THE WOUND RESPONSE IN *ARABIDOPSIS THALIANA* AND *PHYSCOMITRELLA PATENS*

by

CHI-TAI CONAN TANG

A Dissertation submitted to the

Graduate School-New Brunswick

Rutgers, The State University of New Jersey

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

Graduate Program in Plant Biology

written under the direction of

Professor Michael A. Lawton

and approved by

_____

_____

_____

_____

_____

New Brunswick, New Jersey

October, 2007

ABSTRACT OF THE DISSERTATION

The Wound Response in *Arabidopsis thaliana* and *Physcomitrella patens*

By CHI-TAI CONAN TANG

Dissertation Director:
Professor Michael A. Lawton

The wound response in plants is triggered by tissue damage incurred from biotic and abiotic sources. However, identification of wound-specific genes has been complicated by overlap between the wound response and other stress responses. Using representational difference analysis (RDA), an anthocyanidin synthase (*ANS*), steroid sulfotransferase (*SST*) and a tyramine transferase (*THT*) were isolated from wounded *Arabidopsis thaliana*. These genes were expressed in wounded leaves and corresponding systemic tissues. Analysis in wild-type and mutant backgrounds showed that their wound induction was entirely dependent upon jasmonate signaling. SST and putative paralog At5g07000 possess sequence hallmarks of steroid sulfotransferases. The expression of *SST* was silenced using RNAi. A silenced (*SST*::RNAi) line was analyzed for differences in steroid profiles between unwounded and wounded plants. However, differences were not detected between wild-type and *SST*::RNAi plants. Possible reasons for this result are discussed. An RDA-based screen for wounding genes expressed solely in systemic tissues (*SYST*s) revealed, instead, genes whose expression is down-regulated in injured leaves. *SYST1-3* was depleted in wounded leaves within five minutes after wounding and maintained at reduced levels for at least four days. Genetic approaches failed to identify

the signal involved in *SYST1-3* reduction. A *SYST1-3*-like gene, At5g16250, was isolated by homology search and was similarly depleted in wounded leaves indicating a gene family of wound-depleted transcripts. To gain insight into the wound response of a lower eukaryotic plant, the wound response was investigated in the moss, *Physcomitrella patens*. Wounding caused cell death to 1-2 cell layers bordering the wound. Callose was deposited in cells adjacent to the wounded cells. Two putative lipoxygenases (*PpLOX*) were induced by wounding. PpLOX6 and PpLOX7 contain features of lipoxygenases from higher plants. PpLOX7 is predicted to be transported into the chloroplast and a dendrogram showed that PpLOX7 is related to the wound-induced type2-lipoxygenases whereas PpLOX6 clustered with the moss lipoxygenases. Induction of PpLOX7 could be triggered by methyl jasmonate. Wounding also induced putative orthologs of JA biosynthetic enzymes, allene oxide synthase (*AOS*) and allene oxide cyclase (*AOC*) but not an OPDA reductase (*OPR3*). These results suggest that a JA-dependent wound response may be conserved in mosses.

Acknowledgements

I want to first thank my advisor, Michael Lawton, for guiding me through this process and for his financial and intellectual support of my studies. I am grateful for an extremely supportive dissertation committee, composed of Faith Belanger, Wendie Cohick, Tom Gianfagna and Ilya Raskin. I could have not asked for a better group of advisors. Thanks to past members of the lab and current lab mates, Hemalatha Saidasan and Mark Diamond. Special thanks to the other labs in our wing including the Eric Lam lab, the Nilgum Tumer lab, and the Ilya Raskin lab for use of their lab equipment, scientific collaboration and camaraderie. Alex Poulev and Neb Ilic from the Raskin lab were especially helpful in devising a strategy for steroid profiling.

Special recognition is intended for my friends who have kept me sane during my PhD. I owe gratitude to my childhood friends Rob DeAngelis and Matt Tierney for making me laugh, their wives, Sarah and Jenny, for allowing us to engage in our sometimes pre-pubescent behaviors and to my friends from Penn, Amanda Doran & Gil Cordova, Nisha Merchant-Goss & Ray Goss, Omar and Shazaf Moonis. Many thanks for the friends I have made at Rutgers including old house mates from 45 Duke Street (Eric Stiner & Amy Golden, Tomo Kamimoto and Gaai Nakamura, Dan Saad and Iwani Ching), Andre Hudson for late-night-waiting-by-the-centrifuge talks and Ken Ellison for friendship and hosting Heroes night.

I am forever indebted to my aunt and uncle, Chia-jan and Youeh Ou Tzou, for allowing me to live with them. They have taught me about familial obligation, life experiences and have helped me maintain some semblance of conversational Chinese. Thanks to my cousin Mike and his wife Kai. I want to thank my sister Holly. She has

been a tirelessly dedicated supporter. Finally, I want to thank my parents, Chia-ning and Lolita Tang, for stressing the importance of education, personal integrity and humility and the appreciation for the arts.

Table of Contents

Lists of tables

List of illustrations

ABBREVIATIONS

13-HPOT	13S-hydroxyoctadecatrienoic acid
A. thaliana	*Arabidopsis thaliana*
ABA	Abscisic acid
ACC	1-aminocyclopropane-1-carboxylic acid
ACX	Acyl-CoA oxidase
ANS	Anthocyanidin synthase
AOC	Allene oxide cyclase
AOS	Allene oxide synthase
BA	Benzylaminopurine
BnST3	*Brassica napus* sulfotransferase 3
cDNA	complementary DNA
cev	*constitutive expression of VSP*
CK	*Choline kinase*
coi	*coronatine insensitive 1-1*
CS	Chitosan
dad1	*defective in anther dehiscence 1*
dde1	*delayed dehiscence 1*
DP	Difference product
ein2-1	*ethylene insensitive 2-1*
EF1α	*Elongation factor 1α*
ER	Endoplasmic reticulum
EST	Expressed sequence tag
fad	*fatty acid desaturase*
FDA	Fluorescein diacetate
GA_3	Gibberellic acid
GST	Glutathione-*S*-transferase
hEST	human estrogen sulfotransferase

IAA	Indole acetic acid
Ile	Isoleucine
JA	Jasmonic acid
jar1	*jasmonate resistance 1*
JMT	Jasmonic acid carboxymethyltransferase
JR	Jasmonate responsive
KAT	*L-3 ketoacyl CoA thiolase*
kb	kilobase
LOX	Lipoxygenase
LRR	Leucine-rich repeat
MeJA	Methyl jasmonate
mEST	murine estrogen sulfotransferase
mpk4	*map kinase 4*
NahG	Bacterial salicylate hydroxylase
NO	Nitric oxide
npr1	*non-expresser of pathogenesis-related protein*
nt	nucleotides
OGA	Oligogalacturonan
OPDA	*cis*-(+)-12-oxo-phytodienoic acid
OPR	OPDA reductase
OSD	Oligosaccharide-dependent
P. patens	*Physcomitrella patens*
PAPS	3'-phosphoadenosine 5'-phosphosulfate
PCR	Polymerase chain reaction
PDF1.2	*Plant defensin 1.2*
PG	Polygalacturonase
PI	Proteinase inhibitor
RDA	Representational difference analysis
RNAi	RNA interference

ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase PCR
SA	Salicylic acid
SAR	Systemic acquired resistance
SCF	Skp1, Cullin1, F-box, Rbx1
spr	*suppresor of prosystemin-mediated responses*
sqPCR	semi-quantitative polymerase chain reaction
SST	Steroid sulfotransferase
TCH	touch-sensitive genes
THI2.1	*thionin 2.1*
THT	HydroxycinnamoylCoA:tyramine N-hydroxycinnamoyl transferase
UTR	Untranslated regions
VSP	Vegetative storage protein
WR3	*Wound-responsive 3*
XET/XTH	*Xyloglucan endotransglycosylase*

CHAPTER ONE

INTRODUCTION

Survival for plants is dependent upon their ability to withstand challenges posed by changes in the environmental conditions, infection by phytopathogens and infestation by herbivores. Exacerbated by the inability to flee from such dangers, plants appear overmatched. However, plants have evolved mechanisms to counter these abiotic and biotic stresses. 'Homeland security' in plants consists of a two-tiered defense strategy. Passive defenses provide the first line of protection against microbes and herbivores, where anatomical features, such as an outer waxy cuticle or the presence of trichomes, thorns, and toxin-containing glands, offer physical and chemical obstacles to invasion (Wagner, 1991; Eigenbrode and Espelie, 1995; Wittstock and Gershenzon, 2002). The second level is dependent upon the rapid activation of inducible defenses (Maleck et al., 2000; Reymond et al., 2000; Cheong et al., 2002; Moran et al., 2002; Hahlbrock et al., 2003; Eulgem, 2005). A critical aspect of this active defense mechanism is the sensitivity of the plant's recognition system (Van der Biezen and Jones, 1998; Nimchuk et al., 2003; Veronese et al., 2003). Plants are acutely aware of their surroundings and even minor physical disturbances (e.g. shaking) of the aerial tissue can set off a touch response (Braam and Davis, 1990; Braam, 2005). Upon perception of a pathogenic challenge or herbivore attack, cellular signals are dispatched to activate a stimulus-specific counter offensive (Kawano, 2003; Mishra et al., 2006; Kachroo and Kachroo, 2007). These inducible defenses may include, but are not limited to, the *de novo* production of antimicrobial compounds as well as plant volatiles that attract predators to a feeding

insect (Stintzi et al., 1993; Baldwin, 1999; Stotz et al., 1999; Arimura et al., 2000; van Loon et al., 2006).

The wound response has been characterized in a broad spectrum of plants and is conserved among the higher land plants (Bowles, 1993, 1994; Leon et al., 2001; Wasternack et al., 2006). This response is initiated when the integrity of the cell wall is physically violated. Subsequent impact of a wounding event may rupture plant cells and subcellular organelles (Davies, 1987). Tissue destruction can occur by abiotic means, like extreme wind shear or precipitation, or it can be committed biotically by herbivores or invasive pathogens. Thus, the wound response is important because it is a fundamental reaction to a wide variety of external stimuli. Upon the perception of tissue damage, multiple signaling pathways are activated in injured leaves and in remote tissues to confront a variety of ensuing complications, including water loss, loss of photosynthetically-active cells, exposure to opportunistic pathogens, and redirected metabolic flux (Birkenmeier and Ryan, 1998; Chang et al., 2004; Delessert et al., 2005; Quilliam et al., 2006). In addition, wound signaling may be critical for the proper growth and maturation of plants (e.g., leaf senescence and leaf abscission) (Gan and Amasino, 1997; Butt et al., 1998; Orozco-Cardenas and Ryan, 2003). Thus, the versatile role of the wound response in a variety of processes appears to be a consequence of the recruitment of multiple signaling pathways. Largely unexplored is whether these pathways are differentially regulated in the various wound-mediated responses. The composite nature of the wound response can also complicate the identification of genuine wound-specific genes since they may instead function as a secondary pathway. Though efforts have led

to the identity of wound-induced genes, the characterization of wound-specific genes is relatively unexamined.

Physiological responses to wounding

A scar is a visible remnant of a past wounding event and is constructed from a variety of compounds including waxes, suberin, lignin, and wound gums (Priestley and Woffenden, 1922; Pearce, 1996; Biggs, 1997). Surviving cells near the wound site can erect their own barrier made from the deposition of *de novo* callose (Wheeler, 1974; Aist, 1976; Stone et al., 1985; Aloni et al., 1990; Jacobs et al., 2003). The formation of the impervious wound periderm plays a significant role in blocking pathogen ingress through the wound opening (Ride, 1978; Vance et al., 1980). This inhibition can occur through several possible mechanisms (Bostock and Stermer, 1989). The scar can provide a polymeric barrier which is more resistant to degradation by enzymes released by pathogens (Ride, 1978; Bird and Ride, 1981; Ride and Barber, 1987). Moreover, the wound periderm may prevent access to the underlying nutritive tissue and protects the remaining healthy cells from receiving toxins or virulence factors secreted by the pathogen (Bostock and Stermer, 1989). Pathogen entry may also be deterred by scar tissue containing large concentrations of antimicrobial compounds, like phytoalexins (Aist, 1976; Vance et al., 1980; Hammerschmidt and Kuć, 1982).

In response to wounding, subcellular re-organization is observed (Barckhausen, 1978). Dramatic changes occur to chromatin structure in cells of the injured leaf resulting from the active expression of rRNA genes. The endoplasmic reticulum (ER) and ribosomes also increase in the cells of wounded tissues (Barckhausen, 1978; Davies

and Schuster, 1981). These cytoplasmic cues indicate elevated levels of transcription, translation and secretion within affected cells. The formation of wound-associated novel ER bodies in the epidermal cells of wounded *Arabidopsis thaliana* rosettes is a recent discovery (Matsushima et al., 2002; Hara-Nishimura and Matsushima, 2003; Matsushima et al., 2003b). The function of the ER bodies is not well understood but the contents of some ER bodies contain β-glucosidase (PKY10), and these ER bodies eventually fuse with vacuoles (Matsushima et al., 2003a). The wound-triggered fusion of the membrane bodies is speculated to introduce PKY10 to its natural substrate, resulting in the synthesis of wound-induced toxins (Hara-Nishimura and Matsushima, 2003; Matsushima et al., 2003a).

Wound-induced compounds

Early work on the characterization of wound response in higher plants concentrated on the variety of induced compounds (Table 1). Several of the known wound-induced compounds serve as deterrents to herbivory (Mason and Mullet, 1990; Pautot et al., 1991; Koiwa et al., 1997; Shewry, 2003; Liu et al., 2005). For example, the seminal work reported by Green and Ryan (1972) introduced the concept of *de novo* formation of anti-feeding compounds in insect-infested potato and tomato plants. The accumulation of proteinase inhibitors (PIs) was detected in mechanically-damaged and insect-predated tomato and potato plants but was absent in untreated controls. PIs are believed to function as anti-nutritive deterrents against insects by inhibiting digestive proteinases in the midgut of herbivores (Johnson et al., 1989; Urwin et al., 1995). PIs also accumulated in the unwounded leaves (systemic) of injured plants, indicating that a

Gene Product	Process	Plants	Refs
Vegetative storage protein (VSP)	Wounding, nitrogen storage	Soybean, *Arabidopsis*	Berger (1995), Mason (1990)
Proteinase inhibitor (PI)	Wounding, anti-herbivore	*Solanaceous*, alfalfa	Green (1972); Walker-Simmons (1977)
Lipoxygenase	JA signaling	Soybean, *Arabidopsis*	Bell (1991)
Trypsin inhibitor	Wounding	Potato, Soybean	Walker-Simmons (1977)
Leucine aminopeptidase	Wounding, Pathogenesis	Potato, Tomato	Pautot (1993); Gu (1996)
Threonine deaminase	Isoleucine biosynthesis	Potato	Hildmann (1992)
Proline-rich cell wall protein	Cell wall strengthening, Anti-microbial	Soybean	Bradley (1992)
Chalcone synthase	Anti-microbial	Soybean	Lawton (1987)
1-aminocyclopropane-1-carboxylate oxidase	Fruit ripening	Tomato	Barry (1996)

Table 1.1 List of wound-induced genes.

signal must emanate from the locally-wounded leaf to the undisturbed distal or systemic regions of the plant (Green and Ryan, 1972). In tomato, leucine aminopeptidases are induced in local and systemic tissues and predicted to similarly operate in a defensive role (Pautot et al., 1993; Gu et al., 1996).

The vegetative storage protein (VSP) is another classic wound-responsive marker (Mason and Mullet, 1990). VSP was initially described in soybean as a repository for excess nitrogen that is mobilized during plant growth, especially for seed development (Staswick, 1988, 1989a, b). Recent investigation of VSP in *A. thaliana* has shown that it possesses acid phosphatase activity but may not be actually involved in seed development (Liu et al., 2005). Instead, VSP may play a role in the defense response as an anti-pest protein (Berger et al., 2002).

Genome-wide changes in gene expression during the wound response in *A. thaliana*

Wounding in plants causes dramatic transcriptional changes in genes expression (Reymond et al., 2000; Cheong et al., 2002). Identification of the genes affected by wounding can reveal the challenges faced by a wounded plant and the mechanisms by which plants adapt to reduce wound-triggered trauma. Microarray analysis has been helpful in monitoring these vast transcriptional changes in *A. thaliana*, revealing multiple pathways that are activated and repressed by tissue damage (Reymond et al., 2000; Cheong et al., 2002). For example, water deficit is an immediate concern for the wounded plant and water stress has been shown to initiate wound signaling (Reymond et al., 2000). A dehydrin (*XERO2*) and an ethylene-responsive lea-like (*ER5*) gene are both sensitive to changes in water potential and are induced by mechanical damage in *A.*

thaliana leaves (Welin et al., 1994; Zegzouti et al., 1997; Reymond et al., 2000; Cheong et al., 2002). Interestingly, cabbage butterfly (*Pieris rapae*) larvae are able to exploit *A. thaliana* during infestation by inactivating the water-stress signaling pathway while still triggering other wound-responsive pathways (Reymond et al., 2000). Wounding also activates other abiotic stress pathways which affect water availability, including high salinity and cold- and heat-tolerance (Mhiri et al., 1997; Ichimura et al., 2000; Mizoguchi et al., 2000; Christmann et al., 2006).

Microarray analysis determined that wound-induced genes in *A. thaliana* are transcriptionally-activated in two temporal waves (Cheong et al., 2002). The early-responding class of transcripts, up-regulated within 15 minutes of wounding, includes the touch-sensitive genes (*TCH1*, *TCH2*, *TCH3*, and *TCH4*). A contingent (20%) of these early-induced wounding genes correspond to components of signal transduction pathways or transcription factors, including receptor-like kinases (*RLK*), protein kinases, *MYB*-like factors, the *WRKY* class of transcription factors, calcium-binding proteins, and phosphatases. The late-expressing transcripts (up-regulated six hours after wounding) appear to function in pathogen resistance. They include mildew resistance locus o (Mlo)-like genes, non-race specific disease resistance (NDR)-like genes, chitinases, glucanases, non-expresser of PR genes (NPR)-like genes, and genes for enzymes involved in oxidative burst (peroxidases, glutathione-S-transferases, catalases), secondary metabolism (cinnamoyl alcohol dehydrogenase, anthocyanidin synthase) and cell wall modification (xyloglucan endotransglycosylase (XET))-related, pectinerases, glucosyltransferases). The temporal regulation of the two classes of wound-regulated genes suggests a coherent sequence that is first initiated by the early-responding signaling

genes, leading to the subsequent activation of the late-responding genes and the synthesis of important wound-related compounds. Interestingly, some of the effector molecules may possess anti-herbivore activities. For example, the enzymes involved in the tryptophan biosynthesis pathway can be activated by mechanical damage and contribute to the formation of toxic anti-pest compounds called glucosinolates (Rask et al., 2000).

