl_2$, and 50 mM Tris-HCl (pH 8.0) to a final volume of 250 µl. As negative controls, His_6-LAP-A or cleared leaf extract was mixed in the same solution with 20 nM or 20 µM bestatin, respectively. Reactions were performed at 37°C and stopped after 20 min with the addition of 200 µl 5% trichloroacetic acid. After centrifugation for 1 min at 12,000xg, 200 µl of supernatant was added to 400 µl mixture of 80% (v/v) glacial acetic acid, 0.8 M HCl and 47 mM ninhydrin. The mixture was boiled for 10 min followed by cooling on ice. Three hundred µl of the mixture was added to 1 ml of 95% ethanol and ninhydrin-bound free cysteine was measured as the absorbance at 560 nm. Three technical replicates were performed on purified His_6-LAP proteins. Two technical and two biological replicates were performed for leaf extracts.

RNA Isolation and Quantitative RT-PCR Analysis

RNAs were extracted using a hot phenol method as previously described (Pautot et al. 2001). A nano-Drop ND-1000 spectrophotometer was used to quantify RNA and measure 260/280 nm absorbance ratios. Presence of intact rRNA bands by 1.5% formaldehyde gel was determined to

confirm RNA quality. RNAs were stored at -80°C. Total RNA was treated with RQ1 RNase-Free DNase (Promega, Madison, WI) according to the manufacturer's instructions. Reverse transcription (RT) was performed according to the manufacturer's instructions with RNase H^+ iScript reverse transcriptase (Bio-rad Laboratories, Hercules, CA). cDNAs were diluted 1:10 in sterile deionized water for qPCR analysis and stored at -20°C.

Primer3 (Rozen and Skaletsky 2000) was used to design qPCR primers. Annealing temperatures and efficiencies were determined experimentally. Unigene identifications, primer sequences and optimum annealing temperatures are provided in Table 3.4. qPCR reactions were performed in triplicate using iQ SYBRGreen Super-mix (Bio-Rad Laboratories) as described in Fowler et al. (2009). Ct values and reaction efficiencies were determined using real-time PCR miner (Zhao and Fernald 2005). Averaged Ct values of technical replicates and averaged efficiencies of primers were used to calculate mRNA levels of genes of interest and reference genes. mRNA levels of genes of interest were normalized against the geometric mean of *EF1α* (SGN-U580418) and ubiquitin (*Ubi3*; SGN-U580697) as previously described (Fowler et al. 2009). The Ubi3 primers used were previous described (Fowler et al. 2009).

Identification of Putative GSH Metabolism and ROS-Responsive Genes in Tomato

Potential GSH metabolism genes were identified either through literature searches in tomato or through BLASTX search of SGN Unigene database using Arabidopsis homologues (Table 3.2). Protein sequences were analyzed for predicted locations using TargetP (Emanuelsson et al. 2000), MultiLoc (Hoglund et al. 2006), and WoLF PSORT (Horton et al. 2007). Locations shown were identified by at least two of the programs.

Potential ROS-responsive genes identified through a literature search of Arabidopsis and tomato ROS-regulated genes (Table 3.3). Tomato homologues were identified using Basic Local Alignment Search Tool BLASTX (Arabidopsis query sequence) or BLASTN (Tomato query sequence) (Altschul et al. 1990) of the Sol Genomics Network (SGN) Lycopersicon combined Unigene database (http://solgenomics.net/).

Extractions for Thiol and H_2O_2 Measurements

Acid extractions were performed according to Queval and Noctor (2007) with minor modifications. Leaf tissue (0.5 g) was ground in liquid nitrogen with a mortar and pestle and extracted with 6 volumes of 0.2 M HCl. Tissue aliquots for H_2O_2 measurements were diluted to 33.3 mg/ml to increase sensitivity (Queval et al. 2008). Aliquots were spun in eppendorf tubes at 14,000xg for 20 min. Internal standards were added to separate supernatant aliquots (0.5 ml) for recovery measurements (5 nmols N-acetylcysteine, 20 nmols GSH, or 10 nmols H_2O_2). Aliquots for H_2O_2 measurements were flash frozen in liquid nitrogen and processed within 72 hrs. Aliquots were initially neutralized with 50 µl of 0.2 M NaH_2PO_4 (pH 5.6). Aliquots for measurement of GSH oxidation states and H_2O_2 concentrations were further neutralized with 400 µl 0.2 M NaOH, while vortexing, keeping the pH below 5 (Figure 3.6). Aliquots for HPLC thiol analysis were neutralized to reach pH 5-6 (~400-500 µl 0.2 M NaOH).

Enzymatic Determination of Total and Oxidized GSH Concentrations

Total and oxidized GSH were determined according to Queval and Noctor (2007)(Figure 3.6A). Briefly, neutralized supernatants were split into two 0.2-ml aliquots. Half of the aliquots were incubated with 1 µl 2-vinylpyridine (VPD) for 20 min at RT to remove reduced GSH; cleared of excess VPD twice by centrifugation. Triplicate 20-µl aliquots of VPD and non-VPD treated supernatants were added to a 96-well CoStar clear bottom plate wells with 0.11 ml Buffer B (0.2 M NaH_2PO_4, pH 7.2, 10 mM EDTA) and 10 mM NADPH. The reaction was started by the addition of 0.2 U glutathione reductase (GR; Sigma) in Buffer B with 2.4 mM 5,5'-dithiobis(2-nitrobenzoate) (DTNB; Sigma) in a total volume of 50 µl. The reaction was monitored at 405 nm for 5 min with shaking between each reading using a Victor^2 1420 Multilabel Counter (PerkinElmer Life Sciences, Waltham, MA).

Measurement of H_2O_2 concentrations

Neutralized extracts were analyzed for concentrations according to Rao et al. (2000) with minor modifications. Neutralized extracts (0.5 ml) were treated with 1 U ascorbate oxidase (Sigma) for 5 min at room temperature (RT) (Queval et al. 2008). Treated extracts were passed over a column

of 0.5g Dowex 1-X8 50-100 mesh resin resuspended in double distilled water. Extracts were eluted with 3 ml double distilled water. Fifty µl of eluted extract was mixed with 850 µl of 0.2M NH_4OH (pH 9.5) and 30 µM luminol. Luminescence was integrated over a 5-sec period immediately after injection of 100 µl of 0.5 mM potassium ferricyanide in 0.2M NH_4OH (pH 9.5) using Turner Biosystems 20/20n Luminometer (Promega, Madison, WI).

Measurement of GSH and its Metabolites by LC-MS

Bimane label was attached to thiols in the neutralized extracts according to Queval and Noctor (2007) with minor modifications. Neutralized extracts (200 µl) were added to 0.1 ml 0.5 M Ches (pH 8.5). Thiols were reduced with the addition of 10 mM DTT (20 µl) and incubation in the dark for at RT for 30 min. Bromobimane (Sigma) was added to a final concentration of 0.6 mM and incubated for another 15 min at RT in the dark. Excess dye was removed by the addition of 0.5 ml dichloromethane and clearing by 10 min centrifugation at 13,0000xg. Reaction with remaining bromobimane in the supernatant was stopped by the addition of 0.66 ml of 10% v/v acetic acid and 10 min centrifugation at 10,0000xg. Cleared supernatant (0.9 ml) was passed through a 0.2 µM mesh filter (Millipore) and stored at -80°C until analysis.

Cleared extract (10 to 100 µl) was separated on Poroshell 120 EC-C18 column (2.7 µM, 3.0 x 50 mm) connected to a 1260 Infinity liquid chromatography system (Agilent), which was linked to a 6224 Time-of-Flight mass spectrometer (Agilent). Compounds were separated using a six-min gradient program with 0.01% trifluoroacetic acid (solvent A) and acetonitrile (solvent B) at a flow rate of 0.75 ml/min.

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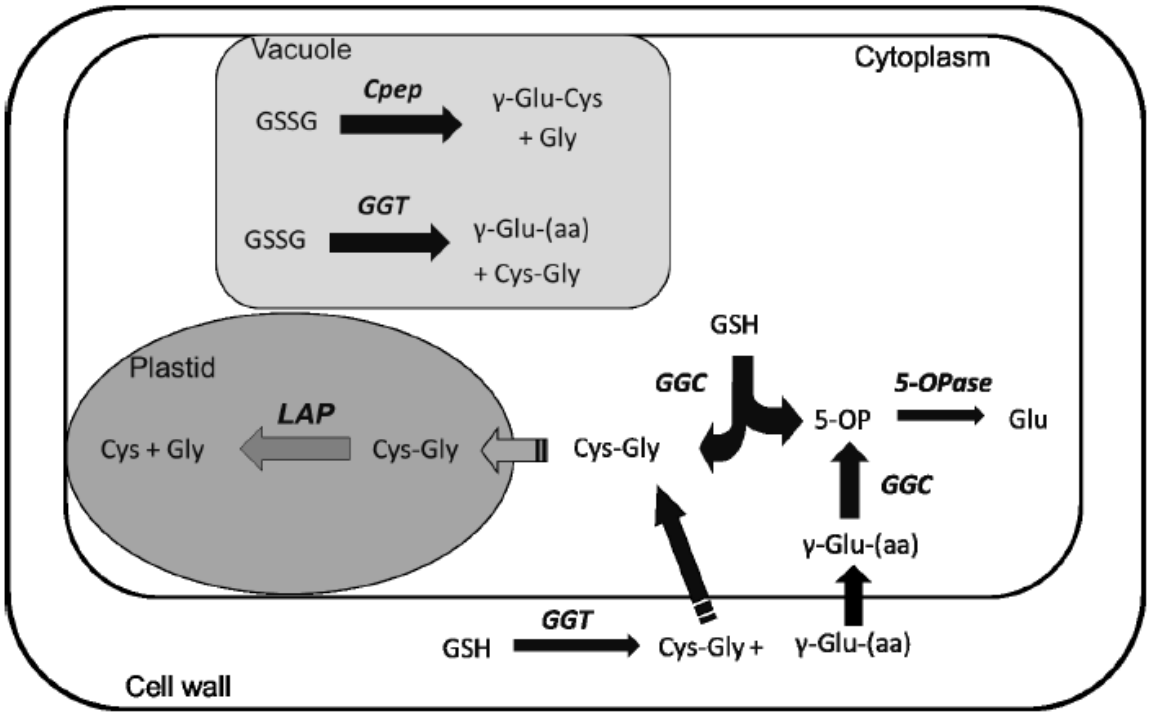


Figure 3.1 Plant GSH catabolism genes and proposed model for LAP's role in plant GSH metabolism. Cpep: carboxypeptidase; GGT: γ–glutamyl transpeptidase; 5OPase: 5-oxoprolinase; GGC: γ–glutamyl cyclotransferase.