Microarray analysis has also documented the critical roles of various plant hormones in the wound response. Previous studies show that the genes encoding for 3-oxo-2-(2'-*cis*-pentenyl)-cyclopentane-1-acetic acid (jasmonic acid, JA) biosynthesis are induced in wounded plants, leading to the accumulation of JA (Creelman et al., 1992). Microarray analysis has also confirmed a central role of JA in regulating the wound response in higher plants (Reymond et al., 2000; Cheong et al., 2002). However, a number of wound-induced genes are independent of JA regulation (Titarenko et al., 1997; Leon et al., 1998; Reymond et al., 2000; Cheong et al., 2002). A large proportion of the JA-independent genes appear to be regulated by water stress (Reymond et al., 2000). Wounding also influences the activity of other plant hormone signaling pathways. For example, salicylic acid (SA) accumulates in pathogen-challenged plants and functions to activate pathogen defense responses (Halim et al., 2006). Two independent microarray studies have documented that wounding may inhibit SA-mediated signaling (Reymond et al., 2000; Cheong et al., 2002). Ethylene is produced in response to tissue injury and senescence (Johnson and Ecker, 1998). Wounding induces the expression of 1-aminocyclopropane-1-carboxylic acid (*ACC*) synthase, an ethylene biosynthetic enzyme (Peck and Kende, 1998; Yu et al., 1998; Tatsuki and Mori, 1999). Ethylene consequently contributes to the late-responding activation of chitinases, glucanases and senescence-

related genes (Cheong et al., 2002). Ethylene-responsive transcription factors (EREBPs), involved in the activation of ethylene-dependent genes, are activated early-on by wounding and may participate in crosstalk with other signaling pathways. Mechanical damage to leaf tissues also represses auxin signaling. The induction of an IAA-glucosyl transferase in injured plants suggests that the bioactivity of indole-3-acetic acid (IAA), an endogenous plant auxin, in wounded tissue is reduced through its modification as a sugar moiety. In addition, wounding may suppress IAA signaling based upon the up-regulation of a putative negative regulator (*NPK1*-like gene).

The activation of the wound response by a wide variety of stimuli demonstrates its importance as a fundamental response to a variety of stresses. However, the recruitment of ancillary pathways obscures the identity of primary wound-specific genes. Current strategies have failed to distinguish wound-specific genes from other general response genes. Thus functions and regulation of wound-specific genes remain relatively unknown.

Jasmonic acid as the wounding signal

JA is ubiquitously found in higher plants and present in virtually all plant tissues (Meyer et al., 1984), although concentrations during development and within different organs may vary (Lopez et al., 1987; Creelman and Mullet, 1995). JA is found in actively growing tissues of a plant (Sembdner and Parthier, 1993; Creelman and Mullet, 1995). Its volatile methylated form (MeJA) was originally extracted as the fragrant oil from *Jasmium* flowers and is used as a component in perfumes. In addition, metabolites of JA and intermediates in its biosynthesis may possess unique bioactive properties (Seo

et al., 2001; Stintzi et al., 2001; Taki et al., 2005). 'Jasmonates' will be herewith used to be inclusive of all the bioactive compounds related to JA. The discovery that jasmonates may also activate defensive and wound responses in plants has fueled additional interest in its versatile functional roles and biosynthesis (Turner et al., 2002; Devoto and Turner, 2003; Schilmiller and Howe, 2005; Delker et al., 2006).

Jasmonates regulate multiple processes in plant growth and development (Staswick, 1992; Creelman and Mullet, 1995, 1997). When applied exogenously, JA stimulates root formation, senescence, ethylene synthesis, petiole abscission, β-carotene production, and tendril coiling (Ueda and Kato, 1981; Saniewski and Czapski, 1983; Czapski and Saniewski, 1992; Weiler et al., 1993). JA also inhibits root growth, seed germination, callus development, pollen germination and chlorophyll production (Ueda and Kato, 1982; Corbineau et al., 1988; Wilen et al., 1991; Staswick et al., 1992; Reinbothe et al., 1993). The apparent contradicting effects of JA treatment on certain processes may be a consequence of species-specific responses to externally applied JA. Alternatively, it is possible that the concentrations of JA applied may not reflect biologically-relevant levels.

The physiological changes moderated by JA are reflected in changes in gene expression. JA was shown to trigger the expression of jasmonate-induced proteins in at least 30 tested plant species demonstrating its nearly universal role as an endogenous plant signal (Herman et al., 1989). Early work characterized several proteins that accumulate upon exposure to JA and MeJA, including the VSP from soybean, seed storage proteins and oil body membrane proteins from *Brassica* embryos, and the PI proteins from *Solanaceous* plants, while levels of proteins representing Rubisco and

plastid polypeptides diminished (Farmer and Ryan, 1990; Staswick, 1990; Wilen et al., 1991).

Several lines of evidence point to JA as the signal responsible for mediating the wound response in higher plants. The first indication came from the similar rates of *PI* expression in *Solanaceous* plants treated with either JA or wounding (Farmer and Ryan, 1990; Farmer et al., 1992; Farmer and Ryan, 1992). In soybean, the accumulation of JA coincided with the expression of *chalcone synthase*, *VSP* and a proline-rich cell wall protein (Creelman et al., 1992). Furthermore, the highest levels of JA and MeJA were found in the wounded sections of soybean hypocotyls. Pre-incubation of tomato and potato tissue with inhibitors of JA biosynthesis prevented the wound-induced expression of *PI,* but expression was subsequently rescued with exogenous JA (Pena-Cortes et al, 1995).

Work with the volatile MeJA also pointed to the role of JA in wound response. The activation of JA- and wound-inducible proteins by MeJA in tomato was elegantly shown in a study where untreated tomato plants were co-incubated with either airborne MeJA or *Artemisia tridentata* plants, which inherently contain high levels of MeJA on their leaf surface (Farmer and Ryan, 1990). Overexpression of the JA carboxylmethyltransferase (*JMT*) gene in *A. thaliana* also resulted in the constitutive expression of the JA-responsive *VSP* and defensin gene, *PDF1.2* (Seo et al., 2001). These early studies demonstrated that wound-responsive genes in plants rely upon the *de novo* production of endogenous jasmonates.

Biosynthesis of JA in *A. thaliana*

The transformation of membrane-derived fatty acids generates a diverse group of related compounds called oxylipins (Fig. 1) (Howe and Schilmiller, 2002; Weber, 2002). JA synthesis is initiated by the lipoxygenase-mediated dioxygenation of α-linolenic acid (18:3) and processed via the octadecanoid pathway (Vick and Zimmerman, 1984). Linolenic acid is primarily found esterified to glycerolipids and phospholipids in the membranes of chloroplasts. The *A. thaliana* triple mutant *fad3-2 fad7-2 fad8*, which possesses negligible amounts of linolenic acid, fails to mount a wound response or accumulate JA after sustaining injury (McConn et al., 1994). Therefore, the availability of free linolenic acid is a critical determinant of the biosynthesis of *de novo* JA. Linolenic acid and lipoxygenases may be physically forced together to trigger JA biosynthesis in wounded tissues due to the compression that occurs during mechanical damage (Creelman and Mullet, 1995). However, wounding also liberates a large amount of linolenic acid from the membrane, possibly though enzymatic action of phospholipases (Conconi et al., 1996). The *dad1* (*defective in anther dehiscence 1*) mutant in *A. thaliana* fails to accumulate JA in response to wounding (Ishiguro et al., 2001). *DAD1* encodes a plastid-localized phospholipase A_1, which regulates JA content in wound-induced plants by functioning as the lipolytic enzyme responsible for liberating linolenic acid from the chloroplast membranes. Several *DAD1*-like genes exist in *A. thaliana* and may contribute to overall wound-regulated lipolytic activity (Beisson et al., 2003). Different plant species may recruit related phospholipases to release linolenic acid. For example, the activity of phospholipase A_2 is important in wound-regulated responses in tomato (Narvaez-Vasquez et al., 1999).

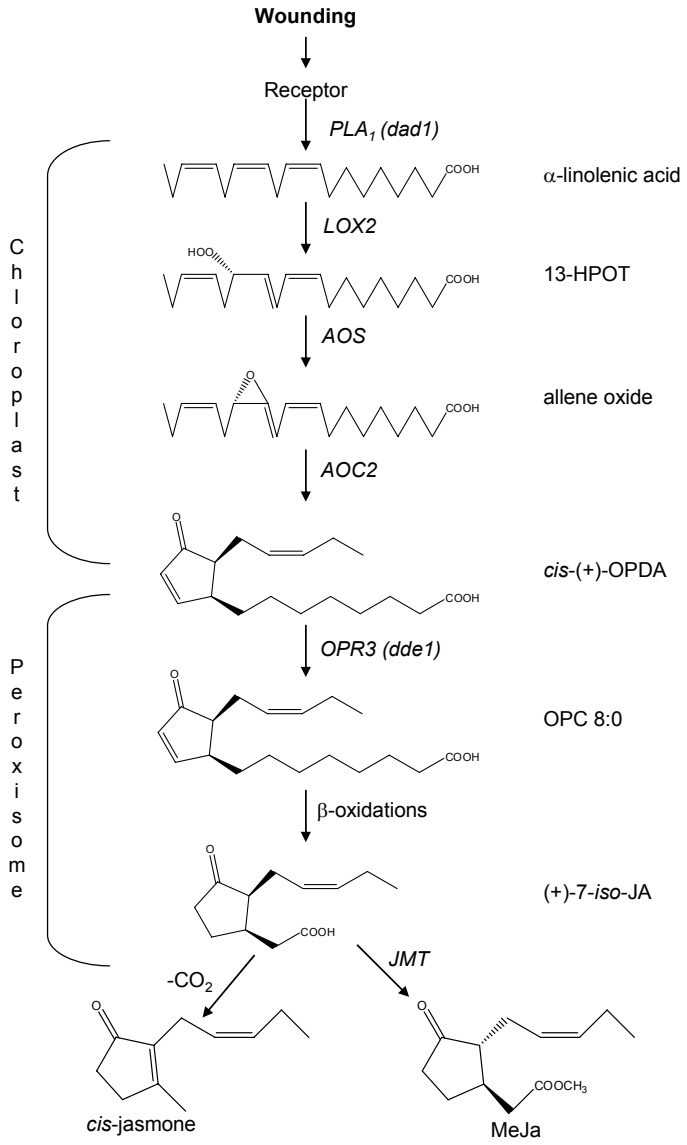


Figure 1.1 Pathway depicting the biosynthesis of JA in *A. thaliana*. Linolenic acid is liberated from plastid membranes by a phospholipase (PLA_1) encoded by *dad1* (*defective anther dehiscence 1*) and dioxygenated by lipoxygenase (LOX2). The resulting 13-hydroperoxyoctadecatrienoic acid (13-HPOT) is dehydrated and circularized in concert by allene oxide synthase (AOS) and allene oxide cyclase (AOC) to form 12-oxophytodienoic acid (OPDA). OPDA is shuttled from the plastid into the peroxisome, where it is reduced by OPDA reductase (*OPR3*), encoded by *delayed dehiscence 1* (*dde1*), and undergoes three successive β-oxidation steps to form JA. Two possible conversions of JA leading to additional active oxylipins are decarboxylation, forming jasmine, and methylation by a jasmonate carboxymethyltransferase (*JMT*) leading to the production of MeJA. Adapted from Turner et al (2002).

A. thaliana contains six lipoxygenase genes (Feussner and Wasternack, 2002). One lipoxygenase (*LOX2*) in *A. thaliana* seems to participate in the generation of endogenous JA (Bell et al., 1995). Expression of *LOX2* is restricted to chloroplasts and its activation is essential for the wound-induced accumulation of JA (Bell et al., 1995). Introduction of oxygen into linolenic acid by LOX2 occurs at C_{13} (13-LOX) resulting in the formation of (13S)-hydroperoxyoctadecatrienoic acid (13-HPOT) (Vick and Zimmerman, 1983; Siedow, 1991; Feussner and Wasternack, 2002). In addition to JA biosynthesis, six other known pathways exploit 13-HPOT to generate an assortment of oxylipins (Fig. 2A) (Schaller et al., 2005). Several products resulting from the metabolism of 13-HPOT are involved in different aspects of defense responses. The hydroperoxide lyase (HPL) pathway metabolizes 13-HPOT into C_6 aldehydes and C_{12}-ω-keto fatty acids (Matsui, 2006). These short-chain aldehydes are volatile compounds that function as antimicrobials and aphid repellants (Croft et al., 1993; Blee, 2002; Nakamura and Hatanaka, 2002). In addition, C_{12}-ω-keto fatty acid can be converted to traumatin, a wound healing molecule involved in mitogenesis (Zimmerman and Coudron, 1979). Divinyl ether (DVE) fatty acids are another metabolite of 13-HPOT, synthesized by DVE synthase (DVS) (Itoh and Howe, 2001; Grechkin, 2002). Recently DVS was shown to mediate responses to elicitation in challenged tobacco plants (Fammartino et al., 2007). Finally, peroxygenases (POX) synthesize the epoxy derivatives of 13-HPOT, which can act as anti-fungal toxins (Lequeu et al., 2003).

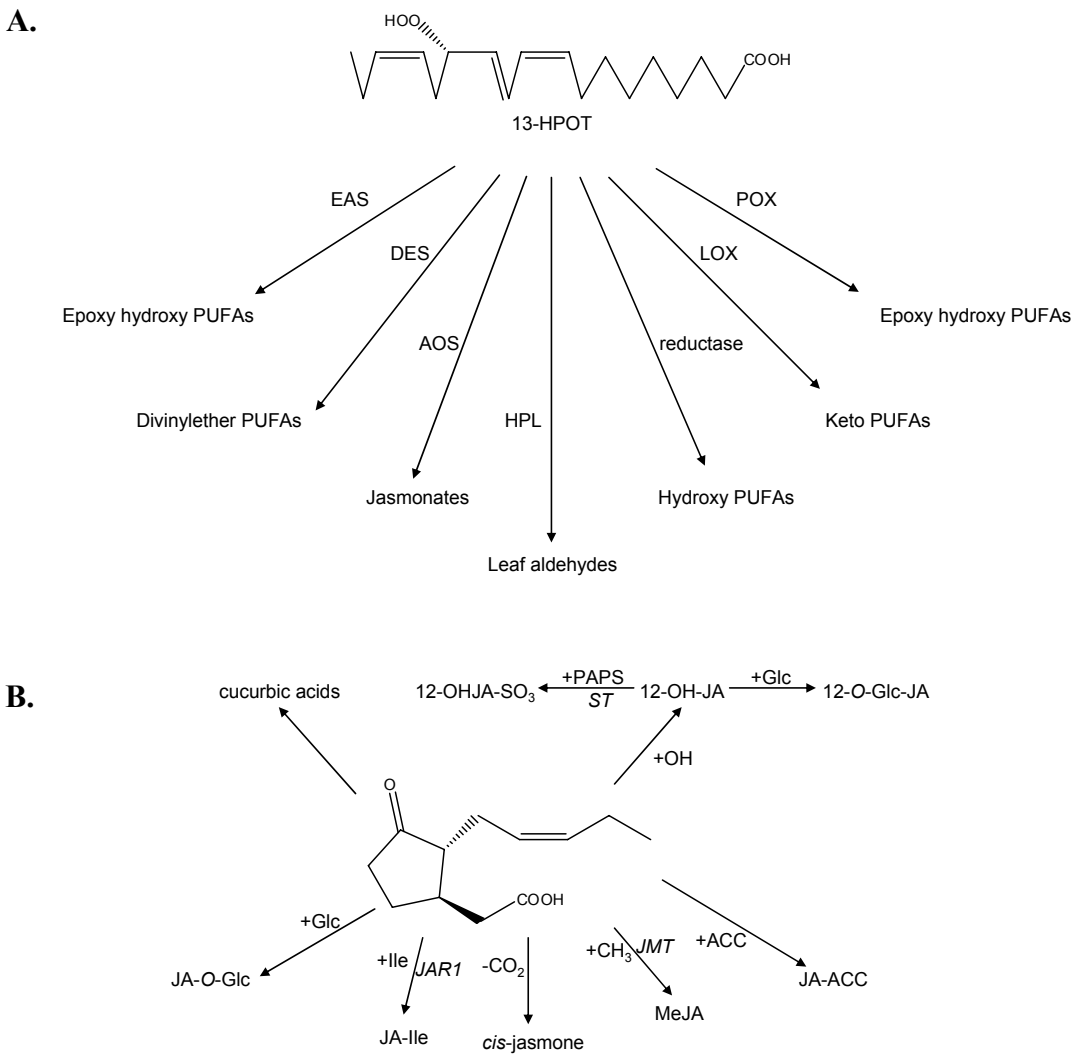


Figure 1.2 Plants generate a diversity of fatty acid-generated compounds.
A. There are seven known enzymatic reactions that modify the product of the lipoxygenase-mediated oxygenation of linolenic acid. Each appears to play a role in defense responses except the ketodiene-forming LOX reaction, whose function to this point is unclear. Modified from Schaller et al (2005). **B.** JA may be further catabolized into several forms. JA may be conjugated to glucose, isoleucine (Ile) or an ethylene precursor (ACC). The JA-Ile conjugate has inherent bioactivity. In addition, JA may be modified into metabolites with unique properties through methylation, decarboxylation and hydroxylation. Hydroxylated JA can be further modified by sulfonation or conjugation as a sugar moiety. EAS, epoxy alcohol synthase; DES, DVE synthase; AOS, allene oxide synthase; HPL, hydroperoxide lyase; LOX, lipoxygenase; POX, peroxygenase; *JAR1*, *jasmonate resistant 1*; *JMT*, *JA carboxylmethyltransferase*; PAPS, adenosine 3'-phosphate 5'-phosphosulfate; ST, sulfotransferase. Modified from Delker et al (2006).

Biosynthesis of JA via the octadecanoid pathway proceeds with the concerted action of allene oxide synthase (AOS) and allene oxide cyclase (AOC), which converts 13-HPOT into *cis*-(+)-12-oxo-phytodienoic acid, or OPDA (Fig. 1) (Hamberg and Fahlstadius, 1990; Laudert et al., 1997; Laudert and Weiler, 1998). Like LOX2, both AOS and AOC localize to the chloroplast (Laudert et al., 1997; Ziegler et al., 2000; Stenzel et al., 2003a). AOS catalyzes the dehydration of 13-HPOT to 12, 13-epoxy-linolenic acid. The genomes of *A. thaliana* and other higher plants possess a single copy of *AOS* (Kubigsteltig et al., 1999). A knockout of *AOS* expression in *A. thaliana* results in the deficiency of JA and the subsequent absence of wound-induced JA formation and JA-regulated gene expression (Park et al., 2002; von Malek et al., 2002). In *A. thaliana*, the AOS-mediated step is a point of regulation (Laudert and Weiler, 1998). In contrast to AOS, *A. thaliana* contains four copies of the AOC enzyme, with preferential induction of *AOC2* in response to wounding (Stenzel et al., 2003a).