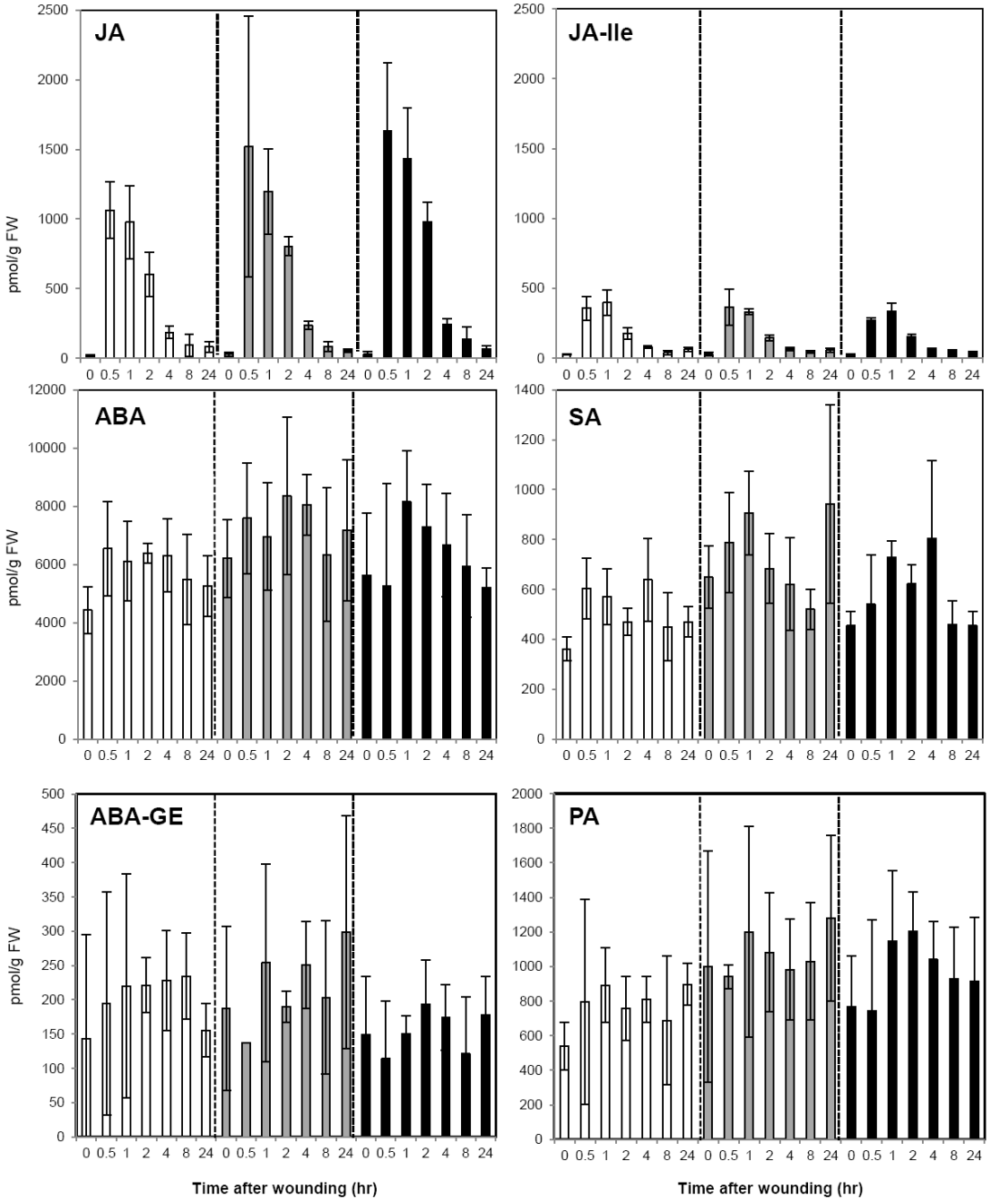


Figure 3.2 LC-MS analysis of key defense phytohormones in WT, *LapA-SI* and *LapA-OX* lines in response to mechanical wounding. Levels of JA, JA-Ile, SA, ABA, ABA-GE, and PA were determined in wounded leaves 0 to 24 hr after injury in WT (white), *LapA-SI* (grey) and *LapA-OX* (black) lines (n=3-4). No significant difference was determined between genotypes [ANOVA, Tukey post-hoc test ($p<0.05$)].

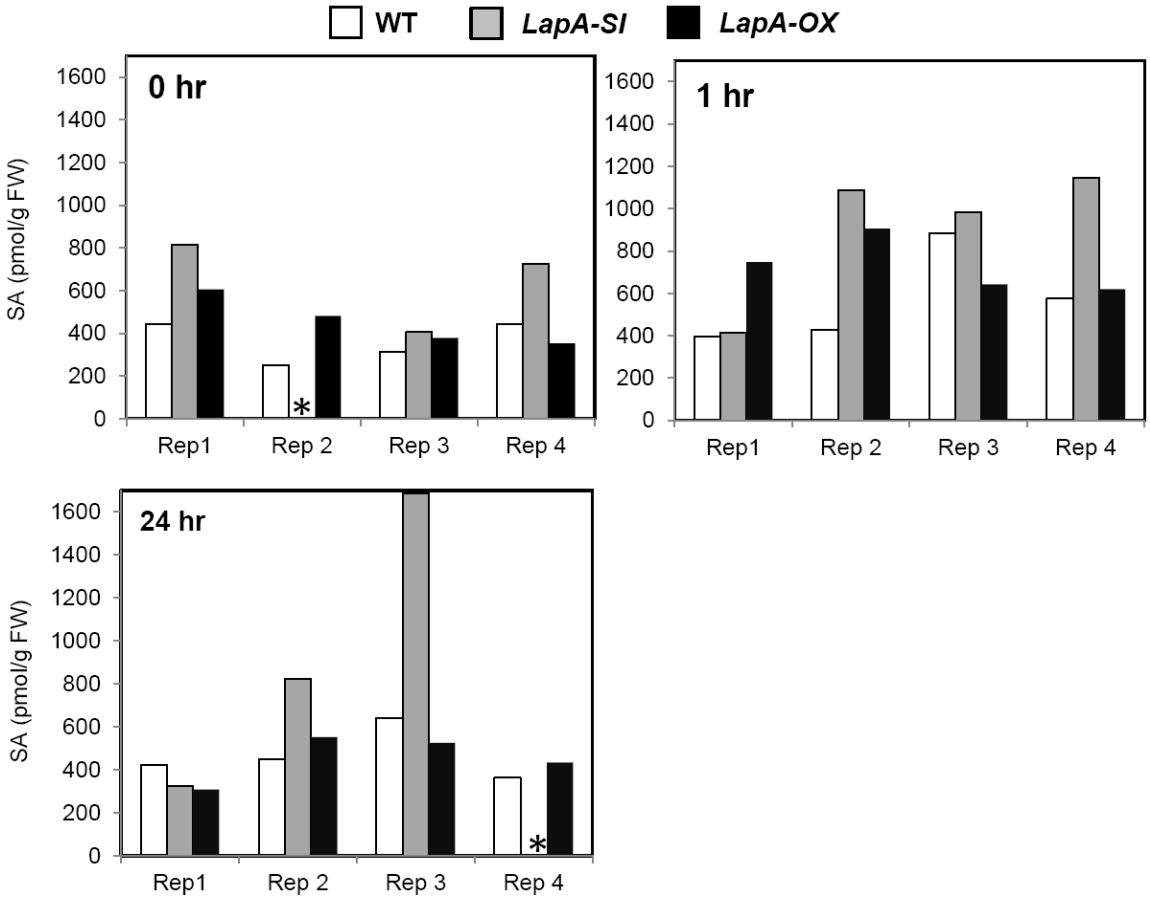


Figure 3.3 Individual measurements of endogenous SA levels in WT, *LapA-SI* and *LapA-OX* lines after wounding. Levels of SA were determined in wounded leaved 0, 1, and 24 hr after injury in in WT (white), *LapA-SI* (grey) and *LapA-OX* (black) lines. Individual replicates (rep) are shown to demonstrate biological variability. Missing values are indicated (*).

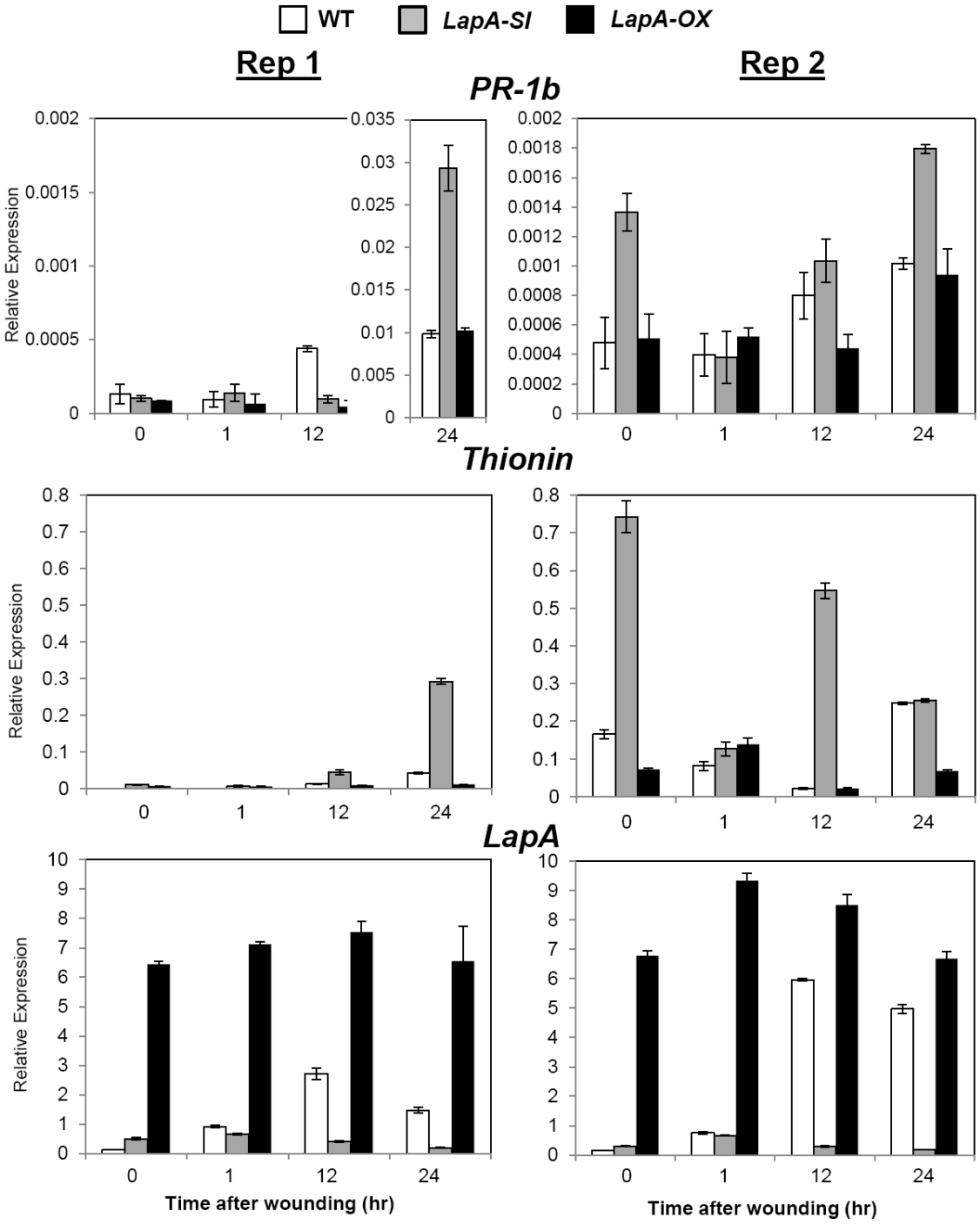


Figure 3.4 Quantitative RT-PCR analysis *PR-1b* and *Thi* mRNAs in WT, *LapA-SI* and *LapA-OX* leaves after wounding. *PR-1b* and *Thi* mRNAs were measured after 0, 1, 12, and 24 hr after wounding from WT (white), *LapA-SI* (grey) and *LapA-OX* (black) lines. Independent replicates are shown separately to demonstrate biological variability. Error bars represent variation from three technical replicates. Genotypes were confirmed by measuring *LapA* mRNAs. Transcript levels were normalized to *EF1α* and *Ubi3*.