Once produced by the concert actions of AOS and AOC, OPDA, or possibly its CoA ester, is transported from the chloroplast to the peroxisome (Theodoulou et al., 2005). An OPDA reductase encoded by *OPR3* converts OPDA from a cyclopentenone into a 3-oxo-2-(2'(*Z*)-pentenyl)-cyclopentane-1-octanoic acid (OPC 8:0) (Fig. 1). Other forms of OPRs exist in *A. thaliana*, but only the mutants of *OPR3* (*opr3*/*delayed dehiscence 1 (dde1*)) are deficient in JA content (Sanders et al., 2000; Stintzi and Browse, 2000; Stintzi et al., 2001; Stenzel et al., 2003b). Interestingly, the *opr3* mutant maintains wild-type resistance to the insect *Bradysia impatiens*, suggesting that OPDA may be the critical molecule involved in some defense responses in *A. thaliana* that were initially attributed to JA (Stintzi et al., 2001). The addition of OPDA to wild-type *A. thaliana*

plants induces genes that are not responsive to JA (Stintzi et al., 2001; Taki et al., 2005). Moreover, tendril coiling in *Bryonia dioica* is more effectively elicited with OPDA than JA (Blechert et al., 1999). Therefore, OPDA possesses signaling properties that are distinct from the JA-mediated responses and represent a branch of bioactive oxylipins.

Following OPR3-mediated reduction, three consecutive rounds of β-oxidation curtail the chain proximal to the carboxylic side to finally form JA (Miersch and Wasternack, 2000). In *A. thaliana*, three enzymes participate in this chain shortening: an acyl-CoA synthase, an acyl-CoA oxidase (ACX1), and a L-3-ketoacyl CoA thiolase (KAT2) (Delker et al., 2006). Both *ACX1* and *KAT2* are induced by wounding, and antisense-based suppression of either prevents the accumulation of wound-induced JA (Cruz Castillo et al., 2004).

Three modes for the regulation of JA biosynthesis have been observed. Expression of the JA biosynthetic enzymes can be restricted to certain target tissues (Kubigsteltig et al., 1999; Delker et al., 2006). For example, promoter studies of the four *AOC* genes in *A. thaliana* showed that they displayed non-overlapping expression domains, suggesting a specific spatial and temporal role for each during growth and development (Kubigsteltig et al., 1999). JA biosynthesis is also regulated by a positive feedback loop. The exogenous application of JA or MeJA to plant tissues up-regulates the expression of genes involved in JA biosynthesis (Reymond et al., 2000; Stenzel et al., 2003b; Cruz Castillo et al., 2004). Mutants which possess elevated or reduced JA levels also have concomitantly higher or lower AOC content than untreated plants, respectively (Stenzel et al., 2003b). Finally, JA biosynthesis is influenced by substrate availability. Overexpression of *AOS* in transgenic plants does not lead to increases in endogenous JA

concentration until the plants are wounded (Laudert et al., 2000; Park et al., 2002). Moreover, despite the abundance of lipoxygenase, AOC and AOS in untreated *A. thaliana* leaf tissue, JA and OPDA are not formed until elicited by stress (Laudert and Weiler, 1998; Stintzi et al., 2001; Stenzel et al., 2003b).

Mutants of JA biosynthesis and signaling

The elucidation of the JA biosynthesis and signaling pathways has been facilitated by mutational analysis in *A. thaliana* (Table 2). Initial screens isolated mutants in root growth assays that were insensitive or possessed attenuated sensitivity to exogenous JA or coronatine, an analog of JA. Several mutants were identified, including *coronatine insensitive 1* (*coi1-1*), *jasmonate resistance 1* (*jar1*) and *jasmonate insensitive 1* (*jin1*) (Staswick et al., 1992; Feys et al., 1994; Lorenzo et al., 2004). An alternative approach involved mutagenesis of plants harboring a construct with a JA-responsive promoter fused to a reporter gene. From this method, the *joe* (*jasmonate overexpression*) mutants were generated using the *LOX2* promoter, *cas1* (*constitutive allene oxide synthase 1*) with the AOS promoter, *cet* (*constitutive expresser of thionin*) mutants using the *THI2.1* promoter and *cev1* (*constitutive expression of VSP1*) with the *VSP* promoter (Ellis and Turner, 2001; Hilpert et al., 2001; Jensen et al., 2002; Kubigsteltig and Weiler, 2003). The mutant plants exhibited increased or constitutive expression of JA-regulated genes. The third approach indirectly resulted from investigations of male-sterility mutants generated by EMS or T-DNA insertional mutagenesis. Several JA biosynthetic mutants in Arabidopsis, including *opr3*, *dad1*, *dde1*, *dde2-2*, and *fad3-2fad7-2fad8*, were initially classified as exhibiting defective anther development (McConn et al., 1994;

Mutant or Transgenic		Phenotype for Mutational Screen	Fertility	Necrotroph Susceptibility	Insect Susceptibility	Encoding gene
Biosynthesis	*fad3-2fad7-1fad8*	Altered fatty acid composition	Sterile	Increased	Increased	Fatty acid desaturases
	dad1	Male fertility	Sterile			Phospholipase A_1
	dde2	Male fertility	Sterile			Allene oxide synthase
	aos	PCR-based screening for T-DNA insertions	Sterile			Allene oxide synthase
	opr3, dde1	Male fertility	Sterile	Reduced	Resistant	12-oxophytodienoic acid reductase
Signaling	*jar1/jin4/jai2*	reduced inhibition of root growth	Wildtype	Increased	Increased	JA-amino acid synthetase
	coi1	reduced inhibition of root growth	Sterile	Increased	Increased	F-box containing, LRR protein
	jin1/jai1	reduced inhibition of root growth	Wildtype	Increased	Wildtype	AtMYC2
	mpk4	dwarf mutant	Reduced	Reduced		AtMPK4
	jai3	reduced root growth inhibition (*ein3* background)				
	jai4/sgt1b	reduced root growth inhibition (*ein3* background)				SGT1b
	jue1-3	reduced *pLOX2::LUC* expression				
Promoter::						
Reporter	*cet1-9*	Constitutive thionin (*Thi2.1:BAR*) expression				
Gene	*cex1*	Constitutive root growth inhibition	Wildtype			
Fusion	*cev1*	Constitutive *pVSP::LUC* expression	Wildtype			AtCeSA3
	joe1	Increased *pLOX::LUC* expression				
	joe2	Increased *pLOX::LUC* expression				
	cas1	Constitutive *AOS1* expression				
Others	*JMT*	Overexpression of JA carboxymethyltransferase				
	cos1	Suppresses *coi1* root growth phenotype				Lumazine synthase

Table 1.2 List of transgenic plants and mutant lines compromised in JA biosynthesis and signaling.
Modified from Devoto and Turner (2005), Delker et al (2006) and Lorenzo and Solano (2005).

(McConn et al., 1994; Sanders et al., 2000; Stintzi and Browse, 2000; Ishiguro et al., 2001; von Malek et al., 2002). Eventual characterization of these mutants demonstrated that the wild-type functions of the mutant genes were engaged in JA biosynthesis. Defective pollen formation in these mutants is rescued by application of JA to immature flowers, suggesting that JA plays an added role in anther development, pollen maturation and dehiscence. Interestingly, the equivalent function of JA of maintaining proper anther development and pollen viability is not observed in tomato. However, failure to perceive JA due to a lesion in the *COI* gene of tomato affects female reproductive development (Li et al., 2004b).

The *jar1* mutant is less sensitive to JA and MeJA than wild-type *A. thaliana* plants (Staswick et al., 1992). Accumulation of JA-responsive VSP proteins is detected in *jar1* mutants though at reduced levels compared to wild-type plants. The *jar1* mutant is more susceptible than wild-type plants to the soil fungus *Pythium irregulare* (Staswick et al., 1998). Thus, some JA responses are responsible for pathogen resistance. The fertility of the *jar1* mutant suggests that *jar1* participates in only a subset of the total JA-dependent responses. The *jar1* locus was cloned and characterized by biochemical assays as an adenylate-forming enzyme belonging to the firefly luciferase superfamily (Staswick et al., 2002). The JAR1 enzyme was later demonstrated to behave as a JA-amino synthetase, catalyzing the conjugation of JA to amino acids, preferably isoleucine (Ile) (Staswick and Tiryaki, 2004). Thus, this modification of JA appears necessary for optimal signaling in *A. thaliana*.

The *coi1* mutant in *A. thaliana* is insensitive to the phytotoxin coronatine, which is a compound secreted from *Pseudomonas syringae* that structurally resembles JA (Feys

et al., 1994). The *coi1* plants are also insensitive to MeJA and are consequently male sterile. Wounded *coi1* seedlings fail to accumulate VSP. They are more susceptible to infestation by *Pythium*, the necrotrophic fungus *Alternaria brassicola* and the necrotrophic bacterium *Erwinia carotova* (McConn et al., 1997; Thomma et al., 1998; Brader et al., 2001) demonstrating once again the importance of JA signaling for resistance against necrotrophic pathogens and insect predation (Farmer et al., 2003).

The *COI* locus encodes a protein containing an F-box motif and leucine-rich repeats (LRR) (Xie et al., 1998). F-box proteins are components of the polymeric SCF complex, comprised of four subunits (Skp1, Cullin1, F-box protein, Rbx1), which acts as an E3-ubiquitin ligase (Lechner et al., 2006). Ubiquitination of a targeted protein leads to its proteolytic destruction by the 26S proteasome (Smalle and Vierstra, 2004). The endogenous substrates for the SCF^{COI} complex are unknown, but yeast models demonstrate that they are first modified by phosphorylation before being recognized by the F-box receptor (Skowyra et al., 1997). Based upon previously characterized models such as auxin signaling (Parry and Estelle, 2006), the substrates targeted for ubiquitination by the SCF^{COI} complex are likely to be the negative repressors of JA signaling.

Yeast-two hybrid experiments identified one substrate for the SCF^{COI} complex (Devoto et al., 2002; Xu et al., 2002). Histone deacetylase (RPD3b) physically interacts with an epitope-tagged COI protein (Devoto et al., 2002). Histone deacetylases regulate the balance of acetylated and deacetylated histones (Murfett et al., 2001; Narlikar et al., 2002). De-acetylation of histones reduces the access of chromatin for transcription. Thus, a proposed model for the wound-induced activation of JA-responsive genes

predicts that mechanical damage of leaf tissue causes the release of JA, which is recognized by a cognate receptor (Devoto et al., 2002; Xu et al., 2002; Devoto and Turner, 2003). The activated JA receptor phosphorylates downstream substrates, including histone deacetylases, which are identified by the SCF^{COI} complex and then subsequently tagged with ubiquitin. The targeted repressors are proteolytically degraded, allowing expression of JA-regulated genes.

JA metabolism

Upon formation, JA may be metabolized into several alternative forms (Fig. 2B). Metabolites of JA may play an important role in plants as they potentially possess unique biological activities. Methylation of JA is executed by JMT. Transgenic *A. thaliana* plants constitutively-expressing *JMT* contain higher levels of MeJA without affecting the JA content relative to wild-type plants (Seo et al., 2001). In addition, transgenic plants overexpressing *JMT* display enhanced diseases resistance, implying that MeJA possesses important bioactive properties. Another modified form of JA, jasmone, is produced by the decarboxylation of JA (Koch et al., 1997). Elevated jasmone levels in plants deters herbivory by aphids (Birkett et al., 2000). Jasmone also triggers the emission of plant volatiles which may attract aphid predators. Some JA metabolites are conjugated compounds as either an *O*-glycosylate at C_{11} and C_{12} or as a gentobiosyl ester, amide-linked amino acid, methyl, or glucosyl at C_1 (Sembdner and Parthier, 1993; Swiatek et al., 2004). Conjugates of JA may play a homeostatic role in controlling levels of available JA. However, the JA-Ile conjugate, formed by the activity of JAR1, rescues the *jar1* phenotype, demonstrating that conjugation may endow novel biological activity

(Staswick et al., 2002; Staswick and Tiryaki, 2004). In addition, tuberonic acid, a tuber-inducing compound first isolated from potato, is a hydroxylated JA at C_{11} or C_{12} (Yoshihara et al., 1989). In *A. thaliana*, the sulfated form of 12-OH JA may represent an inactivate form of the hydroxylated JA, though functions of hydroxylated JA in *A. thaliana* are unclear (Gidda et al., 2003).

Crosstalk between JA and other plant defense pathways

Plants are confronted by a diversity of threats and research has highlighted the intricacy of signaling mechanisms involved in orchestrating specific defense responses to these threats (Reymond and Farmer, 1998; Knight and Knight, 2001; Pieterse et al., 2001; Kunkel and Brooks, 2002). The ability to identify the foreign threat can lead to an appropriate response. Selective activation of specific pathways with restraint of others allows for prioritization of cellular and metabolic processes and helps to conserve resources (Knight and Knight, 2001; Pieterse et al., 2001; Kunkel and Brooks, 2002). Analysis of the interactions among the pathways may illuminate how plants identify and properly respond to different biotic threats.

Though JA is involved in many aspects of growth and development, it is also critical for signaling in response to abiotic stress and for defense responses against herbivores and necrotrophic pathogens (McConn et al., 1997; Thomma et al., 1999; Farmer et al., 2003). On the other hand, the pathogen defense response is also mediated by SA (Delaney et al., 1994; Nawrath and Metraux, Wildermuth et al., 2001), whose accumulation activates systemic acquired resistance (SAR), conferring immunity against any subsequent pathogen attacks (Ryals et al., 1996). The accumulation of SA in the

challenged plant up-regulates the production of pathogenesis-related (PR) proteins, anti-microbial compounds and secondary metabolites (Ward et al., 1991; Cao et al., 1994; Van Loon and Van Strien, 1999).

Pharmacological evidence and mutant analysis have revealed an antagonism between the SA-mediated pathogen defense pathway and the JA-regulated wound response (Fig. 3). Application of SA or related hydroxybenzoic acids to wounded tomato plants prevents the induction of *PI* expression and the accumulation of wound-induced JA (Doherty et al., 1988; Pena-Cortés et al., 1993; Doares et al., 1995b). In wounded tomato, SA suppresses the biosynthesis of JA by blocking the conversion of 13-hydroperoxylinolenic acid to OPDA (Pena-Cortés et al., 1993). Suppression by SA is alleviated only by the addition of JA or OPDA, suggesting that SA inhibits AOS (Pena-Cortés et al., 1993). In *A. thaliana*, SA may inhibit expression of JA biosynthesis at *AtLOX2* (Spoel et al., 2003) or *AOS* (Laudert and Weiler, 1998). In transgenic plants that ectopically express the bacterial gene *salicylate hydroxylase* (*NahG*), SA is converted to catechol, effectively removing active SA from plant tissues (Gaffney et al., 1993; Lawton et al., 1995). The JA-regulated genes in the *NahG*-expressing plants are subsequently hyper-inducible (Reymond and Farmer, 1998).

Several mutants in *A. thaliana* also demonstrate the antagonism between SA and JA signaling pathways. The *enhanced disease susceptibility 4* (*eds4*) mutants are more vulnerable to infection by *Pseudomonas syringae* pv *maculicola* due to the inability to accumulate SA (Gupta et al., 2000); the *eds4* plants also exhibit heightened JA-governed responses. Expression of the JA-induced defensin genes *PDF1*.2 and *Thi2*.1 are dependent upon *MPK4* (*MAP kinase 4*) (Petersen et al., 2000). In the loss-of-function

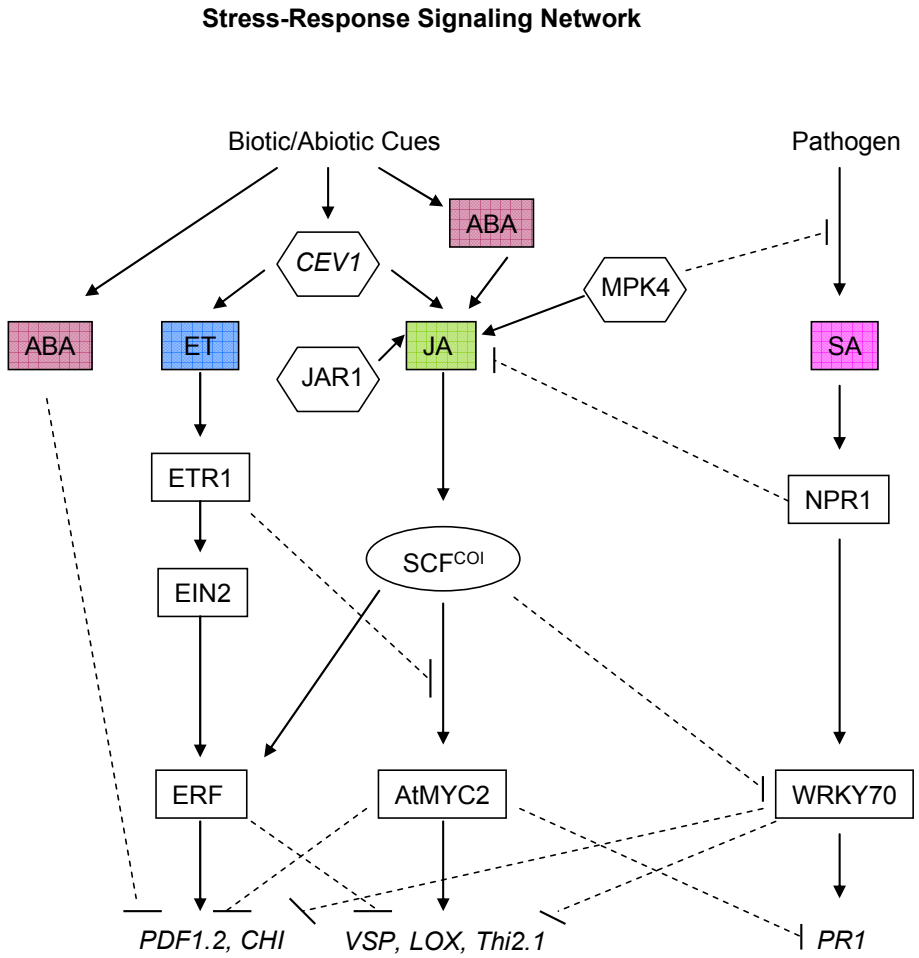


Figure 1.3 Schematic depicting the relationships between the signaling pathways finely tuning the defense response in plants. The primary signaling molecule central to mediating the wound response and some pathogenic defense responses is JA. The *COI* locus functions as a switch for downstream JA–dependent responses. Salicylic acid (SA) antagonizes the JA pathway through NPR1, a regulator of the SA-mediated pathogen defense response, and a transcription factor (WRKY70). The MAP kinase 4 (MPK4) suppresses SA signaling but is required for some JA-regulated genes. Ethylene (ET) and JA pathways are concomitantly triggered by wounding through the action of *CEV1*. Some defense responses require dual input from ET and JA pathways. In contrast, ET antagonizes the JA pathway. Downstream *ERF1* integrates signals from activated JA and ET pathways. Abscisic acid (ABA) does not play a central role in the wound response but regulates responses to desiccation. Modified from Lorenzo and Solano (2005).

mpk4 mutant, JA-dependent gene activation is compromised stemming from elevated levels of SA in *mpk4* plants (Petersen et al., 2000). This suggests that the wild-type function of *mpk4* is to repress SA signaling as it activates the JA pathway. Conversely, the transcription factor WRKY70 concomitantly activates SA-regulated genes while inhibiting JA signaling (Li et al., 2004a). Overexpression of *WRKY70* in transgenic *A. thaliana* plants causes elevated SA levels, conferring increased resistance to pathogens, but in *WKRY70*-suppressed transgenic lines, JA-regulated genes are up-regulated.

Characterization of the *cev1* mutant has uncovered the complex interactions between the JA, SA and ethylene pathways. The *cev1* mutant contains elevated levels of JA and ethylene and constitutively expresses the JA-responsive genes *PDF1*.2, *Thi2*.1 and chitinase (*CHI*) (Ellis et al., 2002b). The mutant consequently possesses enhanced resistance to insects and fungal pathogens (Ellis and Turner, 2001; Ellis et al., 2002a; Ellis et al., 2002b). Application of SA to *cev1* plants suppresses the expression of *PDF1*.2 but activates the expression of *pathogenesis-related protein 1* (*PR-1*), demonstrating SA-mediated inhibition of JA-regulated genes (Ellis et al., 2002a).

The negative influence of SA on JA-mediated activity may be regulated by *NPR1* (Spoel et al., 2003; Dong, 2004). The *npr1* mutant accumulates normal levels of SA in response to pathogens but fails to activate downstream defense genes (Cao et al., 1994; Delaney et al., 1994). In response to pathogen challenge, SA signaling is processed through NPR1, which is then shuttled into the nucleus to bind a TGA/OBF transcription factor for *PR* gene activation (Zhang et al., 1999; Kinkema et al.). However, suppression of JA responses by SA treatment is alleviated in the *npr1* mutant, suggesting that *NPR1*

performs an additional function in pathway crosstalk (Spoel et al., 2003). NPR1 appears to exert its inhibitory influence on JA from the cytosol.

The interaction of the JA and ethylene signaling pathways is critical for some defense responses, as the type of stimulus dictates the nature of the relationship between the two pathways. Cooperation or synergism between JA and ethylene ensues in response to pathogen attack (Xu et al., 1994; Penninckx et al., 1998; Lorenzo et al., 2003). Expression of the antifungal defensin gene *PDF1.2* requires activation by both JA and ethylene (Penninckx et al., 1998). In *A. thaliana* plants, exposure to both JA and ethylene results in enhanced expression of *PDF1.2* compared to plants treated with either signaling molecule alone. The expression of *PDF1*.2 is absent in the double mutant *cev1; etr1* (*ethylene resistant 1*), further confirming that ethylene and JA are both required for *PDF1*.2 activation (Ellis and Turner, 2001; Ellis et al., 2002b). The ethylene response factor 1 (ERF1) integrates the JA and ethylene pathways (Lorenzo et al., 2003). *ERF1*, which requires the concurrent signaling of the JA and ethylene pathways for its activation, regulates the expression of the defense genes *CHI* and *PDF1.2* and confers resistance against necrotrophic pathogens. Compromised signaling in either the JA or ethylene pathway fails to induce *ERF1* activation. In contrast, the expression of *Thi2.1* is constitutive in *cev1; etr1*, meaning that ethylene is required for *Thi2.1* repression in wild-type plants (Ellis and Turner, 2001). This ethylene antagonism of the JA signaling pathway is also observed in early responses to mechanical injury in the wounded leaf (Rojo et al., 1999).

JA-independent wound signals

Abscisic acid (ABA) was an early candidate as the signal responsible for the wound response (Pena-Cortes et al., 1989). Application of ABA to leaf tissue putatively increased *PI* expression in the treated and untreated remote leaves of potato. ABA content was also shown to increase in wounded leaves and systemic tissues (Pena-Cortes et al., 1989). However, wound activation of the *PI* promoter and elevated PI protein were not consistently confirmed by other labs (Ryan, 1974; Kernan and Thornburg, 1989; Birkenmeier and Ryan, 1998). In addition, ABA failed to induce several known wound-response markers (Birkenmeier and Ryan, 1998). Though the accumulation of ABA was observed in the immediate area of the wound site, ABA was speculated to be involved with desiccation, as evidenced by elevated ABA levels in wilted plants. Moreover, ABA repressed oligosaccharide-dependent (OSD) *PI* accumulation (Birkenmeier and Ryan, 1998).

A collection of genes, including *choline kinase* (*CK)* and *wound-responsive 3* (*WR3*), in *A. thaliana* is expressed rapidly upon wounding (Titarenko et al., 1997). Given that expression of *CK* and *WR3* precedes the activation of JA-mediated responses in wounded leaves and that wound induction of *CK* and *WR3* is unaffected in the JA perception *coi1* mutant, it appears that a JA-independent wounding pathway exists in *A. thaliana* (Titarenko et al., 1997). In addition, *CK* and *WR3* are instead activated by treatment with fungal- and plant-derived oligosaccharides (Rojo et al., 1999). Plant-generated oligogalacturonans (OGA) are liberated from the cell wall by mechanical damage or released enzymatically from the pectin component of the cell wall. In tomato, a wound-induced polygalacturonase (PG) releases OGA from the cell wall (Orozco-Cardenas and Ryan, 1999). Fungal OGAs like chitosan also effectively elicit the wound

response and stimulate the expression of oligosaccharide-dependent (OSD) genes (Walker-Simmons et al., 1984; Sanchez-Serrano et al., 1986). The restricted diffusion of oligosaccharides in plant tissue may be the determinant for the local expression of OSD genes (Baydoun and Fry, 1985).

Nitric oxide (NO) was shown to inhibit the JA-mediated wound response in tomato (Orozco-Cardenas and Ryan, 2002). Pre-treatment of tomato plants with NO donors prevent the wound-induced expression of *PI*, while scavengers of NO rescue *PI* expression. NO also inhibits *PI* expression activated by elicitors, systemin and JA. However, transcriptional activation of biosynthetic enzymes for JA is unaffected in NO-treated plants, therefore NO suppresses downstream of JA. Moreover, H_2O_2-induced *PI* expression is not affected by NO, implying that inhibition occurs between JA activation and H_2O_2 generation. Mechanical damage does not trigger the accumulation of endogenous NO in tomato but NO may play a role in antagonizing the accumulation of anti-pest compounds during pathogen attack.

Local and systemic wound signals

Leaf wounding establishes at least two spatial domains for wound expression. Our interpretation defines local expression as the entire wounded leaf and systemic tissues as the unwounded distal leaves. The wounded leaf is confronted by different challenges than the unwounded distal tissues. Injury to the leaf causes dramatic changes in water availability. Wounded leaves must also restore cellular integrity and repair damaged tissues. Therefore, wound signals must be deployed that are exclusive to wounded leaves. The expression of the OSD-induced genes in wounded *A. thaliana*

plants is restricted to the injured leaf (Fig. 4A), most likely a result of the limited mobility of OGAs (Baydoun and Fry, 1985). Furthermore, the OSD pathway inhibits JA-regulated gene expression by recruiting the ethylene pathway to antagonize downstream JA signaling (Rojo et al., 1999). This repression of JA-responsive genes is based on several lines of evidence. First, ethylene accumulates in both the leaves of tomato and *A. thaliana* in response to chitosan, JA or by wounding (Rojo et al., 1999). Additionally, the expression of jasmonate-responsive (*JR*) genes is boosted in ethylene-insensitive mutants (*ein2-1*, *etr1-1*) (Rojo et al., 1999). The inductive effect of JA on *JR* expression is mitigated by co-treatment with chitosan in wild-type *A. thaliana* seedlings, while chitosan is ineffective in suppressing *JR* expression in the *etr1-1* mutant. Antagonism between OGAs and JA signaling is not observed in tomato plants as the interactions among OGA, ethylene and JA are configured differently for the local wound response in *Solanaceous* plants (Fig. 4B) (O'Donnell et al., 1996). In tomato, ethylene acts to potentiate the JA and OGA signals. In addition, JA and OGA work cooperatively in wound signaling within the injured tomato leaf (O'Donnell et al., 1996).

Activation of the wound-induced pathway is not limited to the locally-damaged leaf but also occurs in the undamaged distal systemic leaves (Fig. 5A) (Green and Ryan, 1972). Systemic wound accumulation of PIs was first observed in wounded potato plants and has spurred interest in the non-autonomous signaling pathway that controls distal expression. The transmission of a long-distance wounding signal necessitates the initial recognition of the wounding incident along with synthesis and dispatching of the wound signal(s) to the distal tissues where the transmitted signal is interpreted. Evidence in tomato has implicated jasmonates as candidates for the long-distance wounding signal

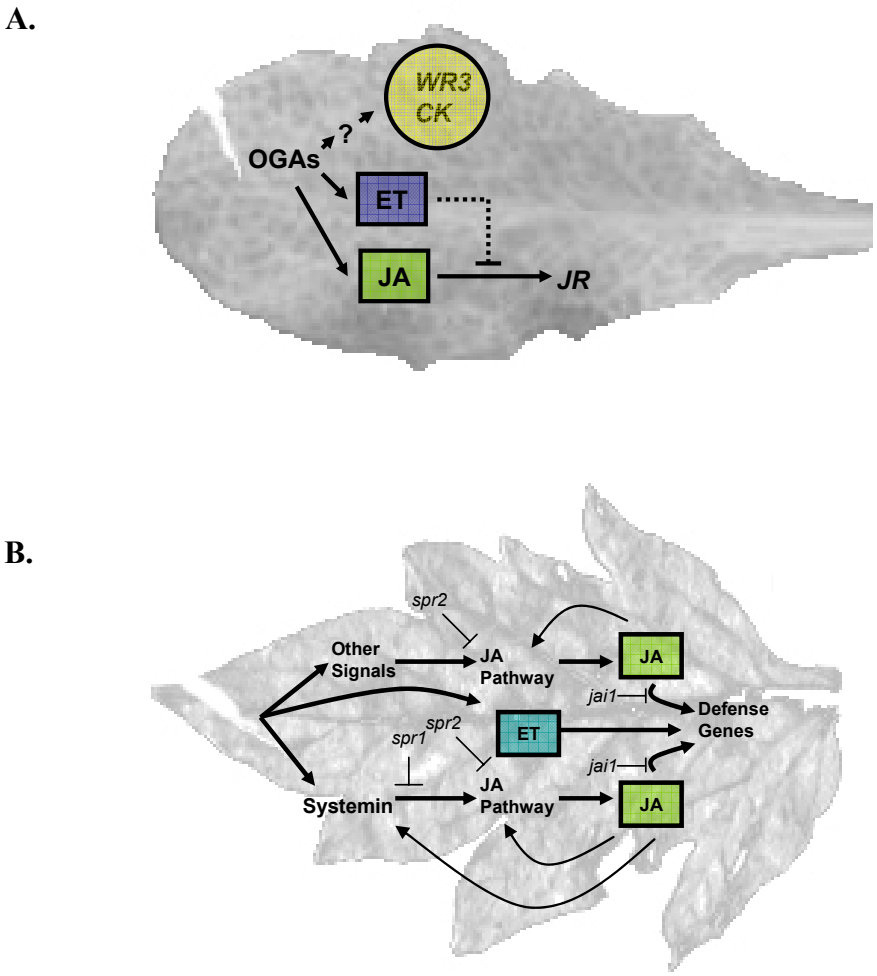


Figure 1.4 Differences in the signaling of mechanically-damaged leaves between *A. thaliana* and tomato.
A. In *A. thaliana* wound signaling, a JA-independent pathway is activated immediately by elicitation from oligogalacturonans (OGAs) released by the cell wall or of fungal origins. This leads to the expression of OGA-dependent genes *choline kinase* (*CK*) and *wound responsive 3* (*WR3*). OGAs activate the synthesis of ethylene (ET) that function to antagonize the JA signaling pathway. **B**. In tomato, wounding activates several pathways, which include a systemin-dependent route that triggers JA accumulation in the wounded leaf and subsequent JA-dependent gene response. Other signals, including electrical, hydraulic and OGAs, can separately induce the JA response. Ethylene works in the wounded tomato leaf to synergistically activate JA-regulated genes. *spr1*, *suppressor of prosystemin-mediated responses 1*; *spr2*, *suppressor of prosystemin-mediated responses 2*; *jai1*, *jasmonate-insensitive 1*. Modified from (Stratmann, 2003).

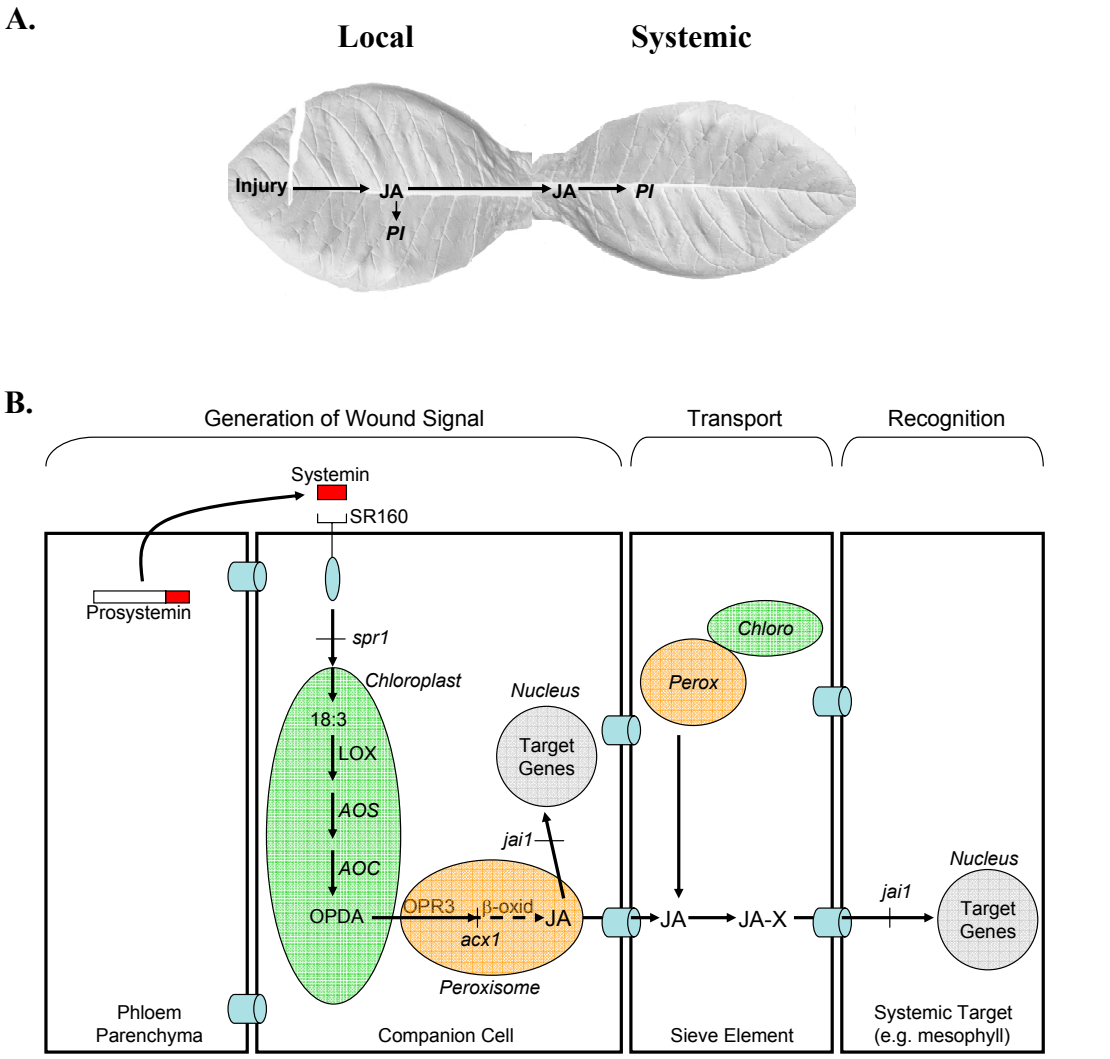


Figure 1.5 The transmission of the wound signal is dispatched to distal tissues through the phloem. **A**. Wounding activates the local and systemic expression of wound-induced genes including proteinase inhibitors (*PI*). Early evidence narrowed candidates of the systemic signal to an octadecanoid molecule that is transmitted distally through the phloem. **B**. The tissue localization of systemin, JA, and enzymes for JA biosynthesis were detected in companion cell-sieve elements of the vasculature. Prosystemin is detected in the phloem of parenchyma cells. Wounding triggers its proteolytic cleavage into active systemin, which binds its cognate receptor (SR160) in an adjacent companion cell, leading to the production of *de novo* JA. The synthesized JA activates local expression of wound-responsive genes or is transported to distal tissues through the sieve elements. JA enters and exits the sieve element through plasmodesmata. Targets of imported JA may include mesophyll cells in systemic leaves. The identity of the octadecanoid molecule is unknown. Adapted from Schilmiller and Howe (2005).

(Farmer and Ryan, 1992; Lightner et al., 1993; Howe and Ryan, 1999; Li et al., 2002c; Li et al., 2004b).

The utilization of tomato has been extremely valuable in characterizing systemic signaling due to the availability of biosynthetic and signaling mutants coupled with the ease of constructing grafting mutants. Pivotal to the identification of the systemic wound signal in tomato were three classes of mutants with compromised functions in systemin signaling, JA biosynthesis, and JA perception (Lightner et al., 1993; Howe and Ryan, 1999; Li et al., 2004b). These mutants failed to induce *PI* expression upon wounding, could not generate a systemic wound response and consequently were more susceptible to herbivory (Li et al., 2002a; Li et al., 2004b). The findings suggest that wound induction of *PI* in systemic leaves is triggered by a JA-related compound involving systemin (Howe, 2004). Grafting experiments with these mutants confirm the identity of the systemic wound signal as a JA-related molecule (Li et al., 2002c). Each of the JA biosynthetic mutants, *acyl-CoA oxidase* (*acx1*) and *suppressor of prosystemin-mediated responses 2* (*spr2*), acting as the wounded root stock fail to trigger *PI* expression in the grafted wild-type scions, suggesting that transmission of the signal requires the *de novo* formation of JA in the wounded leaf (Li et al., 2002c). In contrast, a JA perception mutant *jasmonate insensitive-1* (*jai1*), as the scion to a wild-type root stock, showed that the recognition of a mobile wound signal in the distal tissues is essential for systemic induction of *PI*. These experiments also reveal that *de novo* synthesis of JA in the systemic tissues is not required for *PI* induction as a biosynthetic mutant scion continues to activate *PI* expression when grafted onto a wounded wild-type stock. Thus, mechanical damage of a tomato leaf triggers the local accumulation of JA and generates a

JA-related mobile signal which is perceived in the systemic leaves to activate wound-responsive genes (Li et al., 2002c; Schilmiller and Howe, 2005).