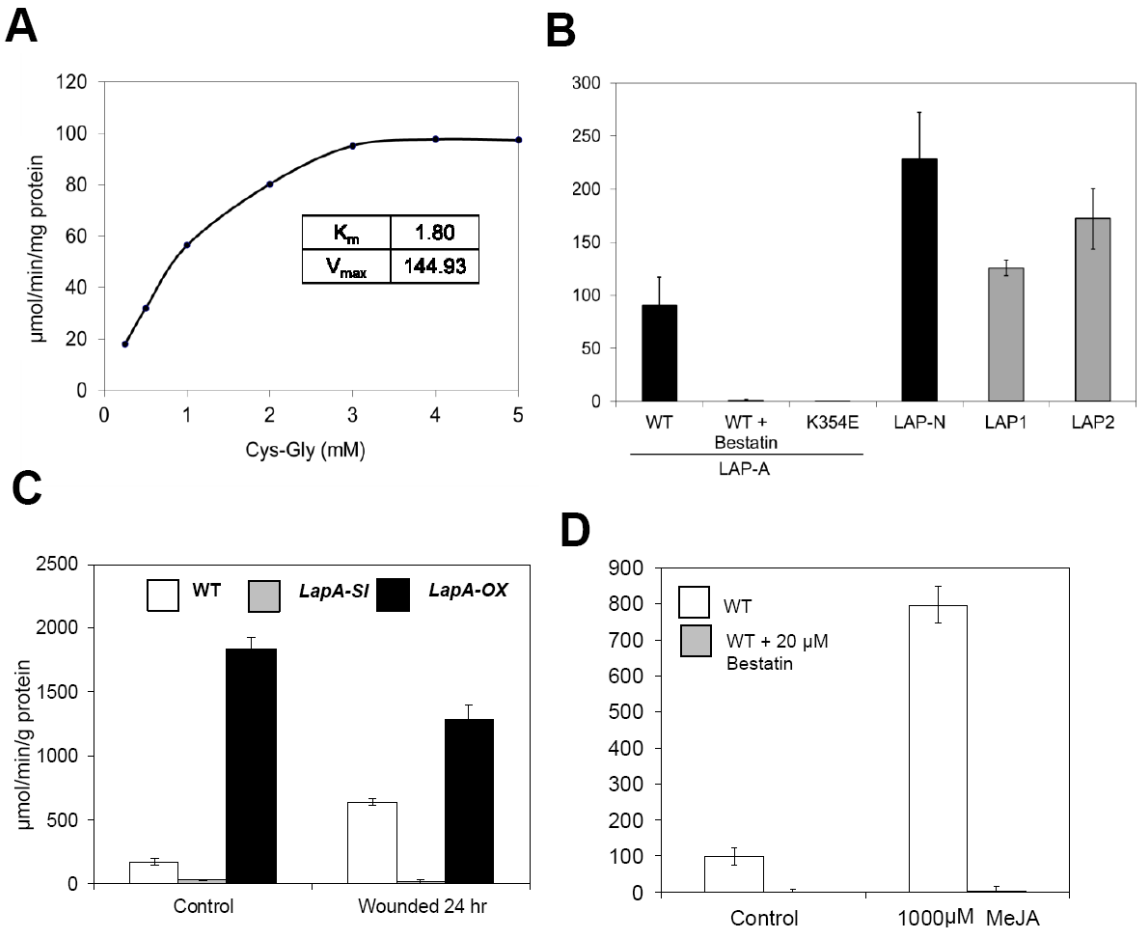
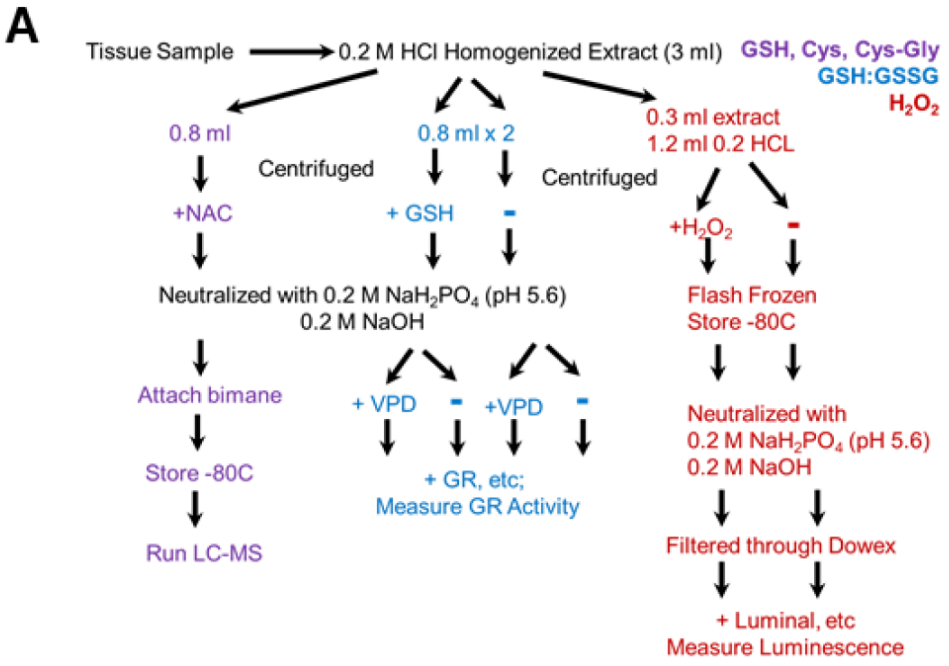
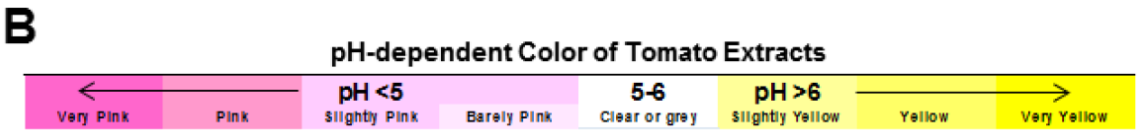


Figure 3.5 Cys-Gly dipeptidase activity of plant LAPs. *A*, The rate of Cys-Gly hydrolysis was determined with purified His_6-LAP-A using 0.25 to 5 mM substrate. *B*, K_m and V_{max} were determined. Cys-Gly dipeptidase activity of His_6-LAP-A, His_6-K354E, His_6-LAP-N, His_6-LAP1, and His_6-LAP2 with 5 mM substrate. Cys-Gly dipeptidase activity of His_6-LAP-A in the presence of 20 nM bestatin was also measured. *C*, Cys-Gly dipeptidase activity of 20 µg of total soluble protein from unwounded and 24 hr wounded WT (white), *LapA-SI* (grey) and *LapA-OX* (black) lines was determined. *D*, Cys-Gly dipeptidase activity of MeJA-treated WT leaves was determined in alone (white) or in the presence of 20 µM bestatin (grey).





C

%GSSG	Color
43	Yellow
17	Yellow--> Grey
9	Slightly Pink
7	Slightly Pink
9	Slightly Pink

D

H_2O_2 nmols/g	Color
2513.28	Barely Pink
1339.43	Clear
2501.17	Barely Pink
2189.68	Barely Pink

Figure 3.6 Diagram of GSH and ROS measurement assays and the effect of pH on GSH oxidation and H_2O_2 recovery. *A*, Overview of steps for acid extraction and measurement of GSH metabolites (purple), GSH oxidation (blue), levels (red). *B*, Coloration of HCl extract in response to changes in pH. *C*, Effect of pH on H2O2 recovery in tomato leaves. *D*, Effect of pH on oxidative state of GSH.

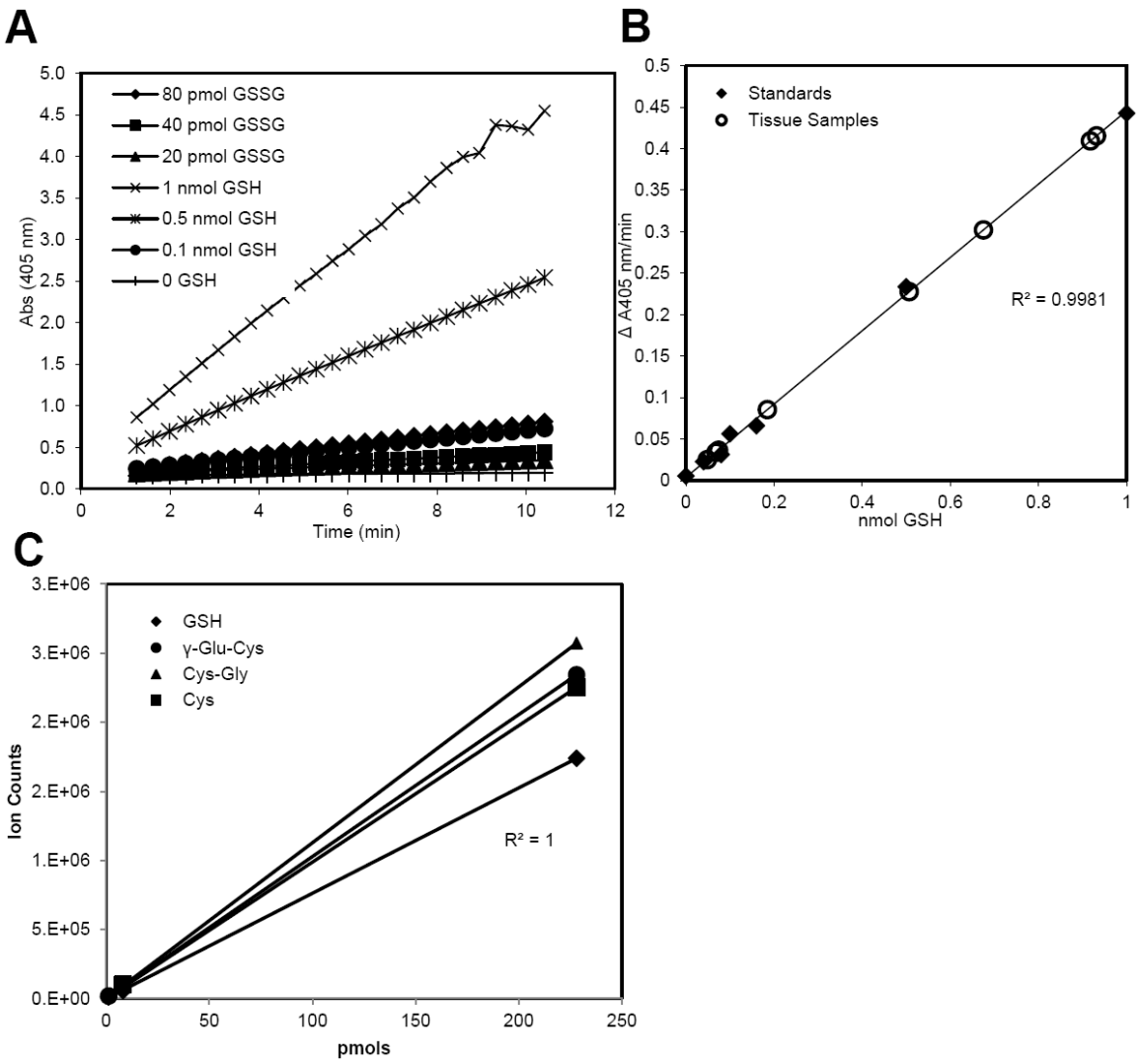


Figure 3.7 Verification of linear GR activity and limit of detection of small thiols by LC-MS. *A*, Linear range of glutathione reductase activity was determined for 0 to 1 nmol GSH and 20 to 80 pmol GSSG. *A-B*, Activity was directly proportional to GSH levels for at least 5 minutes after the start of the reaction. C, Linear range of detection of small thiols by LC-MS analysis as described in *Experimental Procedures*.

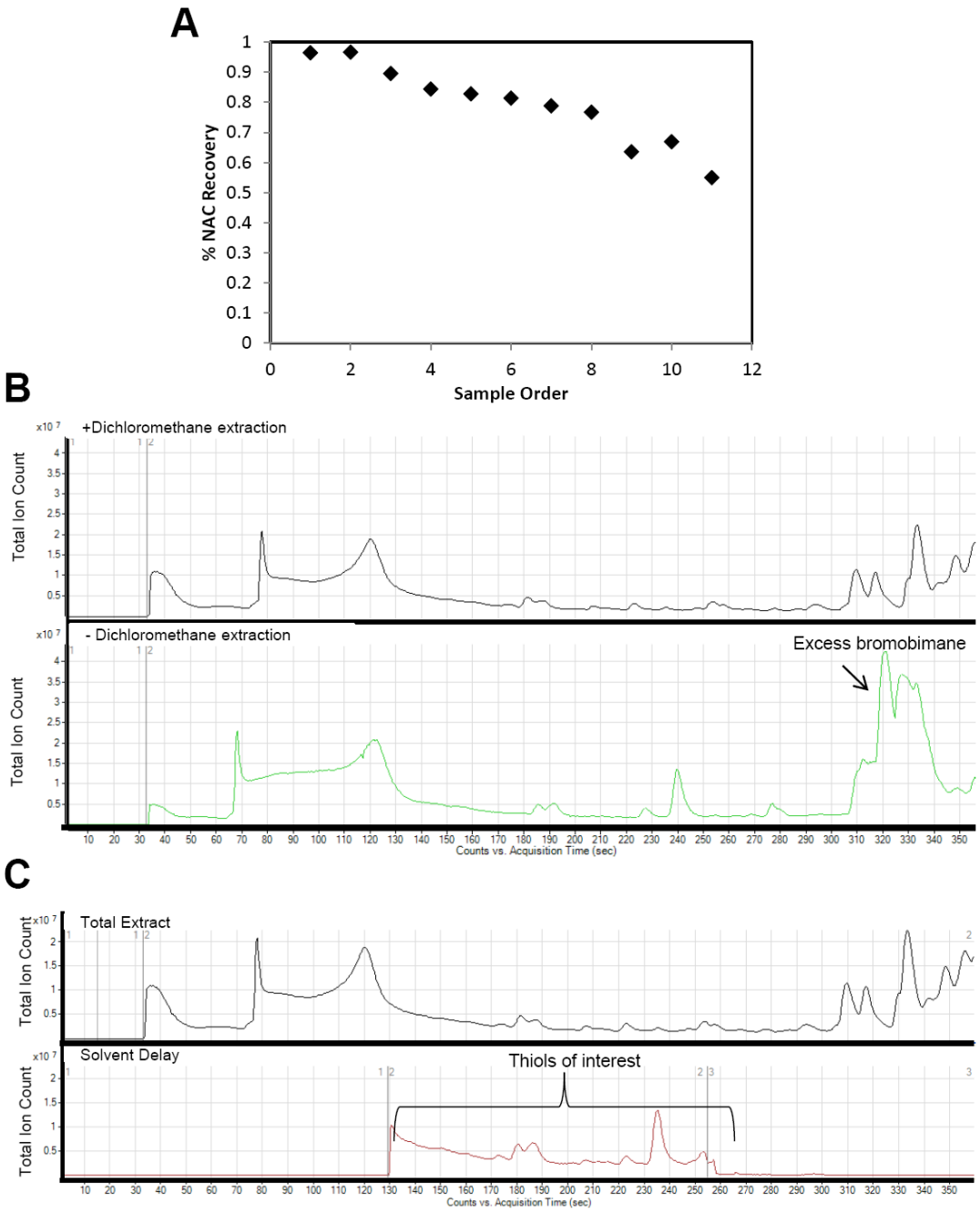


Figure 3.8 Optimization of LC-MS analysis of small thiols. *A*, Percent of internal standard N-acetylcysteine (NAC) recovery was affected by the order measured due to contamination ion source of mass spectrophotometer by excess dye (bromobimane). *B*, Excess unreacted bromobimane removed after dichloromethane extraction. *C*, Removal of excess salts and bromobimane with solvent delay.

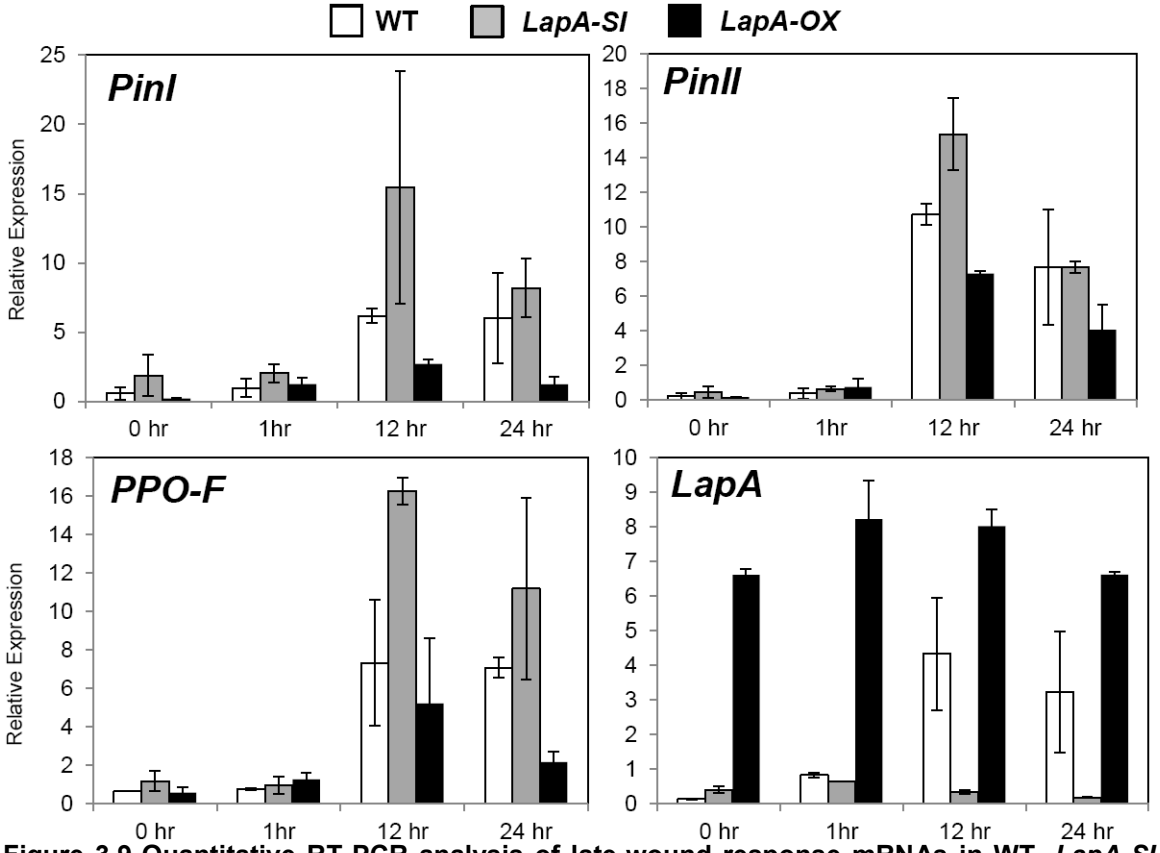
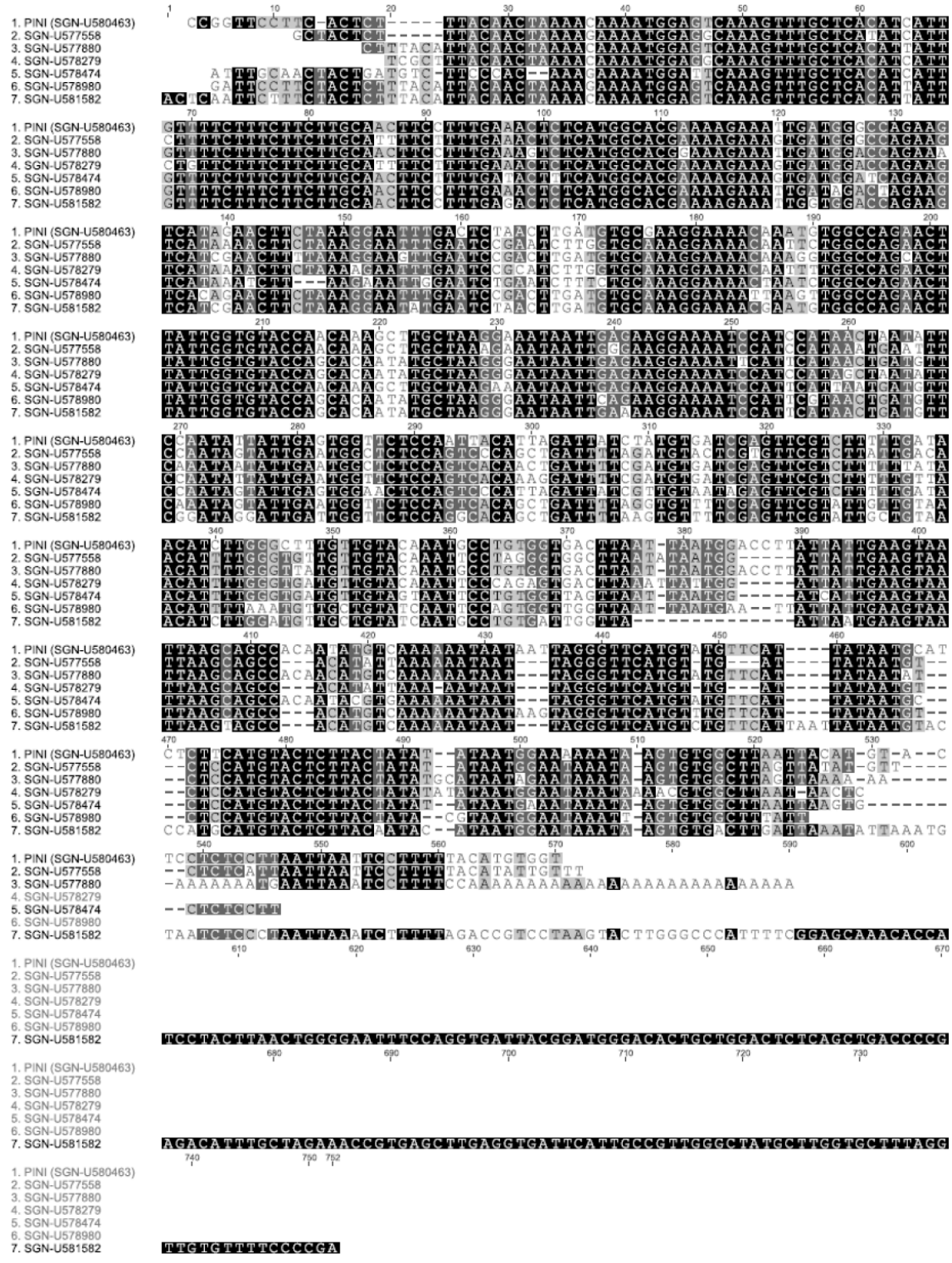