Systemin may act in the wounded leaf to either initiate the JA-specific wound response or amplify the JA signal (Li et al., 2002c). The *spr1* tomato mutant is compromised in the wound induction of *P*I expression (Lee and Howe, 2003). However, this impairment is more aggravated in the distal tissues than in wounded leaves, implying a role for systemin in long-distance signaling. In *spr1* plants, the addition of systemin fails to bring about JA accumulation, and grafting experiments demonstrate that systemin may exert its effects from the wounded leaf (Li et al., 2002c). Wild-type scions fail to systemically express *PI* from a wounded *spr2* rootstock whereas in the reverse orientation, *PI* expression is observed in the *spr2* scion after wounding. Transgenic plants overexpressing prosystemin (35S::*Prosys*) constitutively express *PI*, suggesting that a signal is continuously dispatched that enables systemic *PI* expression (McGurl et al., 1994).

The dispatched JA-related signal traveling from the wound site to the distal tissues is most likely carried through the vasculature (Fig. 5B). The model is based on the detection of the JA biosynthetic enzymes in companion cells and sieve elements of the phloem (Hause et al., 2000b; Hause et al., 2003). Both JA and OPDA are detected in the phloem bundles of *Plantago major* and preferentially in the midrib of tomato leaves (Stenzel et al., 2003a). Furthermore, prosystemin accumulates in the parenchymal cells of the phloem (Narvaez-Vasquez and Ryan, 2004). The proximity of systemin, JA biosynthetic machinery and JA/OPDA accumulation in neighboring cell types implies a linked signaling cascade (Schilmiller and Howe, 2005). The model predicts that after

mechanical damage to a tomato leaf, systemin is released from the phloem parenchyma and recognized by its cognate receptor (SR160) positioned in the cell membrane of an adjoining companion cell (Scheer and Ryan, 2002). Biosynthesis of JA is then activated within the responding companion cell, and this signal is then shuttled through plasmodesmata into the sieve element for systemic transport into the target mesophyll cells and subsequent induction of JA-mediated wound responses. This model may be limited to *Solanaceous* plants since other plant species do not appear to contain systemin. *A. thaliana* utilizes the JA pathway for relaying a systemic wound response (Titarenko et al., 1997; Rojo et al., 1999; Leon et al., 2001; Strassner et al., 2002). However, *A. thaliana* does not contain systemin to amplify the local burst of JA production. Whether systemin-like activity exists in *A. thaliana* is unknown but other factors may play a role in the generation of wound-induced JA formation and transmission of the systemic response.

Thus at least two mechanisms control the spatial regulation of wound-induced genes. The local response is governed by two pathways triggered by released OGAs and JA synthesis (Rojo et al., 1999; Howe, 2004). Systemic expression of wounding genes is dependent upon the JA signal deployed by wounded leaves. The implication of the model predicts that the genes expressed in systemic tissues should mirror the JA-regulated genes in locally-wounded leaves, unless other local signals modify JA induction in wounded leaves. At the moment, no known wound-regulated genes have been isolated which are expressed exclusively in systemic tissues and characterization of these genes have been largely ignored. Conceivably this class of systemically-expressed genes should be regulated by a novel signaling system. In addition, the identities of these

systemic wounding genes could provide helpful molecular marks of systemic expression and their characterization could uncover important functional processes that are pivotal in systemic tissues of wounded plants.

Analogous defense systems between mammals and plants

Aspects of plant and animal defense responses bear striking resemblance to one another (Fig. 6)(Bergey et al., 1996; Staskawicz et al., 2001; Holt et al., 2003). Jasmonates, including JA, OPDA and MeJA (Fig. 6A) are structurally analogous to the prostaglandins in mammals (Bergey et al., 1996). Prostaglandins are powerful signaling compounds involved in inflammation and smooth muscle contraction. Both JA and prostaglandins are similarly generated through lipoxygenase-mediated lipid signaling. Inhibitors of lipoxygenase activity effectively reduce wound responses in plants and inflammation in mammals. In addition, peptide signals are involved in activating defense responses in plants (systemin) and animals. In humans, recognition of injury or an invasive pathogen triggers the release of tumor necrosis factor (TNFα) from a mast cell (Fig. 6B) (Galli and Wershil, 1996). TNFα is a polypeptide cytokine that is carried through the blood to activate phospholipase A_2 in the cells of the brain stem, which releases the arachidonic acid (20:4) from the cell membranes. This liberated fatty acid is converted into prostaglandins to alleviate infection by promoting inflammation and elevating body temperature.

Comparisons of microbial pathogenesis response pathways in insects, vertebrates and plants have also illuminated common features among the kingdoms, though notable differences exist (Ausubel, 2005). Although detection of pathogens is perceived by

similar transmembrane receptors containing LRR domains, plant and animal receptors possess different cytoplasmic domains. Cytosolic receptors associated with microbial responses in animals (CATERPILLER) and plants (NBS-LRR) have similar C-terminal LRR and nucleotide-binding site domains, but differ along the N-terminal region (Holt et al., 2003; Ting and Davis, 2005). Downstream signaling components are not conserved, as plants use WRKY transcription factors, which are absent in animals (Ausubel, 2005). Vertebrates depend on NF-κB signaling, which is a pathway that is absent in plants. Ausubel (2005) argues that existing information does not suggest that similarities between innate immunity in vertebrates and plants developed from a shared ancestor but that they exist as a result of convergent evolution. Phylogenetic studies of innate immunity in different organisms support the notion that innate immunity evolved independently, and that seemingly related components of disease resistance mostly likely developed from generalized signaling elements (Luo and Zheng, 2000; Friedman and Hughes, 2002).

Wound response in bryophytes

The wound response has been extensively studied in higher plants. However, little is known regarding the wound response in bryophytes. Bryophytes, which include hornworts, liverworts and mosses, are primitive land plants that diverged from the higher plants around 450 million year ago (Kenrick and Crane, 1997). Mosses do not produce seed, lack a true vasculature and have a lifestyle that is dominated by the haploid gametophyte stage. In addition, the fatty acid content of the cell membranes from the

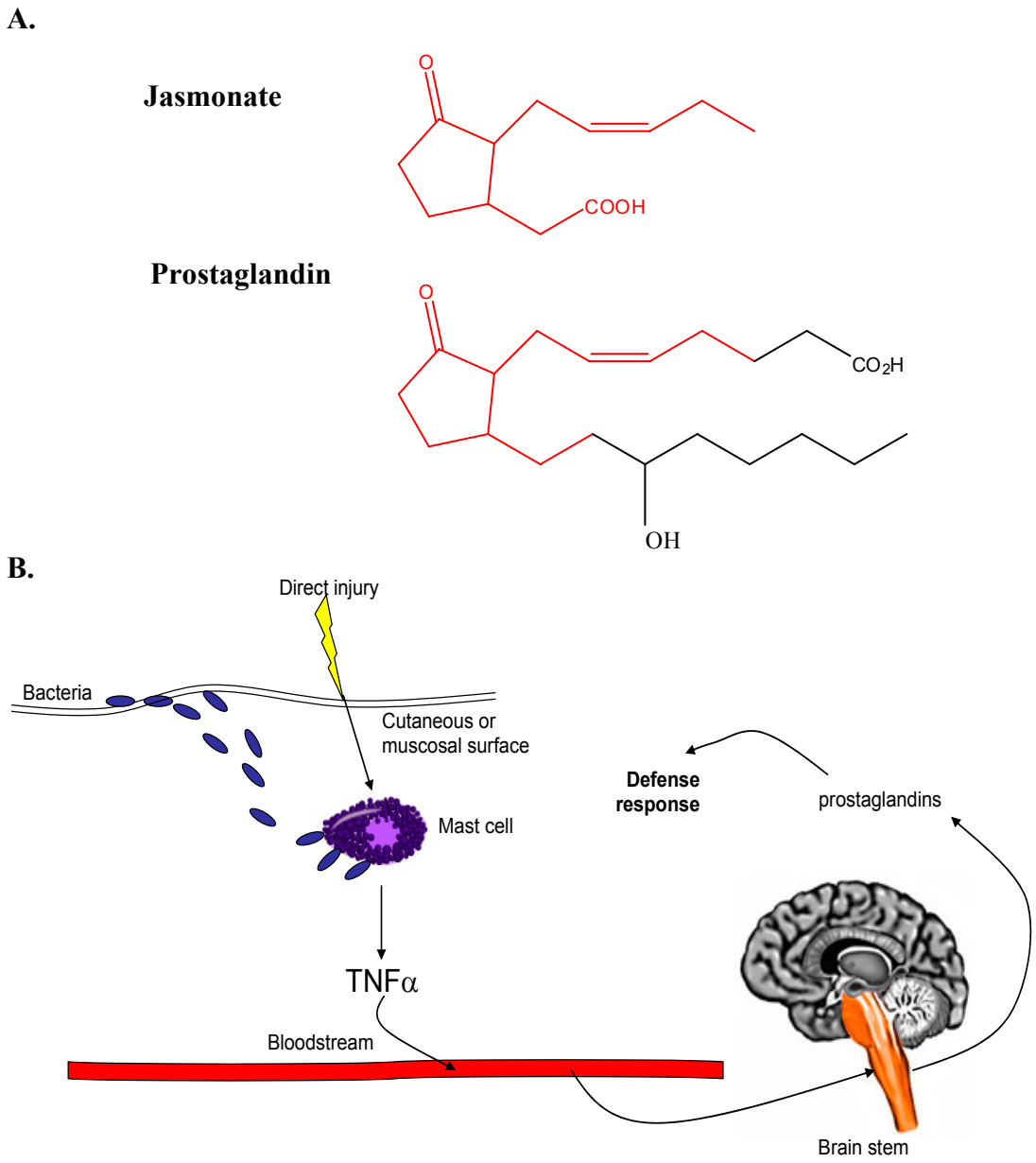


Figure 1.6 Similarities of defense response strategies between plants and animals.
A. The molecular structure of prostaglandins (animals) is similar to JA. The highlighted section (red) of the prostaglandin molecule shown underscores the JA-like component in its structure. **B**. Systemin-mediated long distance wound signaling in *Solanaceous* plants is similar to the proteinaceous cytokine-directed pathogen response in humans. Wounding and pathogen responses are directed by mast cells, which releases TNFα from granules. TNFα is carried through the bloodstream to the brain stem where prostaglandins are synthesized from arachidonic acid (20:4). Prostaglandins trigger defense responses including inflammation and high fever. Modified from Bergey (1996).

moss, *Physcomitrella patens*, contains fatty acids found in both plant (linolenic acid) and animals (arachidonic acid) (Grimsley et al., 1981; Qi et al., 2004).

The wound response in moss has been poorly studied. At the physiological level, mosses do not develop scar tissue in response to injury (Reski, 1998). Recent work has demonstrated that the protonema, or juvenile stage of *P. patens*, is capable of responding to tissue damage (Wichard et al., 2004). Evidence of a wound response in *P. patens* is based on the detection of a *de novo* synthesized mixture of volatile oxylipins from mechanically-damaged protonema. These products are putatively generated from the lipoxygenation of arachidonic acid (Wichard et al., 2004). A lipoxygenase (*PpLOX1*) was later isolated from the protonema stage of *P. patens* (Senger et al., 2005). At the sequence level, PpLOX1 phylogenetically clusters with the lipoxygenases from higher plants, but biochemical characterization of PpLOX1 demonstrates that it resembles animal and algal lipoxygenases. However, a functional role of PpLOX1 in wound response was not demonstrated.

Studies using moss may detail the evolutionary changes that occurred as plants encroached onto land. Comparison of the wound response between moss and higher plants could reveal evolutionarily conserved features, mechanisms that eventually became obsolete or novel aspects of wound response unique to either moss or higher plants. Mosses also offer an interesting opportunity to investigate systemic expression since they lack a true vasculature but still possess a primitive conduit for transport between leaves. Examination of systemic expression could illuminate how multi-cellular plants evolved long-distance signaling from their aquatic unicellular origins. Finally, the functional elucidation of wounding genes can be more directly and efficiently assessed

due to the ease of creating targeted gene knockouts in *P. patens* (Schaefer, 2001). Some features of the wound response appear conserved between higher plants and mosses and defining gene function may be more easily resolved in *P. patens*. In addition, the simple leaf structure offers an easier method to observe the effects using visual tools.

Objectives of this dissertation

This dissertation addresses three important questions regarding the wound response in land plants. The wound response is a composite network of multiple stress responses, but genes committed solely to wounds response are not well known. My goal was to define those components of the wound response which make it unique relative to the pathogen defense response. The isolation and characterization of these wound-specific genes are discussed in Chapter 2. One of those genes, a steroid sulfotransferase, was further characterized by metabolic profiling in RNAi-suppressed lines to determine its functional role in the wound response (Chapter 3). The second section addressed whether any wound-induced genes were expressed strictly in systemic tissues. At the time this work was initiated, known systemic genes were also concomitantly induced in the wounded leaves, but no known systemically-specific genes are reported. Thus, I attempted to isolate systemically-restricted wounding genes (Chapter 4). Finally, the last topic deals with how bryophytes respond to wounding (Chapter 5). Because so little is known regarding stress responses in the lower land plants, I wanted to characterize the wound response in *P. patens* and determine if this response may be different than the wound response in higher plants.

CHAPTER TWO

MATERIAL AND METHODS

Materials

Chemicals were purchased from Sigma® (St. Louis, MO) and Fisher Scientific (Pittsburgh, PA), unless otherwise indicated. Enzymes were obtained from New England Biolabs, Inc® (Ipswich, MA) and Invitrogen™ (Carlsbad, CA). Oligomers were synthesized by Integrated DNA Technologies® (Coralville, IA). A list of the oligomers is included in the Appendix. Plant supplies were purchased from Griffin Greenhouse & Nursery Supplies (Tewksbury, MA).

Protein alignments and dendrogram construction

Protein sequences were aligned with ClustalW .v1.8 and formatted with Boxshade v3.21 (http://www.ch.embnet.org/software/BOX_form.html). For the construction of the evolutionary trees, protein sequences were first aligned with MAFFT v5.8 (http://align.bmr.kyushu-u.ac.jp/mafft/software/) (Katoh et al., 2002; Katoh et al., 2005) and exported to PAUP* v4.0β (Swofford, 2002). A parsimony search was performed on the aligned proteins using a heuristic branch and bound search conditions and equal weighting of characters with gaps regarded as missing data. Support through bootstrap analyses was verified for 100 replicates of the data set.

Arabidopsis Methods

Growth conditions

Arabidopsis thaliana (ecotype Col-O) seeds were sown on Pro-MixB soil (Premier Horticulture LTD; Quebec, Canada) and vernalized in darkness for two days at 4°C. They were then transferred to a growth chamber set at 23°C under continuous fluorescent lighting.

Treatments

Fully-expanded *A. thaliana* leaves from four-week old plants were wounded with forceps along the middle of the leaf, perpendicular to the midvein. The plants were then covered with a dome until collection. For the isolation of wound-responsive genes, aerial tissue from wounded (one leaf wounded per plant) and SA-treated (5 mM) plants were collected at one and three hours after treatment and flash frozen. For the isolation for systemic-specific wounding genes, three leaves were wounded per plant. The wounded leaves and remaining unwounded sections were separately harvested at one, three and six hours post-wounding. Treatments of SA (5 mM) or MeJA (30 μM) were carried out by spraying plants with an aerosol mister.

For the treatments of liquid-grown *A. thaliana* seedlings, sterilized seeds were vernalized for two days in Murashige-Skoog (MS) media (pH 5.8), supplemented with sucrose (3%) and incubated on a shaker (80 rpm). After ten days under a fluorescent lighting regime of 16h light/8h dark, the medium was replaced with fresh MS medium

(pH 5.8) containing sucrose (3%) and either the appropriate hormone (10 μM) or elicitor (150 μg/ml). Seedlings were then collected after two hours on the shaker (80 rpm).

Total RNA and poly-$(A)^+$ RNA extraction

For the subtractive hybridizations, RNA was isolated from vegetative tissues using a large-scale guanidinium thiocyanate (GTC) method (Chomcyznski and Sacchi, 1987). Frozen ground tissue was treated with 50 ml of GTC solution (4M guanidinium thiocyanate, 25mM sodium citrate, 0.5% sarcosyl, 0.1M β-mercaptoethanol, pH 7.0). The mixture was homogenized at full speed using a Polytron® PT3000 (Brinkmann Instruments, Inc) and the debris was pelleted by centrifugation at (13,800 x *g*). The supernatant was collected and treated with 5 ml of 2M sodium acetate (pH 4.0), 50 ml of buffer-saturated phenol and 10 ml of chloroform/isoamyl alcohol (49:1). The samples were vortexed and centrifuged (13,800 x *g*) at 4°C to separate the phases. The overlaying aqueous phase was collected and transferred to a new tube. An equal volume of isopropanol was added and the mixture was stored at -20°C for 1.5 hours. The RNA was pelleted by centrifugation (21,000 x *g*) for 20 minutes at 4°C. To remove carbohydrates and small molecular weight RNA, the RNA pellet was washed with ice-cold 3M sodium acetate (pH 5.2), decanted and then dissolved in ice-cold 0.3M sodium chloride. The supernatant was collected and saved. The insoluble material was re-washed with 3M sodium acetate (pH 5.2) and resuspension repeated with 0.3M sodium chloride. The supernatants were pooled and an equal volume of ispropanol was added before incubation at -20°C and centrifugation (21,000 x *g*) at 4°C for 30 minutes. The purified RNA was washed with 70% ethanol, air dried and resuspended in water.

For northern blot analysis and RT-PCR, total RNA was extracted using Trizol® reagent (Invitrogen). Frozen ground tissue (100 mg) was treated with 500 µl of Trizol® buffer and incubated at room temperature for 10 minutes. Tissue was homogenized and the insoluble material was pelleted by centrifugation (8,161 x *g*) for 5 minutes at room temperature. The supernatant was transferred to a fresh tube and 100 µl of 5M sodium chloride was added followed by 300 µl of chloroform. After vortexing, the mixture was separated by centrifugation (8,161 x *g*) for 5 minutes. The overlaying aqueous phase was transferred to a fresh tube and an equal volume of isopropanol was added. The sample was incubated at room temperature for 10 minutes. The RNA was pelleted by centrifugation (13,379 x *g*) at 4°C for 25 minutes. Following a 70% ethanol wash, the RNA pellet was dried briefly and resuspended in 80 µl of water. To remove genomic DNA and other contaminants, 20 µl of 10M lithium chloride was added and stored at -20°C for 20 minutes. The RNA was precipitated by centrifugation (13,379 x *g*) for 25 minutes at 4°C and then washed with 70% ethanol. The pellet was air dried for 5 minutes and resuspended in water.

For the subtractive hybridization, messenger RNA was purified from GTC-isolated total RNA using the PolyATtract® mRNA Isolation Systems according to the manufacturer's instructions (Promega Corporation, Madison, WI).