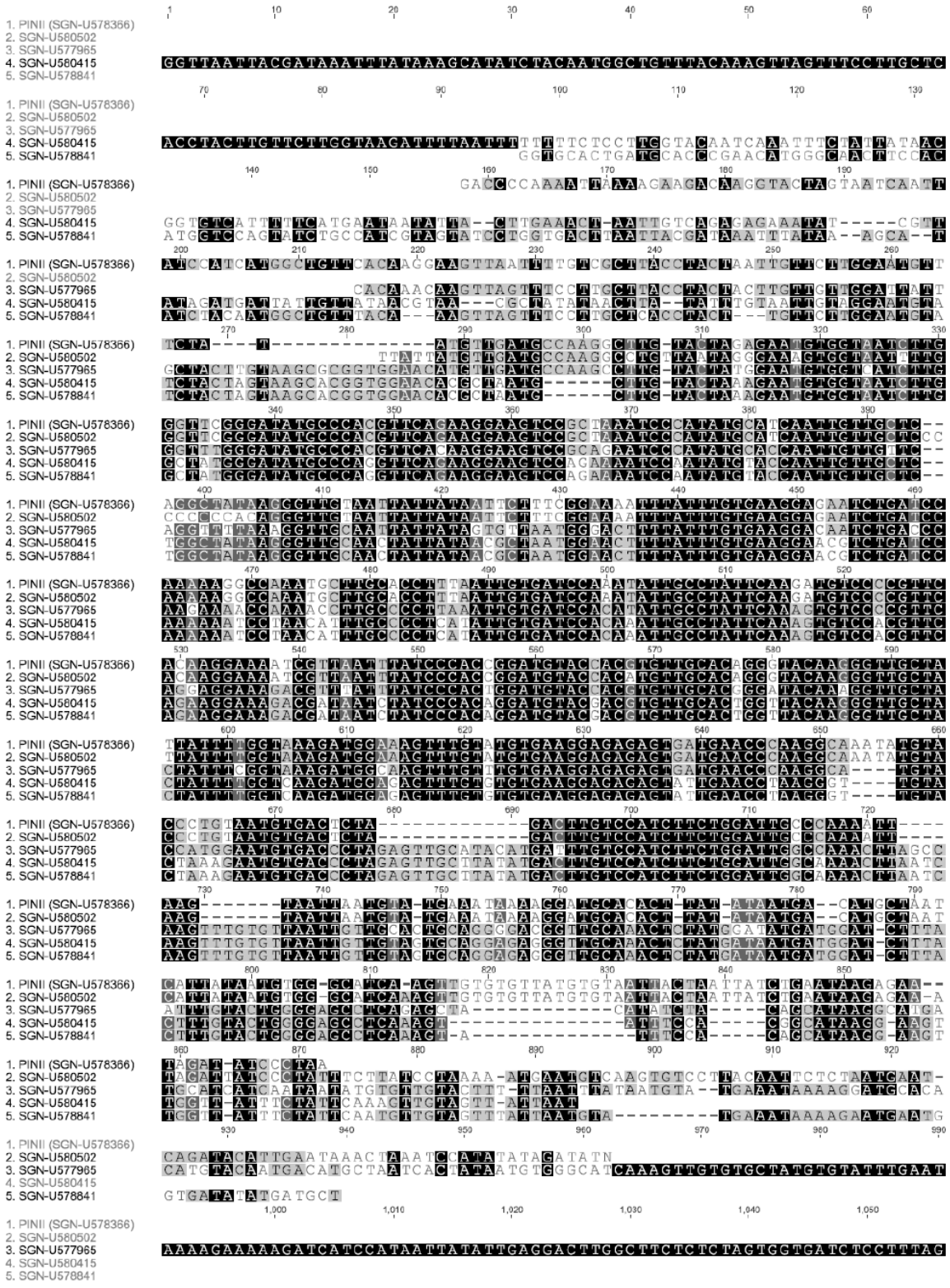
Figure 3.9 Quantitative RT-PCR analysis of late-wound response mRNAs in WT, *LapA-SI* and *LapA-OX* tomato leaves after wounding. *PinI* (SGN-U580463), *PinII* (SGN-U578366) and PPO-F (SGN-U577900) mRNAs were measured after 0, 1, 12, and 24 hr after wounding in WT (white), *LapA-SI* (grey) and *LapA-OX* (black) lines (n=2). Combined *LapA* mRNA levels from Figure 3.f are shown. Transcript levels were normalized to *EF1α* and *Ubi3*.

Figure 3.10 Nucleotide alignment of tomato *PinI* and *PinII* unigenes. BLASTN searches of *PinI* (SGN-U580463) and *PinII* (SGN-U578366) were performed against SOL tomato Unigene database. Homologous sequences (>60% identity) were aligned using Geneious alignment (Kearse et al. 2012).

A


1. PINI (SGN-U580463)
2. SGN-U577558
3. SGN-U577880
4. SGN-U578279
5. SGN-U578474
6. SGN-U578980
7. SGN-U581582

B


1. PINII (SGN-U578366)
2. SGN-U580502
3. SGN-U577965
4. SGN-U580415
5. SGN-U578841

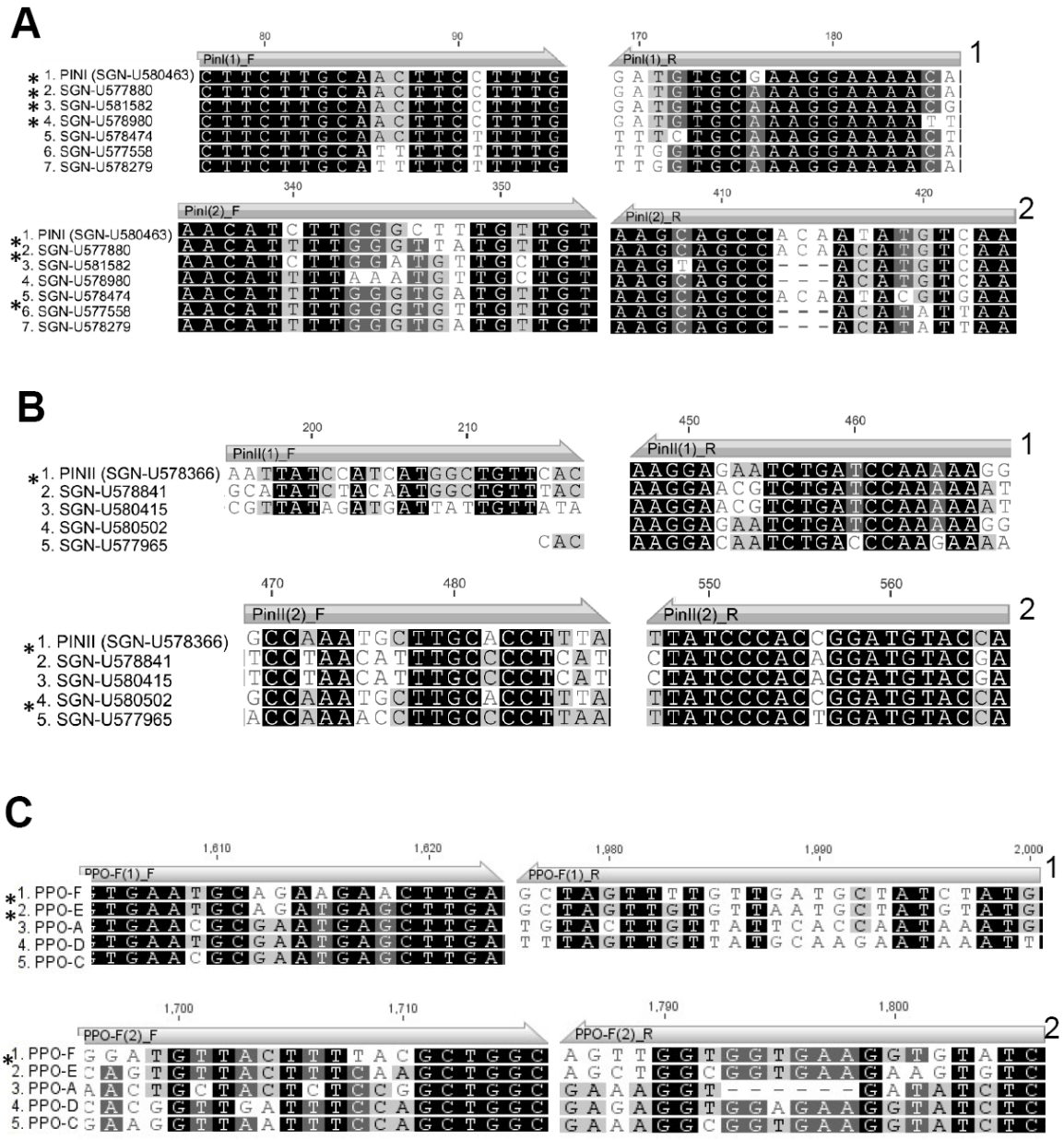


Figure 3.11 Specificity of the tomato *PinI*, *PinII*, and *PPO* qPCR primers. Previously used qPCR primers were aligned with *PinI*, *PinII*, and *PPO* family members to verify specificity (Top; 1; (Fowler et al. 2009). Primers used in current study were also aligned (Bottom; 2). Genes high identity to qPCR primers are indicated (∗).

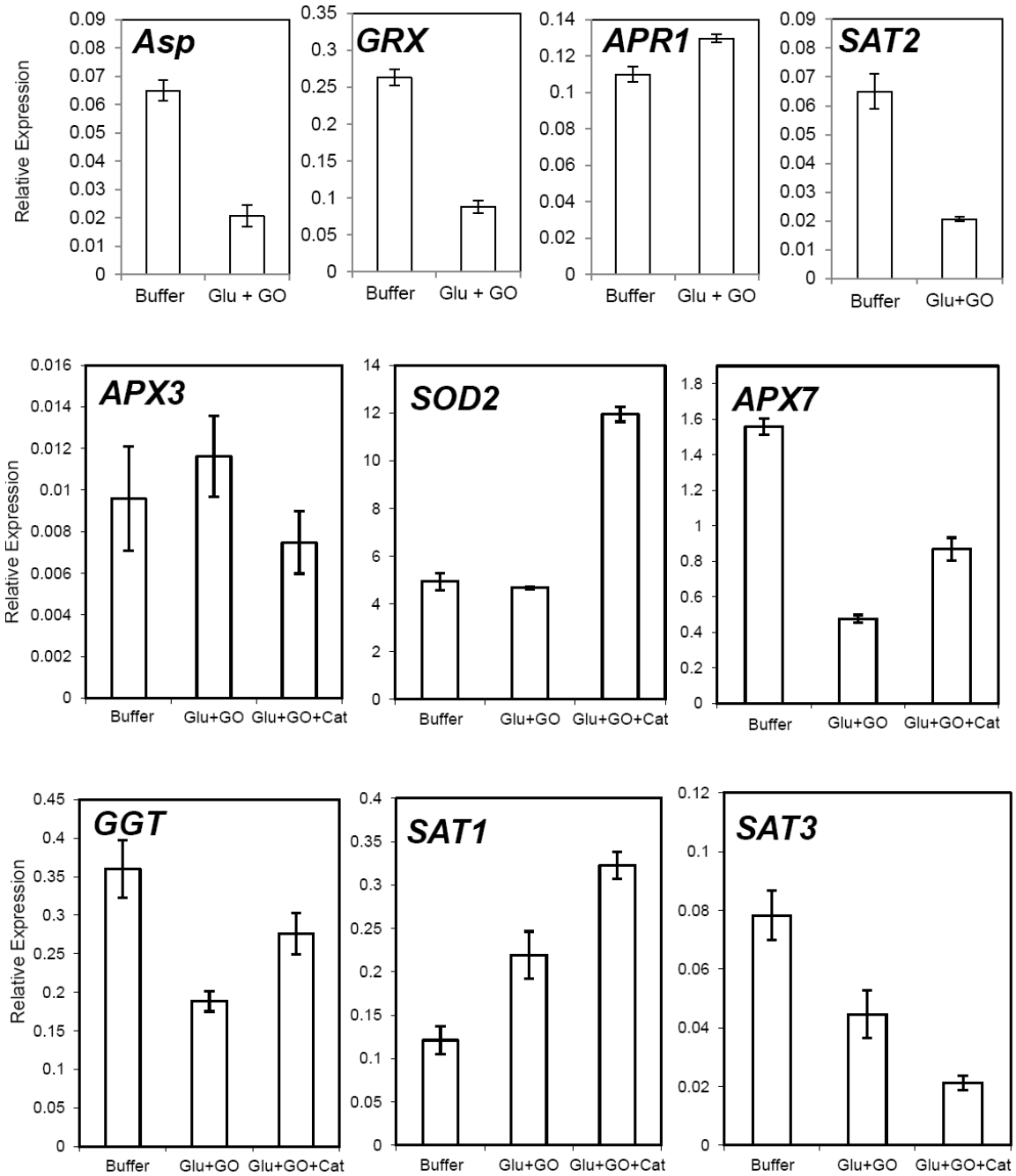


Figure 3.12 Quantitative RT-PCR analysis of potential ROS-responsive genes in WT tomato leaves after H_2O_2 treatment. Changes in ten potential ROS-responsive gene RNAs were analyzed by qPCR in leaves 10 hr after treatment with 50 μM glucose (Glu) and 2.5 U/ml glucose oxidase (GO). Six genes that showed ROS-responsiveness were also tested for ROS-dependency by measuring RNAs in leaves 10 hr after treatment with 50 μM Glu, 2.5 U/ml GO, and 2.5 U/ml catalase (Cat). Transcript levels were normalized to *EF1α* and *Ubi3*.

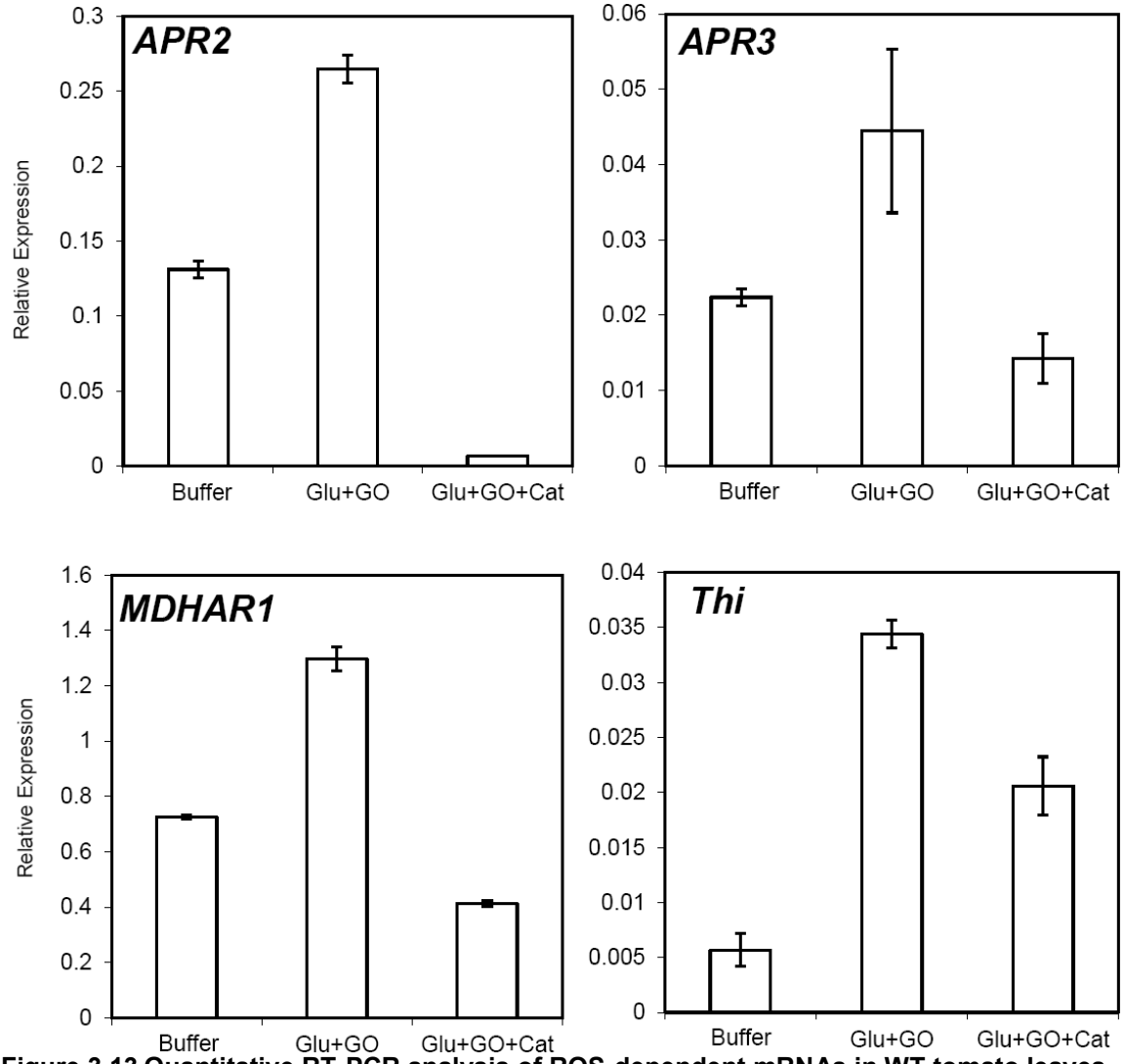


Figure 3.13 Quantitative RT-PCR analysis of ROS-dependent mRNAs in WT tomato leaves after H_2O_2 treatment. Four ROS-responsive genes RNAs were measured by qPCR after Glu + GO treatment with and without catalase as described in Figure 3.12.

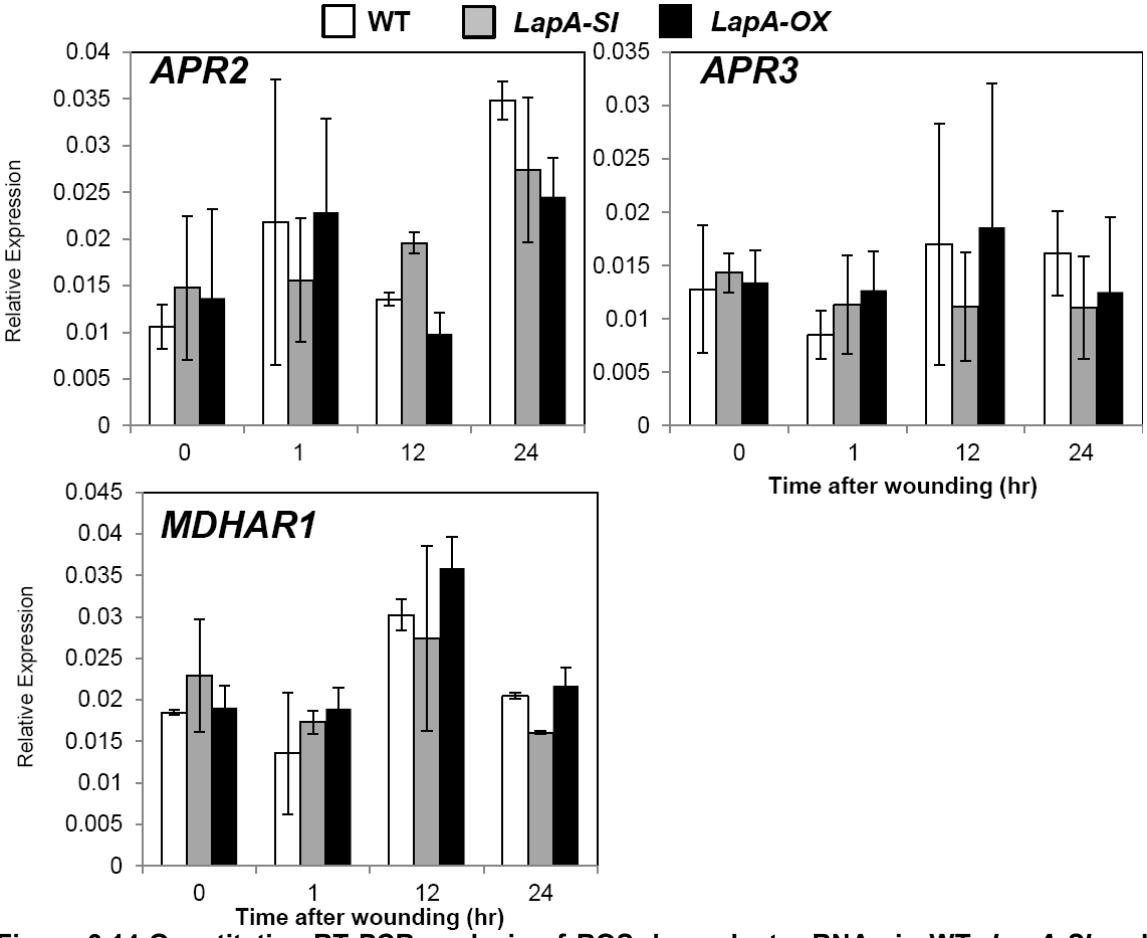


Figure 3.14 Quantitative RT-PCR analysis of ROS-dependent mRNAs in WT, *LapA-SI* and *LapA-OX* tomato leaves after wounding. Three ROS-dependent mRNAs were measured after 0, 1, 12, and 24 hr after wounding in WT (white), *LapA-SI* (grey) and *LapA-OX* (black) (n=2).