Representational Difference Analysis (RDA)

RDA was performed as described (Hubank and Schatz, 1994). For the tester and driver samples, double-stranded (ds) cDNA was synthesized using poly-$(A)^+$ RNA extracted from plant tissues with the Superscript™ Choice System (Invitrogen), per

manufacturer's recommendation. The ds cDNAs were digested with *Dpn*II at 37°C for 2 hours followed by heating at 65°C for 20 minutes to denature *Dpn*II. Ligation of 2 μg of annealed R-oligos (R-12mer/R-24mer) to *DpnII*-digested cDNA was performed overnight at 14°C. The ligations were diluted 3.3-fold in TE (10 mM Tris, pH 7.4, 1 mM EDTA, pH 8.0) and then amplified by bulk PCR (12 x 50 μl reactions) with 1 x PCR buffer, 0.32 mM dNTPs, 10 μg/ml R-24, and 0.5 μl diluted ligation/50 μl reaction volume. The R-12mer was liberated by heating for 3 minutes at 72°C and the overhanging ends filled by heating for 5 minutes at 72°C in the presence of 5 units *Taq* DNA polymerase. The representations (amplicons) were amplified by cycling 20 times for 1 minute at 95°C, 3 minutes at 72°C and with final extension of 10 minutes at 72°C. Amplification resulted in the generation of 0.25-0.50 mg of each amplicon. After phenol/chloroform purification of each sample and sodium acetate/isopropanol precipitation, the driver (240 μg) and tester (20 μg) were digested with *Dpn*II for 4 hours at 37°C and subjected to another round of phenol/chloroform purification and sodium acetate/isopropanol precipitation. The tester cDNAs were electrophoresed on a 1.2% agarose-TBE gel and extracted with Qiaex® II resin (Qiagen Inc; Valencia, CA). The annealed J-oligo (J-12/J-24, 8 μg) was ligated to the gel-purified, *Dpn*II-digested tester cDNAs overnight at 14°C and was diluted three-fold in TE.

For subtractive hybridization, the driver amplicon (40μg) was mixed with J-ligated tester (0.4 μg), extracted with phenol/chloroform, and precipitated with ammonium acetate/EtOH. The pellet was resuspended in 4μl 3X EE buffer (30 mM EPPS, pH8.0; 3 mM EDTA). The driver-tester mixture was denatured for 5 minutes at

98°C and cooled to 67°C whereupon 1 µl of 5M NaCl was added. Heating at 67°C continued for 20 hours and the cDNAs were diluted to 400 µl with TE. The tester was selectively amplified in a 200 µl reaction (1X *Taq* buffer, 0.32 mM dNTPs, 20 µl diluted hybridization mix) by heating at 72°C for 3 minutes, adding 5 units of *Taq* polymerase and continued heating at 72°C for 5 minutes. Primer J-24 (2µg) was added and amplification was performed with 10 cycles of 1 minute at 95°C, 3 minutes at 70°C and an extension of 10 minutes at 72°C. The PCR products were extracted twice with phenol/chloroform/isoamyl alcohol, precipitated with sodium acetate/isopropanol and resuspended in 0.2 x TE (40 µl). The PCR product was digested with 20 units of mung bean nuclease in 1X mung bean nuclease buffer and 20 µl of DNA and incubated for 35 minutes at 30°C. The reaction was stopped by a five-fold dilution with 50 mM Tris-HCl (pH 8.9) followed by heating at 98°C for 5 minutes. Amplification was repeated with 2 µg of J-24 (2 µg), 0.32 mM dNTPs, 1X PCR buffer, 20 µl of DNA. The reaction was heated at 95°C for 1 minute, cooled to 80°C whereupon 5 units *Taq* polymerase were added. The sample was subjected to PCR amplification for 18 cycles, consisting of 95°C for 1 minute, 70°C for 3 minutes and 1 cycle for 10 minutes at 72°C. The samples were combined and extracted twice with phenol/chloroform/isoamyl alcohol and once with chloroform/isoamyl alcohol. The PCR product was precipitated with sodium acetate/isopropanol and centrifuged (13,793 x *g*). The pellet was resuspended in TE at approximately 0.5 mg/ml and designated as difference product one (DP1).

The J-adapters were replaced by digesting with *Dpn*II for 4 hours at 37°C and repeating the phenol/chloroform/isoamyl alcohol extraction and sodium

acetate/isopropanol precipitation. The pellet was resuspended at 0.5 mg/ml in TE. An aliquot (1 μl) was diluted 1:10 (50 ng/μl) in TE and 200 ng of DNA were used for ligation with the N-annealed oligos (8 μg) overnight at 14°C. The ligation was diluted to 1.25 ng DNA/μl and 50 ng of J-ligated DP1 was mixed with 40 μg of driver. Subtraction and amplification steps were repeated as described above for the generation of the second difference product (DP2). The third difference product (DP3) was enriched by diluting 1 μl of J-ligated oligo (1 ng DNA/μl) with 39 μl TE and mixing with 40 μg of driver. The subtraction and amplification was repeated. PCR products were run on 1.3% agarose-TBE gel, excised and purified with Qiaex® II resin.

Subtracted DP candidates were ligated into either pBluescript® II (SK+) (Stratagene; La Jolla, CA) or pZero™ (Invitrogen) as a *Bam*HI-fragment and subsequent ligations transformed into competent *E. coli* (DH5α™, Invitrogen) cells by heat shock and selected on kanamycin- or zeocin-containing LB agar plates, respectively. Colonies were picked from plates and grown in 4 ml of selective media overnight in a 37°c shaker (150 rpm). Plasmids were extracted by alkaline lysis accompanied including a phenol/chloroform purification step (Sambrook and Russell, 2001).

Sequencing

Sequencing of the DP2 and DP3 cDNA fragments was performed manually using Sequenase™ version 2.0 (USB® Corporation, Cleveland, OH)). Sequencing of mini-prepped plasmid DNA was performed in the presence of ^{35}S-dATP using the M13 reverse, T7 or T3 primers**.** Reactions were run on a denaturing 6% acrylamide/bis-acrylamide gel containing 7M urea and 1X TBE. The gel was dried and then exposed to

film at -70°C. Additional sequencing was completed with an ABI Prism® 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) at the DNA Core facilities located at UMDNJ (Piscataway, NJ).

Northern blot analysis and probe radiolabeling

For northern blot analysis, total RNA (10 μg) was denatured by heating at 65°C, size fractionated by electrophoresis in a 1.2% agarose, 0.6 % formaldehyde/MOPS denaturing gel run in 1X MOPS buffer. The RNA was transferred to a Nytran membrane (Schleichel & Schuell®; Keene, NH) by capillary transfer overnight in 20X SSC. The RNA was fixed to the membrane by UV cross-linking (Stratagene).

Probes were radiolabeled with ^{32}P-α-dCTP using random primer labeling. The template DNA (25-50 ng) and random primers (6 μg) were heat denatured by boiling for 5 minutes and then promptly chilled on ice. Klenow 10X buffer (5 μl), 2 μl of 0.5 mM dATP, 0.5 mM dGTP, 0.5 mM dTTP, ^{32}P-α-dCTP (5 μl, 3000 Ci/mmol) and *E. coli* DNA polymerase I (2-4 units) were added and incubated for 1 hour at 37°C. The reaction was stopped by the addition of 1 μl of 0.5M EDTA and heating at 75°C for 15 minutes. Unincorporated label was removed by centrifugation (735 x *g*) through a Sephadex G50 spin column (GE Healthcare; Piscataway, NJ).

Hybridizations of radiolabeled probes to membranes were based upon the Church method (Church and Gilbert, 1984). The blots were pre-hybridized at least 1 hour at 65°C in hybridization buffer (1% BSA, 1 mM EDTA, 0.5 M $NaHPO_4$ (pH 7.2), 7% SDS). The radiolabeled probe was denatured by boiling for 5 minutes and added to the hybridization buffer (10^6cpm/ml). The blot was hybridized overnight at 65°C. The blots

were washed twice for 5 minutes at 65°C in 0.5% BSA, 1 mM EDTA, 40 mM NaHPO4 (pH7.2), 5% SDS and then four times for 5 minutes at 65°C in 1mM EDTA, 40 mM NaHPO4 (pH 7.2), 1% SDS. Blots were exposed to film in -70°C.

RT-PCR

The synthesis of first-strand cDNA with Superscript™ II was performed as outlined. For *A. thaliana*, 5 µg of total RNA was primed with 500 ng of oligo$(dT)_{12-18}$ and heated with 1 µl of 10 mM dNTPs at 65°C for 5 minutes. After quick chilling for 2 minute on ice, 4 µl of 5X first strand buffer, 2 µl of 0.1 M DTT, 1 µl RNaseOUT™, and 200 units of Superscript II™ were added. The reaction was incubated at 42°C for 50 minutes and then inactivated for 10 minutes at 70°C.

For PCR, 0.4 µl of first-strand cDNA was added in a reaction volume of 10 µl containing 1X Taq polymerase buffer, 0.2 mM dNTPs, 0.2 mM of each primer, and 0.5 units of *Taq*. Products were electrophoresed on 1.2% TBE agarose gels and visualized with a Bio-Rad Gel Doc™ 2000 (Bio-Rad Laboratories Inc; Hercules, CA). Quantitation of bands was determined relative to loading controls using Scion Image software (ver. alpha 4.0.3.2) (Scion Corporation; Frederick, MD).

Generation of RNAi-silenced *SST* plants

Gene silencing of *SST* (At5g07010) was carried out using the pHANNIBAL RNAi vectors (Wesley et al., 2001; Helliwell and Waterhouse, 2003). A 329 bp cDNA representing the RDA-subtracted fragment of *SST* was amplified with flanking primers introducing *Xba*I-*Xho*I (5'-ATC TAG ACT CGA GGA TCC CAA GTT TCT CCA TGT-

3') recognition sites on one end and *Kpn*I-*Cla*I (5'-AAT CGA TGG TAC CGA TCA AAC CGA TAA CGG TTA-3') on the opposite. A PCR fragment was generated in a reaction containing 10 pg of the cloned *SST* fragment, 1X *Taq* buffer, 0.4 mM dNTPs, 1 μM each of primer, and 1 unit of *Taq*. The amplification conditions were: 94°C 5 minutes, 30 cycles of 94°C for 30 seconds, 65°C for 30 seconds, 72°C for 30 seconds and 1 cycle of 72°C for 7 minutes. An *Xho*I-*Kpn*I digested PCR product forming one end of the stem loop was first introduced into a *Xho*I-*Kp*nI digested pHannibal. The antisense arm was then ligated as an *Xba*I-*Cla*I digested PCR product. The *SST* silencing construct was released from pHANNIBAL with *Not*I and shuttled into binary vector pART27 as a 3.7-kb cassette.

The vectors were introduced into *Agrobacterium tumefaciens* (strain GV3101) by freeze-thaw method and transformed into *A. thaliana* with Silwett L-77 (OSi Specialties, Inc; Danbury, CT) (Clough and Bent, 1998). Transgenic plants were selected on kanamycin (50μg/ml)-containing MS plates supplemented with 0.25% Phytagel™ (Sigma). After 10 days, kanamycin-resistant seedlings were transferred to soil.

Extraction of Sterols from Plant tissues

For metabolic profiling of wounded plants, steroid extraction from *A. thaliana* tissues was partially based on extant methods for brassinosteroid extraction with modifications (Noguchi et al., 1999). Frozen ground tissue (2 g) of the unwounded and wounded leaves from wild-type and the transgenic *SST*::RNAi plants was treated with 20 ml of methanol: chloroform (4:1) and homogenized. The tissue was placed on a shaker overnight at room temperature. The debris was pelleted and the supernatant was dried by

vacuum. The dried material was resuspended in 8 ml of water. An equal volume of hexane was added and then vortexed. Phase separation was achieved by spinning at 4°C at 3750 X g for 15 minutes. The lower (aqueous) phase was collected and 8 ml of butanol was added to this aqueous phase. The samples were vortexed and spun. The overlaying butanol partition was removed and dried in by vacuum. The dried samples were weighed and resuspended in MeOH/chloroform (4:1) at concentration of 10 mg/ml.

Extracts were separated and examined with a Waters LC-MS Integrity™ system (Milford, MA) composed of a solvent delivery system, comprising of a W616 pump and W600S controller, W717plus auto-sampler, W996 PDA detector and a Waters TMD Thermabeam™ electron impact (EI) single quadrupole mass detector. Data was gathered & analyzed with software (Waters Millennium® v3.2) coupled to the Wiley Registry of Mass Spectral Data (6th edition). Compounds were separated through a Phenomenex® Luna C-8 reverse phase column (Torrance, CA) size 150x2 mm, particle size 3 μm, pore size 100 Å, outfitted with a Phenomenex® SecurityGuard™ pre-column. The mobile phase was composed of 2 constituents: Solvent A (0.5% acetic acid in double distilled de-ionized water, pH 3-3.5), and Solvent B (100% acetonitrile). The flow rate for the mobile phase was regulated at 0.25 ml/min and typically a gradient mode was employed for each analysis. The gradient points were designated for time: 0 min. – 95% A and 5% B; time 25 min. – 5% A and 95% B; sustained isocratic for 2 minutes, 27 to 30 min. – returned to original conditions of 95% A and 5% B. A column equilibration time of 15 minutes was established between successive injections.

Genomic DNA isolation and Southern Blot

Frozen ground *A. thaliana* tissue (1g) was treated with 5 ml of UEB extraction buffer (7M urea, 30mM NaCl, 50mM Tris-HCl, pH8; 2mM EDTA, pH8; 1% sarcosyl) and mixed thoroughly. An equal volume of phenol:chloroform (1:1) was added and the mixture was shaken. The mixture was spun down and the aqueous phase was collected. Isopropanol (5 ml) was added and then spun to pellet the DNA. After discarding the supernatant, the pellet was treated with 0.7 ml TE supplemented with RNaseA (20 μl/g). The solution was spun to remove the debris. To the supernatant, 72 μl of 3M sodium acetate and 0.72 ml isopropanol were added and then mixed. The genomic DNA was pelleted by spinning at 8000 rpm for 5 minutes. The pellet was then washed with 80% EtOH. The genomic DNA was gently removed and transferred into a fresh tube and dried. The genomic DNA was resuspended in TE.

For Southern blotting, genomic DNA (1 μg) was digested for 6 hours with restriction enzymes and then loaded onto a 0.7% agarose-TBE gel. The digested genomic DNA was size fractionated overnight by electrophoresis. The agarose gel was then immersed in 0.25 HCl for 10 minutes with gentle agitation. After rinsing in water, the gel was soaked in 1.0M NaCl, 0.5M NaOH twice in 15 minutes intervals and then neutralized in 0.5M Tris-HCl, pH7.4/1.5M NaCl. Transfer of the genomic DNA occurred overnight onto Nytran using 10X SSC. The DNA was immobilized onto the membrane by UV cross-linking. Probe preparation and hybridization conditions were as described with northern blotting.

P. patens Methods

Propagation

Culture techniques for moss were based on (Schaefer et al., 1991). Homogenized protonema of *Physcomitrella patens* (Gransden) was inoculated onto cellophane discs set on $PpNH_4$ medium supplemented with ammonium tartrate (2.7 mM) and agar (7%). Protonema and gametophore were cultured at 24°C under 16h light/8h dark regime. Gametophore plants were initiated from two-week old protonema cultures by removing and directly implanting individual gametophores into fresh $PpNH_4$ agar media.

Treatments of *P. patens*

The leaves of four to six-week old *P. patens* gametophore were wounded by cutting off the tips of gametophore leaves with a double-edged razor blade. Approximately half of the total leaf area was removed. Each wounded gametophore plantlet had about one-third of its leaves wounded. The wounded gametophores were harvested at the designated times and 35 to 50 wounded gametophores were collected for each time point. For MeJA treatment of *P. patens*, several drops of 10 μM MeJA were applied directly unto the aerial portion of each implanted gametophore.

RNA isolation and first-strand cDNA synthesis

Isolation of total RNA from *P. patens* was similar to the extraction from *A. thaliana* with noted differences. Approximately 125 mg of frozen ground gametophore tissue was used for each RNA sample. Frozen tissue was homogenized with an Arrow 1750 electric stirrer (Arrow Engineering Co, Inc; Hillside, NJ) outfitted with a pellet

pestle (Kimble/Kontes; Vineland, NJ) Total RNA was extracted using the Concert™ Plant RNA reagent (Invitrogen) as per the manufacturer's directions. Further purification to remove genomic DNA and impurities was performed by an additional lithium chloride precipitation. Generation of first-strand cDNA from total RNA isolated from *P. patens* was similar except 0.2-1 μg of total RNA was used as template for cDNA synthesis.

Fluorescein diacetate (FDA) and aniline blue staining

For cell viability studies, wounded leaves were detached from the main body of the gametophore. Wounded leaves were then stained with FDA solution (0.01% w/v) for 5 minutes and then observed by fluorescence illumination with a Nikon EF-D Optiphot epifluorescence microscope fitted with a blue light, excitation filter 450-500 nm and a dichroic 495 nm, barrier 525 nm.

Callose was detected by staining with aniline blue. *P. patens* gametophore leaves were wounded for at least 12 hours before overnight fixation in FAA solution (5% formaldehyde, 5% glacial acetic acid, 50% ethanol). After several washes with water, the leaves were then stained overnight with aniline blue solution (0.01% aniline blue in 0.07M Na_2HPO_4, pH9). Callose was detected with an epifluorescent microscope with an excitation filter 420-490 nm.

CHAPTER THREE

ISOLATION AND CHARACTERIZATION OF WOUND-SPECIFIC GENES FROM *ARABIDOPSIS THALIANA*

Introduction

Wounding of *A. thaliana* vegetative tissues triggers a multifaceted response requiring gene activation to execute a variety of functions (Reymond et al., 2000; Cheong et al., 2002). These wound-related genes are involved in various processes including wound healing, mounting a defense response against opportunistic pathogens, coping with desiccation, dispatching wound signals to the undamaged parts and re-allocating energy and resources to subsidize these processes (Bostock and Stermer, 1989; Reymond et al., 2000; Leon et al., 2001; Matsuda et al., 2003; Quilliam et al., 2006). JA is the principal signal mediating the wound response in higher plants (Leon et al., 2001; Howe, 2004). However, exquisite fine-tuning of the relationships among the competing and cooperative signaling pathways is required to orchestrate an appropriate response to wounding (Reymond and Farmer, 1998; Knight and Knight, 2001; Pieterse et al., 2001; Kunkel and Brooks, 2002; Delker et al., 2006). For example, salicylic acid (SA), the signaling compound responsible for pathogen defense response in higher plants, is an antagonist of JA-mediated signaling (Doherty et al., 1988; Pena-Cortés et al., 1993; Doares et al., 1995b; Reymond and Farmer, 1998; Spoel et al., 2003). We used a subtraction hybridization technique, called RDA (representational difference analysis), to identify novel wound-specific genes from *A. thaliana* by exploiting the suppressive effects of SA on JA signaling (Hubank and Schatz, 1994). Consequently, an

anthocyanidin synthase (*ANS*), steroid sulfotransferase (*SST*), cytochrome P-450-like gene (*CYT*), glutathione-*S*-transferase (*AtGST17*), lipoxygenase (*LOX2*) and tyramine *N*-hydroxycinnamoyl transferase (*THT*) were isolated from wounded tissues. Only *ANS*, *SST*, and *THT* demonstrated both wound induction and insensitivity to SA. Based on MeJA treatment and expression analysis in JA signaling mutants, JA is sufficient and necessary for the wound activation of *ANS*, *SST* and *THT*. Finally, expression for *ANS*, *SST* and *THT* is observed in the injured leaves and the unwounded distal tissues.

Results

Strategy using RDA to isolate wound-specific genes

Several criteria were important in designing a strategy to isolate wound-specific genes. The strategy should lead to the discovery of novel wound-specific genes. Although microarray technology depicts the large-scale changes in gene expression and assists in the identification of novel functions for genes, this approach nonetheless has limitations. The earliest designer cDNA arrays were limited in efficacy due to their bias in the selection of representative genes affixed on the chip (Reymond et al., 2000). As a result, unknown genes and genes presumably unassociated with the wound response were not excluded. This limitation is now remedied with the whole genome chip, which permits global evaluation of the changes in gene expression. However there are additional complications associated with the use of the whole genome arrays. For smaller labs, the cost of these large-scale gene expression studies using a genome chip is often prohibitive. In addition, initial gene identification was based upon an outdated annotation, which is continuously being amended (Bennetzen et al., 2004; Brent, 2005).

Thus, the inventory of genes affixed on the genome chip may not reflect current gene annotation. More importantly, large-scale experimental techniques may neglect rare transcripts, small genes or genes that do not encode proteins (e.g. snRNAs, miRNAs). Weakly-induced genes are overlooked in microarray experiments and by other gene discovery techniques because their levels of up-regulation are below the cutoff for significance, commonly used in such experiments (typically >1.5-2.0 fold above basal levels). Such genes nonetheless may play an important role in the wound response, irrespective of their modest induction since they may represent wound-specific genes. Therefore, the isolation strategy must be sufficiently sensitive to detect and isolate the rare wound-specific genes.

RDA is a subtractive hybridization technique that offers a highly sensitive method (Hubank and Schatz, 1994; Lisitsyn, 1995) that satisfies these criteria because it enriches for transcripts characteristic to a particular tissue of interest (tester). Tissue where the genes-of-interest are absent (driver) is used as a comparative sample during the subtraction hybridization steps. After three rounds of subtractive hybridization using progressively increasing concentrations of driver, a final 40,000-fold enrichment of genes native to the tester is expected (Hubank and Schatz, 1994). RDA also offers a more sensitive method of isolating the rare tester-specific transcripts due to the integration of PCR into the subtraction protocol. Therefore, RDA was adopted to furnish a collection of wound-specific genes from *A. thaliana*.

The selection of appropriate sources for tester and driver was also critical. Tissue from wounded *A. thaliana* (ecotype Col-O) plants was used to generate the representation of tester-specific genes. JA-treated tissue was not considered a viable option as a tester

because wounding also activates a JA-independent wound-response pathway (Titarenko et al., 1997). In addition to wound response, JA serves functional roles in development and defense response against necrotrophic pathogens (Staswick, 1992; Creelman and Mullet, 1995, 1997; Staswick et al., 1998; Vijayan et al., 1998). Therefore, a JA-derived tester not only would lack the full complement of wounding genes but also contain JA-induced genes not associated with tissue damage. Therefore, the tester was generated from wounded *A. thaliana*.

Mechanical damage of *A. thaliana* leaves induces at least 650 transcripts, many of which have been implicated in other stress responses including pathogen resistance (Cheong et al., 2002). As a result, the capability of distinguishing novel wound-specific genes may be complicated by the activation of these non-wound specific genes. We anticipated that using stressed tissue as a driver would selectively remove these multifunctional stress-response genes from the tester. A second reason for a SA-treated driver takes advantage of the observed SA-mediated suppression of JA-regulated responses (Doherty et al., 1988; Doares et al., 1995b; Reymond and Farmer, 1998; Spoel et al., 2003). We predicted that detection of some wound-induced genes in the tester may be enhanced during the subtraction process if their expression is inhibited in the SA-treated driver. This may especially contribute in isolating those rare wound-specific transcripts.

Identities of subtracted cDNA from DP2 and DP3

The tester representation was generated from four- to six-week old *A. thaliana* plants that were collected at one and three hours after wounding. Wounding was

executed mechanically using forceps to crush a section of a leaf perpendicular to the vasculature. The wounded section accounted for approximately 20% of the leaf area. For the tissue used for RDA subtraction, one leaf was wounded per plant. The entire aerial sections, including the remaining undamaged leaves, were harvested. For the driver, plants were sprayed with SA (5 mM) and also collected at one and three hours after treatment. From the driver and tester tissues, poly-$(A)^+$ RNA was isolated and converted to double-stranded cDNA with Moloney Murine Leukemia Virus (MMLV). The tissue was processed for RDA as described (see Chapter2-Methods).

From the DP2 round of subtracted cDNA, 21 different genes from 45 sequenced inserts were isolated (Table 1). Due to the large complexity of the DP2 population, identification of the sequencing inserts was limited to 45 cloned cDNA inserts. A fragment encoding a putative anthocyanidin synthase (*ANS*) (At2g38240) was most frequently encountered, accounting for 15 of the 45 sequenced inserts (30%). A lipoxygenase (*LOX2*) gene was the second most prevalently sequenced in six out of the 45 inserts (13.3%). Most genes were represented once (2.2%) in the subtracted library, but singletons cumulatively accounted for 16/45 (35.6%) of the total sequenced population. Four of the DP2 genes are implicated in abiotic stress responses (*COR15A*, *AtGSTF10*, *RD19*, and *HSC70-1*) (Wu et al., 1988; Yamaguchi-Shinozaki et al., 1992; Kiyosue et al., 1993; Baker et al., 1994) whereas six genes participate in metabolism or metabolic salvaging (*FNR*, *CAB*, *FBA*, *ATR1*, and *EF1*α,) (Bender and Fink, 1998; Lucinski et al., 2006; Lintala et al., 2007). The *LOX2* gene is involved in JA biosynthesis and the function of *OPR1* is not clear although an isoform of *OPR* (*OPR3*) is required for JA biosynthesis (Bell and Mullet, 1991, 1993; Biesgen and Weiler, 1999). The *PRP4*

Subtracted DP2 cDNA	*n*	Locus Tag	Function
Anthocyanidin synthase (ANS)	15	At2g38240	2-oxoglutarate dependent dioxygenase
Lipoxygenase (LOX2)	6	At2g38240	JA biosynthesis
Cold-regulated protein (Cor15a)	3	At2g42540	cold-regulated, chloroplast
Chlorophyll a/b protein (CAB)	3	At1g61520	photosynthesis, chloroplast
AtGSTF10 (ERD13)	1	At2g30870	dehydration-induced gene
AtGST17 (ERD9)	2	At1g10370	early-responsive to dehydration
Ferredoxin-NADP+ reductase (FNR)	1	At5g66190	electron transport, chloroplast
polyubiquitin (UBQ11)	1	At4g05050	protein catabolism
heat shock protein (HSC70.1)	1	At5g02500	responds to heat and virus
Synaptobrevin (STB)	1	At2g33120	vesicle-associated membrane, transport
Fructose-bisphosphate aldolase (FBA)	1	At4g38970	pentose phosphate shunt, chloroplast
Cysteine protease (RD19)	1	At4g39090	drought and salt stress inducible
Germin-like protein (GER1)	1	At1g72610	exported to extracellular matrix
Guanine nucleotide exchange protein (GNE)	1	At3g43300	vesicle trafficking
ATR1 (MYB34)	1	At5g60890	transcription factor, Trp biosynthesis
12-oxophytodienoate reductase (OPR1)	1	At1g76680	upregulated by senescence and JA
Ein-binding F-box protein2 (EBF2)	1	At5g25350	part of SCF complex, represses ethylene
Elongation Factor 1-alpha (EF1α)	1	At5g60390	translation elongation factor
Proline-rich protein (PRP4)	1	At4g38770	localized to cell wall
AtRER1C1	1	At2g23310	ER protein, protein localization
Unknown protein	1	At4g15630	integral protein

Table 3.1 Collection of cDNAs isolated from DP2 round of subtraction.
Inserts from cloned DP2 inserts were sequenced and identified from BLAST searches of the *Arabidopsis* genome. *n*-number of times the cDNA was recovered in the sequenced population.

gene encodes a proline-rich protein which localizes to the cell wall of growing aerial tissues (Fowler et al., 1999). Two genes are involved in ubiquitin-mediated protein degradation (*UBQ11*, *EBF2*) (Sun and Callis, 1997; Potuschak et al., 2003). Three of the genes function in transport (*AtRER1C1*, *GNE, STB*) (Sato et al., 1999).

Three rounds (DP3) of enrichment generated six unique cDNA fragments from a total of 34 clones (Table 2). Based on the degree of heterogeneity in DP2 and DP3, a nearly three-fold decrease (from 21/45 (46.7%) to 6/34 (17.6%)) in heterogeneity in the transition from second to third rounds of subtractive hybridization was observed. As in DP2, *ANS* was the most highly represented cDNA (22/34, 64.7%) in DP3, having undergone a greater than two-fold enrichment from DP2 (30%). Three subtracted genes of the DP3 population (steroid sulfotransferase (*SST*), cytochrome P450 mono-oxygenase (*CYT*), and tyramine *N*-hydroxycinnamoyl transferase (*THT*)) were not uncovered during sequencing of DP2. A GST (*AtGST17*) was represented in both second (4.4%) and third rounds (5.5%) with minimal enrichment. Lipoxygenase (*LOX2*) was less enriched (2.9%) in the DP3 than in DP2 (13.3%). *LOX2* is a known wound-induced gene and therefore served as an internal wound control for subsequent experiments (Bell et al., 1995). The reduction of *LOX2* during the transition from DP2 to DP3 may have been a consequence of the low expression of *LOX2* in SA-treated plants. As a result, presence of *LOX2* transcripts in the driver may have effectively removed some of the *LOX2* from the tester during subtraction. The sequencing effort was then ceased because the four cDNAs (ANS, CYT, THT, GST17) represented the majority of the sequenced inserts (32/34)

Gene	Symbol	*n*	Biochemical Function	Locus Tag
Anthocyanidin synthase	ANS	22	2-oxoglutarate dependent dioxygenase	At2g38240
Steroid sulfotransferase	SST	1	Sulfonates steroids by transferring SO_3^- from PAPs to a steroid	At5g07010
Tyramine transferase	THT	5	HydroxycinnamoylCoA:tyramine *N*-hydroxycinnamoyl transferase	At2g39030
Cytochrome P450-like protein	CYT	3	Terminal oxidases in multi-component electron transfer chain	At3g48520
Glutathione-S-transferase	GST17	2	Conjugates glutathione to an acceptor molecule	At1g10370
Lipoxygenase	LOX2	1	Introduces molecular oxygen into fatty acids	At3g45140

Table 3.2 List of subtracted cDNAs from the DP3 round.
Inserts from cloned DP3 fragments were sequenced and identified from BLAST searches of the *Arabidopsis* genome. *n*-number of times the cDNA was recovered in the sequenced population.

whereas the singletons (*SST*, *LOX2*) were represented by a low proportion (2/34). This suggested that a relative level of homogeneity was achieved by the DP3 round and that discovery of additional novel genes would be extremely unlikely without rigorous sequencing.

Validation of the wound-specificity of DP3 transcripts by northern blot analysis

To validate that the subtracted cDNAs represent genuine wound-specific transcripts, northern blot analysis was performed on *A. thaliana* plants that were either mechanically damaged or spray-treated with SA (5 mM) in a time-course experiment. Total RNA was isolated from treated plants, size fractionated on a denaturing agarose gel and transferred to nylon membrane. The subtracted cDNAs representing the DP2 and DP3 rounds were radiolabeled with α^{32}P-dCTP by random-primed labeling and used as probes.

Northern blot analysis demonstrated that nearly all of the DP3 genes were induced by wounding in *A. thaliana* leaves (Fig. 1). Within the first three hours after wounding, accumulation of the DP3 transcripts was detectable. Three of the cDNAs (*ANS*, *SST*, and *THT*) were both discernibly induced by wounding and not up-regulated in SA-treated plants. The timing of wound induction was consistent with the original time points (one and three hours post-wounding) for the collection of wounded and SA-treated plants for RDA. The *AtGST17* transcript was triggered by wounding but also transiently activated by SA treatment. Accumulation of the *CYT* transcript was extremely weak in wounded tissues and difficult to confirm consistently by northern blot analysis. This suggested that the RDA subtraction was not entirely reliable in isolating wound-specific genes.

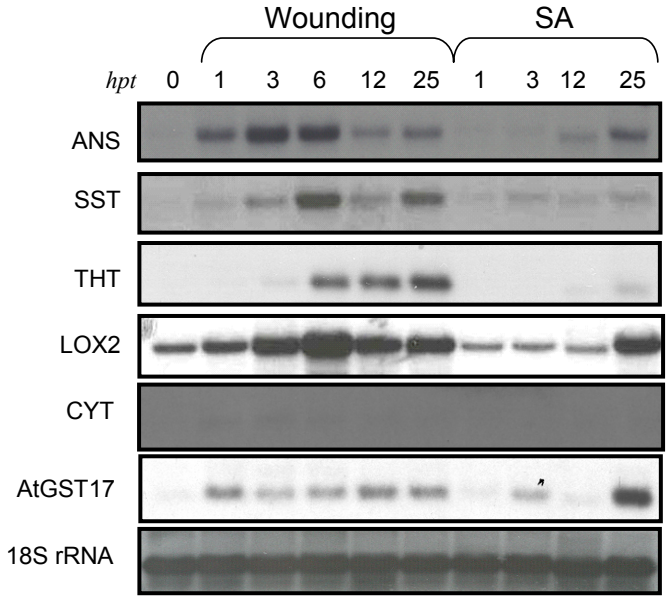


Figure 3.1 Expression of the subtracted DP3 genes in wounded and SA-treated *Arabidopsis* plants. Wounded or SA (5mM)-treated plants were harvested in a time-course assay. Approximately five micrograms of total RNA was loaded per sample. Loading was determined by 18S rRNA. *hpt*-hours post-treatment.

Prolonged exposure (25 h) to SA in *A. thaliana* eventually activated some of the DP3s. This could represent an indirect effect stemming from elevated levels of and prolonged exposure to SA which in turn triggers JA-regulated defense responses. Some wound-induced genes are also characterized by delayed activation by SA (Yamada et al., 2004).

The expression of the internal control, *LOX2*, peaked by 6 hours after wounding. Exposure to SA may have suppressed the expression of *LOX2* as the level of *LOX2* transcripts in SA-treated plants fell below the basal levels observed in untreated *A. thaliana* plants. SA has been shown to suppress transcription of JA biosynthetic enzymes (Pena-Cortés et al., 1993; Spoel et al., 2003). With the exception of *LOX2*, basal expression of DP3 genes was not detectable in the undamaged control plants. Thus, the *de novo* accumulation of *ANS*, *SST* and *THT* transcripts in wounded plants coupled with their insensitivity to SA indicate that these genes may represent wound-specific genes.

Validation of the wound-specificity of DP2 by northern blot analysis

Wounded and SA-treated *A. thaliana* plants were also examined for expression of DP2 cDNAs. Six of the DP2 cDNAs were randomly selected as probes for northern blot analysis. The results from northern analysis demonstrated that some of the DP2s were differentially regulated by wounding relative to SA treatment (Fig. 2). Several transcripts were up-regulated relative to the unwounded levels (e.g. *STB*, *HSC70.1* and *RD19*) but three transcripts (*GER1*, *FBA* and *CAB*) did not appear to be influenced by either wounding or SA treatment. The probe encoding the cysteine protease (*RD19*) detected two transcripts suggestive of splice number variants. Both *RD19* transcript isoforms

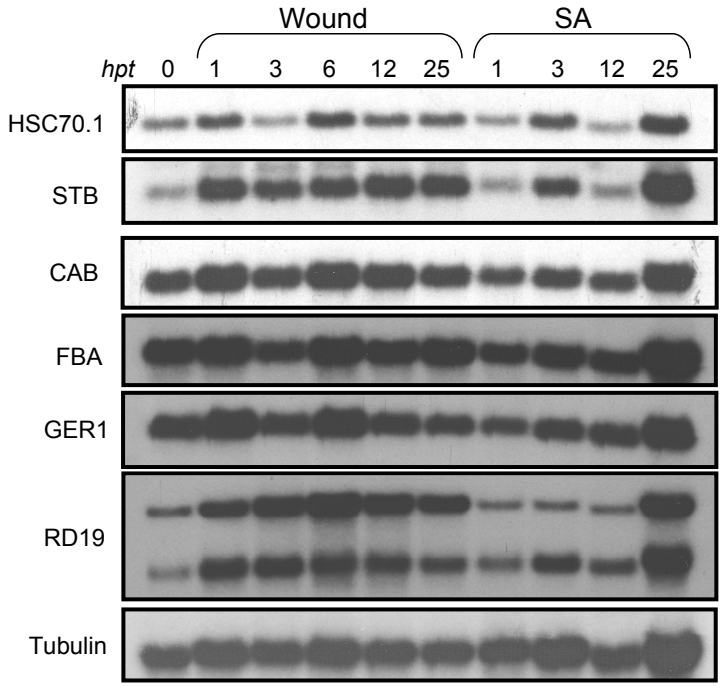


Figure 3.2 Expression of selected DP2 genes in wounded and SA-treated *Arabidopsis* plants. Wounded or SA (5mM)-treated plants were harvested in a time-course assay. Approximately five micrograms of total RNA was loaded per sample. Tubulin was used to determine loading discrepancies between samples. *hpt*-hours post-treatment.

behave similarly, showing a progressive increase in *RD19* transcripts after wounding. These results show that wound-regulated genes were also constituents of the second round of subtraction and that additional putative wound-induced transcripts could be potentially extracted from the more diverse DP2 population.

There were significant differences in gene expression profiles between genes isolated in DP2 and DP3. Genes selected in the DP3 population (*ANS*, *SST*, *AtGST17* and *THT*) were undetectable in unwounded tissues (Fig. 1); in contrast, genes selected in the DP2 exhibited minor or significant expression in unwounded plants (Fig. 2). In addition, wound-induced DP2 (*HSC70.1*, *STB*, and lower *RD19* isoform) genes were activated and peaked by 1 h while the expression of wound-induced DP3 genes (*ANS*, *SST*, *THT*, and *LOX2*) gradually increased and peaked at 6 h. Expression of DP2 genes was also detected in the SA-treated plants within an hour after treatment, whereas DP3 genes showed little or no expression in response to SA (except for *AtGST17*). This indicates that there was a significant enrichment for wound-specific genes in the tester-specific library between the DP2 and DP3 rounds of subtraction. By the third round of subtractive hybridization, most genes that were expressed in SA-treated plants were eliminated.