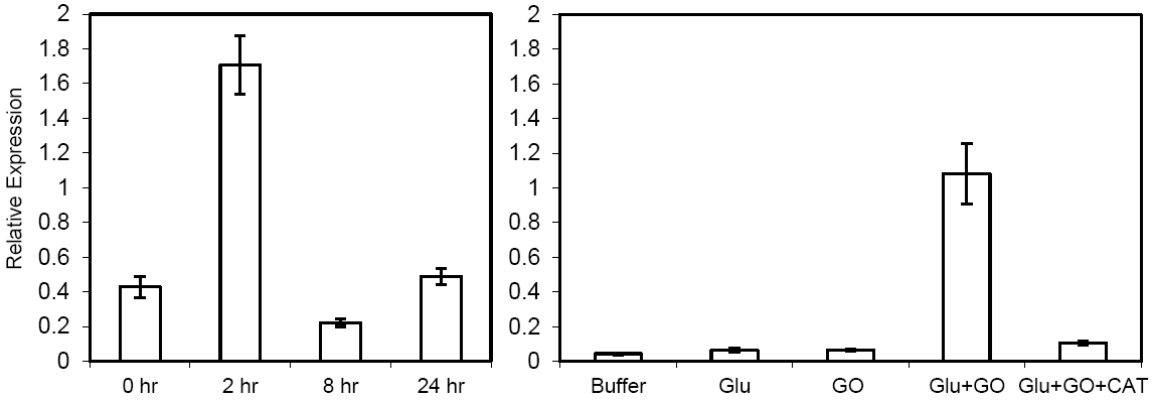


Figure 3.15 Relative expression of *PinI* after H_2O_2 treatment in WT tomato leaves. *A*, WT plants were treated with Glu+GO as described in Figure 3.12 for 2 hr. For 8 hr and 24 hr treatments, plants were transferred to water after 2 hr treatment as described in Orozco-Cárdenas et al. (2001). *PinI* transcript levels were measured and normalized as described in Figure 3.12. *B*, WT plants were treated with Glu+GO as described in Figure 3.12 for 10 hr. Expression was compared to Glu or GO only control as well as to Glu+GO and catalase.

Table 3.1 Multiple-Reaction Monitoring and spectrophotometer conditions used for Quattro Premier XE tandem quadrupole mass spectrometer.

	Standard	Molecular weight	Parent ion (m/z)	Daughter ion (m/z)	Cone Voltage (V)	Collision Energy (eV)	Retention Time (min)
Labeled	d2-GA4	334.41	333.4	215.4	50	28	2.88
	d4-ABA	268.34	267.3	156.1	28	10	2.06
	d3-PA	283.33	282.3	142.1	34	16	1.37
	d5-ABA GE	431.49	430.4	292.4	28	10	1.37
	d3-DPA	285.35	284.3	240.3	28	16	1.09
	d4-SA	142.15	141.1	97	28	10	1.57
Non-labeled	GA1	348.39	347.3	273.3	46	28	1.25
	GA3	346.38	345.3	143.2	22	22	1.27
	GA4	332.39	331.4	257.3	46	28	2.87
	ABA	264.32	263.3	153.1	28	10	0.98
	PA	280.32	279.3	139.1	28	10	1.39
	ABA GE	426.46	425.4	263.3	28	10	1.41
	DPA	282.33	281.3	237.3	34	16	1.01
	SA	138.12	137.1	93	34	22	0.89

GA: Gibberelic Acid; ABA: Abscisic Acid; PA: phaseic acid; ABA-GE: ABA-glucose ester; DPA: dihydrophaseic acid; SA: salicylic acid

Table 3.2 Potential Cysteine Biosynthesis and GSH Metabolism Genes in Tomato.

Gene Name		Tomato ID	Tomato Predicted Localization[A]	Arabidopsis ID	Arabidopsis Localization	Arabidopsis Study
Cys Biosynthesis	*APR1*	SGN-U580331	Chloro	AT4G04610	Chloro	Rev in Martin et al., 2005
	APR2	SGN-U580235	Chloro	AT1G62180	Chloro	Rev in Martin et al., 2005
	APR3	SGN-U578339	Chloro	AT4G21990	Chloro	Rev in Martin et al., 2005
	SAT2.1-like	SGN-U582407	Chloro	AT1G55920	Chloro	Noji et al., 998
GSH Biosynthesis	*γ-ECS (GSH1)*	SGN-U563104	Chloro	AT4G23100	Chloro	Wachter et al., 2005
	GSH-S (GSH2)	SGN-U575017	Chloro	AT5G27380	Chloro/Cyto	Wachter et al., 2005
GSH Degradation	*GGT1*	SGN-U580790	Secret/PM	AT4G39640	Secret/AP/Vacuole	Ohkama-Ohtsu et al. 2007a-b
	Phytochelatin synthase	SGN-U564555	Cyto	At5G44070	Cyto	Blum et al., 2007
	5-Opase	SGN-U575862	Cyto	AT5G37830	Cyto	Ohkama-Ohtsu et al. 2008
M1 Proteases	*MPA1-like*	SGN-U562601	Mito/Chloro	AT1G63770	Cyto/AP	Kaffarnik et al., 2009
	APM1-like	SGN-U581472	Cyto	AT4G33090	Secret/PM (Membrane)	Peer et al., 2009
	Leukotriene A4 hydrolase-like	SGN-U569832	PM	AT5G13520	Cyto/Nuc	Hosein et al., 2010

[A]Tomato locations based on identical calls made by at least two protein localization programs: TargetP, MultiLoc, or WoLF PSORT. Proteins were predicted to be localized to the chloroplast (Chloro), secretory pathway (Secret), plasma membrane (PM), apoplast (AP), cytosol (Cyto), mitochondria (Mito), or nucleus (NUC).

Table 3.3 Potential ROS-regulated genes in tomato and their expression after a 10-hr H_2O_2 treatment.

Gene	SGN	10 hr H_2O_2 Treatment WT Tomato[A]	ROS Regulation in Previous Studies	Organism in Previous Study	Source
Asp	SGN-U583152	Down-Regulated	Up-Regulated	Tomato	Sagi et al. 2004
Thi	SGN-U577810	Up-Regulated	Up-Regulated	Tomato	Sagi et al. 2004
APR1	SGN-U580331	Not regulated	Up-Regulated	Arabidopsis	Queval et al. 2009
APR2	SGN-U580235	Up-Regulated	Up-Regulated	Arabidopsis	Queval et al. 2009
APR3	SGN-U578339	Up-Regulated	Up-Regulated	Arabidopsis	Queval et al. 2009
SAT1-like	SGN-U566396	Not regulated	Not regulated	Arabidopsis	Queval et al. 2009
SAT2.1-like	SGN-U582407	Down-Regulated	Up-Regulated	Arabidopsis	Queval et al. 2009
SAT3-like	SGN-U573965	Down-Regulated	Not regulated	Arabidopsis	Queval et al. 2009
γ-ECS (GSH1)	SGN-U563104		Not regulated	Arabidopsis	Queval et al. 2009
GSH-S (GSH2)	SGN-U575017		Not regulated	Arabidopsis	Queval et al. 2009
GGT1	SGN-U580790	Down-Regulated	Up-Regulated	Arabidopsis	Destro et al. 2011
LeGR	SGN-U562842		Not regulated	Tomato	Ioannidi et al. 2009
GRX	SGN-U583365	Down-Regulated	Up-Regulated	Tomato	Guo et al. 2010
SOD1	SGN-U581590		Not regulated	Tomato fruit	Ioannidi et al. 2009
SOD2	SGN-U578588	Down-Regulated	Up-Regulated	Tomato fruit	Ioannidi et al. 2009
SOD3	SGN-U583863		Not regulated	Tomato fruit	Ioannidi et al. 2009
MDHAR1	SGN-U583672	Up-Regulated	Up-Regulated	Tomato fruit	Ioannidi et al. 2009
MDHAR2	SGN-U315877		Not regulated	Tomato fruit	Ioannidi et al. 2009
APX3(AtAPX2)	SGN-U579887	Not regulated	Up-Regulated	Arabidopsis	Ball et al., 2004
APX7	SGN-U574728	Down-Regulated	Up-Regulated	Tomato fruit	Ioannidi et al. 2009; Najami et al. 2002

[A]Expression levels shows in Figure 3.12-13

Table 3.4 Tomato qPCR primers.

Gene	Sequence (5'→3')	Tm (°C)
Asp	CGCTCTTCGCTCTTCTCTAC	55
	GCCTCCATTTCCACTGTTCT	
Thi	GTTTGGCTTGGTGCTTTGC	55
	TCACTCATTCCATGGCTCGTTC	
APR1	CGGCAGTGAAGCCATTGTAT	55
	GAGGGGAAGCATTTTGAAGC	
APR2	CTCATCAACACCCCAAAGTG	55
	TAGTGGCTTCACAGCACAAC	
APR3	GCAGTGGAGAAGCACTATGGG	55
	GCCCTTGTTCCTCACTAAAG	
SAT1-like	GCACTTCAATCCCGAATCTC	55
	TTCACCAACAACCACTCCTG	
SAT2.1-like	CCCAAACAAGCCACAAATCG	55
	CAGGAAACAAGGTCAGGGAA	
SAT3-like	AACACCGCTTCTACTCTTGG	55
	TATTGGCTCCTTCTCTGCCT	
GGT1	GATGTGAAGAGAGCAGGTGG	60
	AATCAGAGAAATCCCGAGCG	
GRX	GGCGACCTTCAACATCTCAT	55
	AGTGGTGGTTCTGGTTTTGG	
SOD2	ATACACACCACTCCTCACCA	55
	GCCTAATACTGACTGCTTCCC	
MDHAR1	TCGGATTTCAAGGGTTTCGG	55
	TTCCTCCTCCAACTACCACA	
APX3	CTTTTGGAGCCGATCAAGGA	55
	TTCTGGAGGTGGTTCTGTCT	
APX7	AATGCCCAGAAGAAGGAAGG	55
	AAAGTGTGAGCCCCAGAAAG	
PinI	AACATCTTGGGCTTTGTTGT	55
	TTGACATATTGTGGCTGCTT	
PinII	GCCAAATGCTTGCACCTTTA	55
	TGGTACATCCGGTGGGATAA	
PPO-F	GGATGTTACTTTTACGCTGGC	55
	GATACACCTTCACCACCAACT	
EF1α	ACCCTTGGTGTCAAGCAAAT	63
	GGGGATTTTGTCAGGGTTGT	

DISCUSSION

Far from being mere housekeeping proteins, aminopeptidases direct roles in the regulation of a wide array of processes in the cell including growth, development, homeostasis and stress response (Barrett et al. 2004; Walling 2006). In most of these cases, target substrates and mechanisms of action remain unknown. However, the default presumption is that these aminopeptidases act through their peptidase function to affect the stability, activity, or localization of peptides and proteins (Graciet and Wellmer 2010; Tasaki et al. 2012; Varshavsky 2011; Walling 2006). However, multifunctional peptidases are known. In particular, LAPs from animals and bacteria are also known to be Cys-Gly dipeptidases and the LAP *E. coli* homologue mediates site-specific DNA recombination and can act as a transcription factor in *carAB* regulation (Cappiello et al. 2004; Colloms 2004; Jösch et al. 2003; Suzuki et al. 2001).

In plants, LAP-A is unique from the neutral LAP-N. While LAP-N is found in all plants and is constitutively expressed, LAP-A is only found in a subset of the Solanaceae (Bartling and Nosek 1994; Chao et al. 2000; Dammann et al. 1997; Hartl et al. 2008; Herbers et al. 1994; Tu et al. 2003). In addition, *LapA* transcripts accumulate in response to wide variety of biotic and abiotic stress (Chao et al. 1999; Jwa and Walling 2001; Pautot et al. 2001; Pautot et al. 1993). In particular, *LapA* accumulates in response to mechanical wounding and in response to wound-response elicitors (systemin, JA, and ABA) (Chao et al. 1999; Dammann et al. 1997).

In addition, LAPs are important in defense against *Manduca sexta* in tomato and nightshade (Fowler et al. 2009; Hartl et al. 2008). Increased insect feeding and growth was seen when LAP was transgenically silenced in tomato or transiently silenced in nightshade (Fowler et al. 2009; Hartl et al. 2008). Reciprocally, decreased feeding and delays in insect growth and development were seen in tomato plants with constitutively high expression of LAP-A (Fowler et al. 2009). In addition, LAP-A modulated the expression of other JA-dependent late wound-response genes while not affect early wound response genes in tomato. However, the mechanisms by which LAP

affects both insect growth and development and modulates late wound gene expression remain unknown.

Previous studies have provided some clues to how LAP-A may act in insect defense and wound signaling. LAP-A is the most abundant protein within the insect midgut and has a pH optima similar to the alkaline gut (Chen et al. 2007; Dow 1992; Gu et al. 1999; Pautot et al. 1991). However, preliminary studies demonstrated that LAP-A does not act alone to directly affect insect growth or development (Fowler et al. 2009). In addition, LAP-A is localized to the plastid (Narváez-Vásquez et al. 2008). Therefore, LAP-A must act through a retrograde signal or signaling pathway to affect the nuclear gene expression of the late wound-response pathway.

My dissertation studies focused on potential mechanisms by which LAP-A may affect insect defense, wound signaling, or both in tomato. Unlike in other kingdoms, the only known function for plant LAPs was as aminopeptidases. However, in my dissertation I demonstrate that plant LAPs have at least two other potential molecular functions. Chapter 1 demonstrates that plant LAPs have molecular chaperone activity; while Chapter 3 demonstrates that plant LAPs can also hydrolyze the dipeptide Cys-Gly.

While two aminopeptidases have demonstrated chaperone activity in *E. coli* (Heat shock protein 31; Hsp31) and yeast (Aspartyl aminopeptidase; (Lee et al. 2009; Malki et al. 2005), Chapter 1 is the first study to demonstrate a plant aminopeptidase or a LAP from any kingdom has chaperone activity. Both acidic (LAP-A) and neutral forms (LAP-N, LAP1, and LAP2) of plants demonstrated chaperone activity to various degrees and with varying preferences to the three model substrates used. In addition, LAP-A chaperone activity was independent of ATP, as well as LAP-A's peptidase activity.

Chapter 1 also demonstrated that some mutations within the peptidase active site disrupted LAP-A's ability to form a hexamer and disruption of LAP-A's homohexameric structure increased chaperone activity. Therefore, crystal structures were generated of the WT LAP-A and the disruption mutant K354E (DuPrez et al., unpublished). These crystal structures illustrated that the K354E substitution within the peptidase active site caused a loop to become disordered, which in

turn impeded LAP-A's ability to form a hexamer. In addition, these structures revealed that LAP-A has a highly hydrophobic dimer interaction surface buried within its hexameric structure. This hydrophobic patch is exposed when LAP-A cannot form a hexamer, which was confirmed by *in vitro* analysis of total exposed hydrophobic residues (data not shown).

Increased exposure of hydrophobic residues is consistent with small heat shock proteins, which also form higher order oligomeric structures. However, in response to stress, these proteins increase their exchange rate or dissociate into smaller oligomeric forms, exposing hydrophobic residues that bind to denature protein substrates (Basha et al. 2010; Haslbeck et al. 2005). Together these data suggest that the structural integrity of LAP-A can also affect its chaperone activity. Therefore, if LAP-A is present in smaller oligomeric structures *in vivo*, this would suggest that these smaller forms have a high potential to act as molecular chaperones. In fact, preliminary data demonstrated that LAP-A can be present as both a hexamer and a trimer in the insect midgut (Bhattacharya and Walling, unpublished). Therefore, future work will focus on LAP-A's chaperone activity within the midgut. Some potential targets may be other midgut stable plant proteins (Chen et al. 2007). These plant proteins originate from all compartments in the cell but must maintain their stability within the harsh alkaline environment of the lepidopteran midgut (Chen et al. 2007; Dow 1992). This may require the aid of molecular chaperones such as LAP-A.

Structural data of the LAP-A WT protein also demonstrated that access to LAP-A's active site is constraint. If polypeptides are hydrolyzed, the residues in the 4-6th positions must be smaller (DuPrez et al., unpublished). Alternatively, LAP-A may act on di- or tri-peptides substrates. Within the plastid, there are no known active peptides. Most peptides are likely Clp protease degradation products that LAP may aid in further catabolizing to recycle amino acids (Sakamoto 2006). However, the regulatory tripeptide glutathione (GSH) is synthesized and highly abundant within the plastid (Noctor et al. 2012).

GSH is an attractive target for LAP due to GSH's role in stress response and signaling (Noctor et al. 2012). In addition, LAP homologues in animal and bacteria have shown Cys-Gly dipeptidase activity (Cappiello et al. 2004; Chu et al. 2008; Jösch et al. 2003; Suzuki et al. 2001).

Cys-Gly metabolism is important in GSH metabolism, sulfur metabolism, and removal of excess Cys-Gly, which is a damaging oxidant (Baudouin-Cornu et al. 2012; De Donatis et al. 2010; del Bello et al. 1999; Del Corso et al. 2002; Enoiu et al. 2007). No Cys-Gly dipeptidase has been identified in plants to date (Noctor et al. 2012). Therefore, Chapter 3 provided evidence that LAP-A could be the major Cys-Gly dipeptidase in tomato. Studies performed demonstrated that LAP-A, as well as neutral plant LAPs, could readily hydrolyze Cys-Gly. In addition, *LapA* transcript levels correlated with Cys-Gly dipeptidase activity in tomato and Cys-Gly dipeptidase activity could be inhibited with the LAP inhibitor bestatin. Methods were optimized to measure GSH and H_2O_2 levels, as well as GSH redox states in the leaves of wounded and healthy WT and *LapA* mis-expression lines. In addition, based on Arabidopsis literature, I identified ROS-responsive genes as an indirect, but potentially ultra-sensitive measure of oxidative stress by identifying tomato homologues of ROS-responisive Arabidopsis genes. While 14 genes were tested for responses to H_2O_2, only four ROS-dependent genes were identified (*Adenosine 5'-Phosphosulphate Reductases* (*APR2, APR3*), *Monodehydroascorbate reductase 1*, and *Thionin*). This suggests that ROS gene regulation in tomato differs substantially from Arabidopsis. Future work will focus on understanding the relationship between LAP-A Cys-Gly dipeptidase activity and GSH and ROS metabolism in tomato.

Previous studies of LAP-A gene regulation only focused on a small set of JA-responsive genes that were established markers for the early and late branches of the tomato wound response (Fowler et al. 2009). However, wounding modulates a wide set of stress and defense-related genes that are in turn modulated by a complex network of signaling molecules (Erb and Glauser 2010; Erb et al. 2012; Pieterse et al. 2009; Robert-Seilaniantz et al. 2011; Robert-Seilaniantz et al. 2007). Many of these signaling molecules are localized and/or begin their synthesis within the plastid (Dudareva et al. 2006; Torres et al. 2006; Uppalapati et al. 2007; Vranova et al. 2012; Wasternack 2007). Therefore, in Chapter 3, LC-MS analysis was performed to determine if the metabolism of key plastid signals was altered. In addition, in Chapter 2 microarray analysis was performed on WT and *LapA-SI* lines before and after wounding to

determine if LAP-A modulated the expression of other genes that may be controlled by one or more of these plastid signals. This data could guide studies to narrow down potential signaling pathways by which LAP-A modulates wound signaling.

Key phytohormone signals that have well-established roles in wound signaling and modulation that originate from the plastid include JA, SA, GA, and ABA (Erb et al. 2012; Uppalapati et al. 2007; Vranova et al. 2012; Wasternack 2007). Therefore, LC-MS methods were optimized to measure these compounds as well as the active JA conjugate JA-Ile. GA levels were too low to detect by this method, while JA, JA-Ile, and ABA levels were not modulated by LAP-A. However, SA levels were slightly elevated in the *LapA-SI* line, though subject to wide biological variability. In addition, SA-responsive *PR-1* and *Thi* transcript levels are also elevated in *LapA-SI*. *PR-1* and *Thi* genes also regulated by ROS. This is consistent with preliminary data that demonstrated that whitefly (*Bemisia tabaci B*) feeding caused a hypersensitive response (HR) in the *LapA-SI* line (Holzer, Borhorquez, and Walling, unpublished results). HR is regulated by both SA and ROS (Gechev and Hille 2005; Liu et al. 2007; Vlot et al. 2009; Zurbriggen et al. 2009). Together this data suggested that LAP-A may modulate SA-JA antagonism or ROS metabolism. Due to the complex relationship between SA, H_2O_2, and GSH, and the fact that LAP-A may be involved in GSH metabolism, it is not clear if LAP-A acts through GSH and/or H_2O_2 signaling to affect SA signaling or if LAP-A affects SA signaling and metabolism through an alternate pathway. Further experiments are required to verify these results and determine how LAP-A affects each of these pathways.

Since no studies have looked at global gene expression in tomato in response to wounding, in depth analysis of the types of genes and biochemical pathways regulated after early and late as well as locally and systemically was performed. Like other plant species, tomato regulated many different biological pathways after wounding and these pathways were differentially modulated by time and location. For example, like other studies, systemic responses were delayed compared to local (Fowler et al. 2009; Strassner et al. 2002). Moreover, comparison with *LapA-SI* responses demonstrated that LAP-A modulation of wound responses is more expansive

and complex than previously known. Compared to WT plants, *LapA-SI* lines had a delayed response early after wounding. In addition, LAP-A negatively modulated the expression of late-wound dehydrins as well as other *PR-1*s (*PR-1c*, *PR-1A2*). Up-regulation of late-wound dehydrins (*TAS14*, *Dhn3*) may be a compensatory response due to the lack of LAP-A chaperone activity. However, since the regulation of several *PR-1* genes in tomato is unknown; it is not clear how LAP-A may affect this gene family. These data are in contrast to previous studies that showed LAP-A only positively modulated late-wound gene expression (Fowler et al. 2009). Finally, while there was a clear enrichment of stress-response genes that are differentially expressed in the *LapA-SI* line, identity of these genes did not clearly indicate a particular signaling pathway that was modulated by LAP-A. Therefore, future work should verify further targets for LAP-A modulation to potentially use in chemical complementation studies to determine if LAP-A acts through other signaling molecules, particularly through SA-JA signaling.

In my dissertation studies, I have demonstrated that LAP-A regulation of wound responses in tomato is more extensive and complex than previously known. LAP-A can affect both early and late wound responses and can be both a positive and negative regulator of these responses. In addition, previous studies have shown that LAP-A affects insect growth and development (Chen et al. 2007; Fowler et al. 2009). While my studies have not provided direct mechanism of action for either of these roles, these studies have provided essential clues to how LAP-A may function. My studies have demonstrated that like LAPs from other kingdoms, plant LAPs have many potential functions and may execute many different roles. Beyond being an aminopeptidase, LAP-A *in vitro* can also function as a Cys-Gly dipeptidase and as a molecular chaperone. It is possible that LAP-A may also have multiple functions *in vivo* that contribute to its role in wounding signaling and defense. For instance, LAP-A's Cys-Gly dipeptidase activity may regulate GSH or ROS signaling to affect nuclear gene expression after wounding, while LAP-A's chaperone activity may protect plant defense proteins within the insect midgut to suppress insect growth. It will be interesting to determine which of LAP-A's functions is responsible for its roles in wound signaling and defense.

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