Role of the JA signaling pathway in DP3 expression

The JA biosynthetic pathway plays a pivotal role in the wound response of higher plants (Leon et al., 2001; Howe, 2004). Mutants compromised in JA biosynthesis fail to respond to wounding and are consequently more susceptible to herbivory (McConn et al., 1997). Exogenous JA also positively regulates the expression of the JA biosynthetic

enzymes (Reymond et al., 2000; Stenzel et al., 2003b; Cruz Castillo et al., 2004). To examine whether wound activation of DP3 genes was regulated by JA, *A. thaliana* plants were treated with MeJA (30 μM) and tested in a time course experiment for DP3 induction by northern blot analysis.

Exogenous treatment with MeJA triggered the expression of the *ANS*, *SST* and *THT* genes (Fig. 3). Upregulation was observed within three hours of the treatment. The timing of this up-regulation by MeJA emulated the induction by wounding. Some differences were observed between wound- and MeJA-induction of *ANS*, *SST* and *THT*. Activation by MeJA was weaker than that triggered by wounding and the kinetics of expression behaved differently. This suggests that additional MeJA-independent factors may contribute to the nature of wound induction. The internal control, *LOX2*, was also strongly induced by MeJA activation. Its accumulation indicated that there was effective uptake of the MeJA solution by the treated plants and represents positive activation of the JA pathway.

Control plants sprayed with water also showed a partial induction of *LOX2* transcripts by ten hours after treatment. This mild induction in control plants may be due to the touch response, a mechanical stress associated with application of water droplets (Braam and Davis, 1990; Braam, 2005), which is known to activate a number of genes that are also wound-regulated. This touch-induced activation was not responsible for the activation of *ANS*, *SST* and *THT* transcription, whose inductions preceded the touch-specific accumulation of *LOX2* transcripts. These results indicate that the JA signaling pathway may play a regulatory role in the wound-specific induction of *ANS*, *SST* and

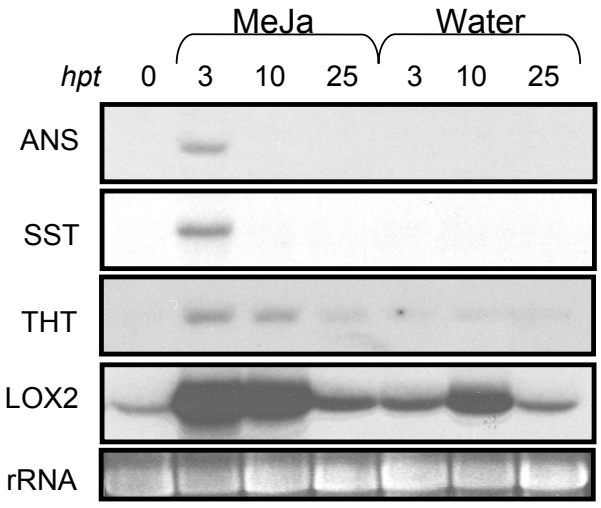


Figure 3.3 Activation of DP3 genes in *Arabidopsis* plants treated with MeJA.
Plants were sprayed with 30 μM MeJA or water control and then harvested in a time-course assay. Total RNA was extracted from tissues and loaded at approximately ten micrograms per lane. Loading was assessed by quantifying rRNA determined by ethidium bromide stained gels. *hpt*-hours post-treatment.

THT. Moreover, the comparable rate of *ANS*, *SST* and *THT* induction by either MeJA or wounding suggests that their coordinate wound activation is regulated by JA.

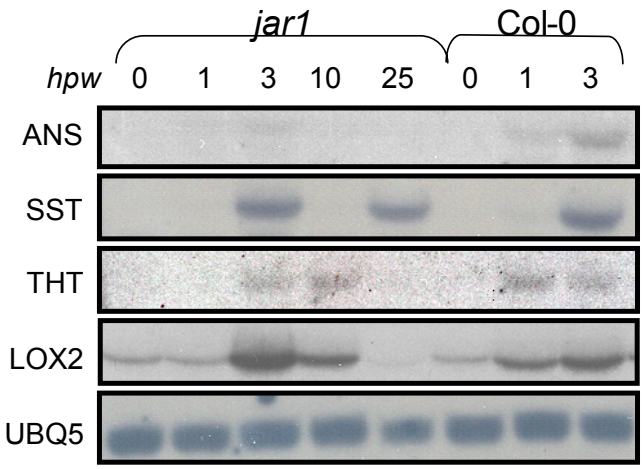
Response of DP3 genes in the jar1 *mutant*

To confirm the dependence of *ANS*, *SST* and *THT* on JA signaling for wound induction, their expression was tested in a JA-insensitive mutant, *jar1* (Staswick et al., 1992). The wild-type *jar1* gene encodes an acyl adenylate-forming enzyme that activates JA for conjugation to amino acids (with preference for Ile) and may function in *A. thaliana* to regulate endogenous JA levels (Staswick et al., 2002; Staswick and Tiryaki, 2004). The JA-Ile conjugate may also possess intrinsic bioactivity and may be involved in wound signaling. The expression of *ANS*, *THT*, and *SST* was monitored by northern blot analysis in wounded and MeJA-treated *jar1* mutant plants to determine if the induction of DP3 expression was dependent on the functional *JAR1* gene product.

In wounded *jar1* plants, the wound-specific activation of *ANS*, *SST* and *THT* expressions mimicked the induction of these genes in wounded wild-type plants (Fig. 4A). Induced transcripts in both the wild-type and *jar1* plants were observed by three hours after mechanical wounding. Moreover, the expression levels in the *jar1* mutant and wild-type plants were approximately equal, although *ANS* expression was somewhat reduced in the *jar1* mutant. The internal control for JA-responsive genes, *LOX2*, was also robustly induced in the wounded *jar1* mutant. These results indicated that the *JAR1* gene may not regulate the wound-induced expression of *ANS*, *SST*, and *THT*.

The exogenous treatment of *jar1* plants with MeJA also triggered the weak expression of *ANS*, *THT*, and *SST* (Fig. 4B). However, the extent of accumulation in the

A



B

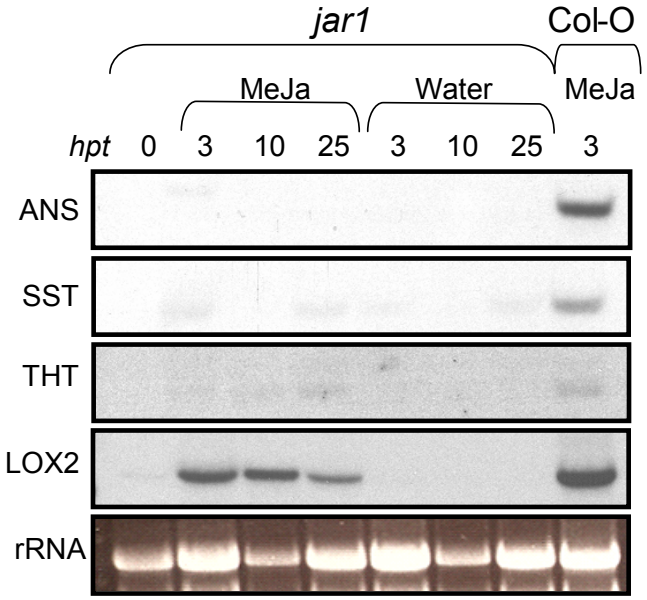


Figure 3.4 Expression of DP3 genes in wounded and MeJA-treated *jar1* mutants. **A.** Wild-type and *jar1* mutant plants were wounded. Tissue was harvested at the times indicated. Ten micrograms of total RNA was loaded per sample. Ubiquitin (*UBQ5*) expression was used to monitor loading. **B**. Wild-type and *jar1* mutants were treated with MeJA (30μM) or water. Loading was measured with ethidium bromide-stained rRNA. *hpw*-hours post wounding, *hpt*-hour post-treatment.

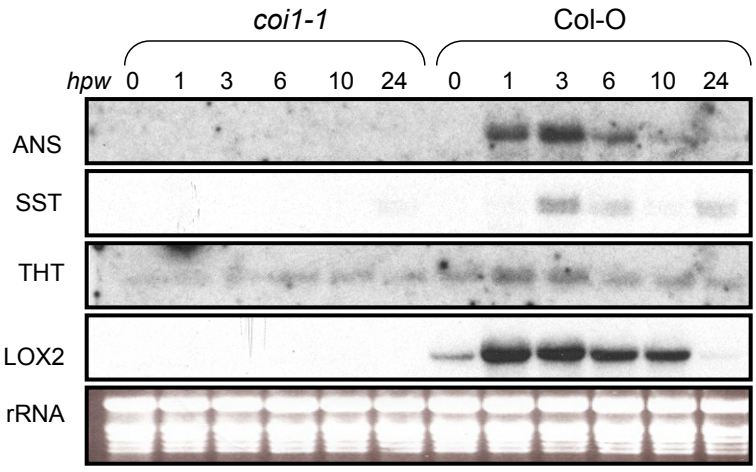
MeJA-treated *jar1* mutant was diminished relative to the MeJA-treated wild-type plants. The timing of MeJA induction was also unaffected in the mutant. This result suggests that the response of the *ANS*, *SST*, and *THT* genes to MeJA may be partially regulated by JAR1 signaling. Alternatively, there may be additional signaling pathways that operate during the wound response which can compensate for suboptimal efficacy of the MeJA/*JAR1* mediated pathway.

Expression of DP3 genes in the coi1-1 *mutant*

The *JAR1* gene regulates a subset of JA-dependent responses. The *COI1* gene, on the other hand, appears to govern all JA-dependent responses (Xie et al., 1998). The *coi1-1* mutant was originally isolated as a coronatine-insensitive mutant (Feys et al., 1994). *COI1* encodes an F-box protein, which is a component of the SCF complex (Feys et al., 1994). The SCF complex is involved in the selective degradation of regulatory proteins modified by ubiquitination (Smalle and Vierstra, 2004; Lechner et al., 2006). To ascertain the requirement of JA signaling for wound-specification of the DP3 genes, *coi1-1* plants and wild-type plants were wounded in a time-course assay. Expression of DP3 transcript levels was assessed by northern blot analysis.

Wounding of the vegetative leaves of *coi1-1* mutant plants failed to activate the expression of *ANS*, *SST* and *THT* (Fig. 5A). The *LOX2* internal control was not up-regulated by mechanical damage in *coi1-1* plants. This result suggested that the activation of *ANS*, *SST* and *THT* is absolutely dependent upon *COI*-regulated JA signaling for wound activation. Furthermore, expression of the *ANS*, *SST* and *THT* gene was not induced by MeJA in *coi1-1* plants (Fig. 5B). As was observed for the wound

A



B

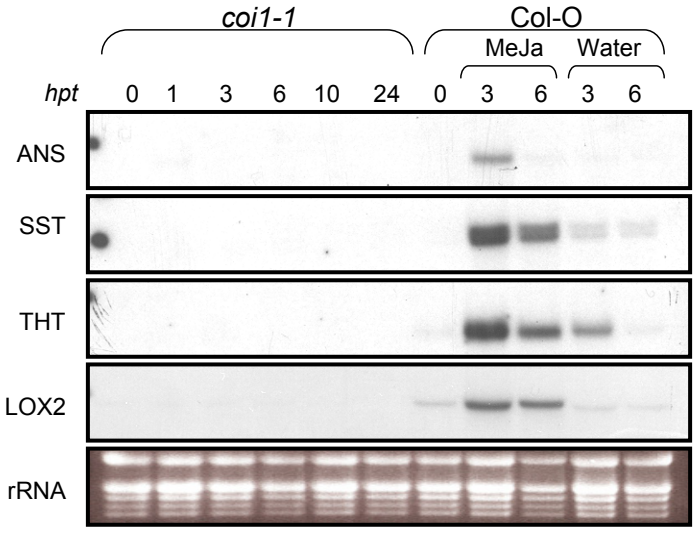


Figure 3.5 Expression of DP3 genes in wounded and MeJA-treated *coi1-1* mutant. **A**. Wild-type and *coi1-1* mutant plants were analyzed for wound-induced expression of DP3. **B**. Wild-type and *coi1-1* mutants were examined for activation of DP3 expression rescued by 30 μM MeJA. Loading amounts (~10 μg/sample) were determined by ethidium bromide stained rRNA. *hpw*-hours post-wounding, *hpt*-hours post-treatment.

induction of this gene in the *coi1-1* mutant, expression of *LOX2* was completely absent in MeJA-treated *coi1-1* plants. These two results from the analysis of the *coi1-1* mutants demonstrated that the wound-specificity of *ANS*, *SST* and *THT* activation is completely dependent upon the JA signaling pathway.

Spatial regulation of DP3 expression in wounded plants

In addition to the immediate wounded (local) leaf, wound-induced genes may be expressed in undamaged distal (systemic) tissues of damaged plants (Green and Ryan, 1972; Schilmiller and Howe, 2005). In *Solanaceous* plants, the activation of wound-induced genes in systemic tissues has been attributed to an unidentified JA-related molecule that is dispatched from the wounded leaf to the remote leaves (Li et al., 2002c). The spatial expression of the *ANS*, *SST* and *THT* genes was investigated to determine whether their respective mRNAs were restricted to wounded leaves, expressed remotely in systemic leaves, or activated in both. To test this, wounded *A. thaliana* leaves and corresponding unwounded (systemic) aerial tissues were separately harvested in a time-course experiment. To prevent cross contamination of the samples, exactly three leaves were damaged per plant and accounted for during the tissue collection. Preference for local or systemic expression of DP3s was tested by northern blot hybridization.

Northern blot analysis demonstrated that the selected DP3s were induced rapidly in the wounded leaf within one hour after wounding (Fig. 6). In wounded leaves, the expression of *ANS*, *SST* and *THT* peaked by three hours after wounding and receded within 12 hours after wounding. Expression of these genes was also up-regulated in unwounded systemic tissues. The pattern of DP3 induction in the systemic leaves was

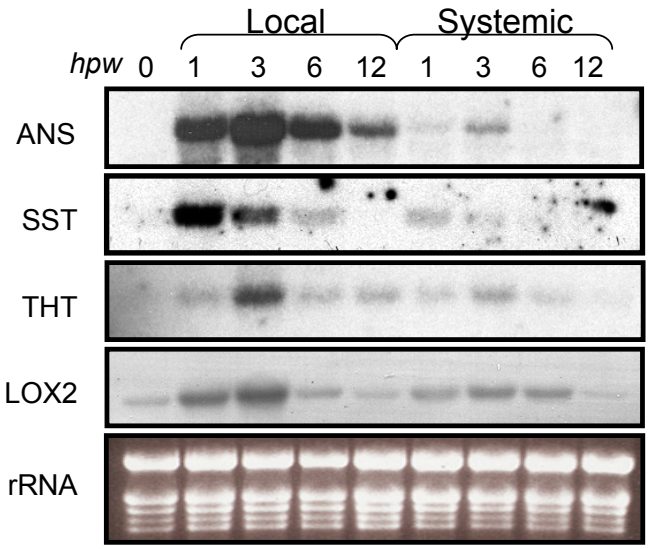


Figure 3.6 Expression of DP3 genes in locally-wounded and systemic tissues.
In a time course assay, three leaves were plants were wounded and collected separately from the remaining undamaged aerial tissues. Approximately ten micrograms of RNA were loaded per sample. Loading was measured by rRNA. *hpw*-hours post-wounding.

similar to that observed in locally wounded tissues. However, transcript levels in the systemic leaves were weaker than those observed in the injured leaves. Thus, expression of *ANS*, *SST* and *THT* was not restricted to the wounded leaf but spread distally to the unwounded leaves. The similarity in the induction rates of the DP3 genes in the local and systemic tissues suggests that a systemic signal is dispatched rapidly to the outlying unwounded leaves.

Discussion

The wound response in plants is an intricate signaling network, which mediates a diversity of pathways engaged in wound healing, pathogen defense, water stress, auxin signaling, secondary metabolism, and JA biosynthesis (Bostock and Stermer, 1989; Reymond et al., 2000; Cheong et al., 2002). The enlistment of these ancillary pathways during the wound response may obfuscate the isolation of wound-specific genes. A modified RDA design applying a stressed (SA-treated) driver was used to expurgate these ancillary genes from a wound-specific tester. Consequently, six cDNA fragments were isolated by RDA from tissues that were differentially regulated by wound induction and SA treatment (Table 2, Fig. 1). Three of these genes (*ANS*, *SST*, and *THT*) were not detected in uninjured plants and were rapidly induced within three hours of wounding. Treatment of SA did not activate their expression. One cDNA, *CYT*, was only feebly activated by wounding and its induction was difficult to confirm. The *AtGSTF17* gene was induced by wounding but expression was also observed in SA treated plants. Finally, a subtracted cDNA encoding *LOX2*, a known molecular marker for wound response, was isolated (Bell and Mullet, 1991, 1993; Bell et al., 1995). It was robustly

expressed in wounded plants. The *ANS*, *SST* and *THT* genes were consequently the focus of subsequent characterization for their inherent specificity to wounding. Thus the goal for the isolation of wound-specific transcripts was achieved.

Assessment of the RDA strategy

The proposed RDA strategy was successful in identifying three wound-specific genes from *A. thaliana* and the sensitivity of RDA enabled the isolation of weakly-expressed wounding genes like *THT*. Analysis of the transition from DP2 to DP3 demonstrates that a three-fold level of enrichment occurred and selectivity for wound-specific genes was achieved. However, two cDNAs (*CYT*, *AtGST17*) failed to meet wound-specificity requirements suggesting that RDA is not a foolproof technique. Though *ANS* was enriched almost three-fold, the enrichment of *AtGST17* was less robust Representation of *LOX2* fell in DP3. The weak expression of *LOX2* in SA-treated plants may have reduced its representation in DP3 and the resulting presence of *LOX2* and *AtGST17* in DP3 may have been a consequence of a more robust or cumulative induction by wounding. Three cDNAs (*SST*, *CYT*, and *THT*) were never uncovered in DP2. The complexity of DP2 may have played a role in masking their isolation. Weak induction of these genes in wounded plants may have also played a role as their respective transcripts were poorly represented and were also out-competed by highly expressed genes in the complex DP2. Consequently, *SST* and *THT* could not emerge until the majority of the SA-induced genes were eliminated.

The wound response is unlikely to be defined solely by three genes. Thus in some instances, RDA may be limited by the effort to sequence the DP3 cDNAs. Even though

RDA can detect the weakly-induced gene, they may still represent a small proportion of the DP3. For example, *SST* and *LOX2* represented only 2 of 34 determined sequences. Therefore, other wound-induced genes are likely to exist that have yet to be isolated from the DP3. Though RDA may increase the likelihood of isolating novel genes, a concerted sequencing effort is required to discover the majority of the DP3 transcripts. Similarly, to assess the level of enrichment between the DP2 and DP3 requires rigorous sequencing to accurately determine the complexity of DP2. The three-fold level of increase of homogeneity from DP2 to DP3 may underestimate the exact level of enrichment since a limited amount of characterization of the DP2 was performed.

Among the DP3, the number of times a specific cDNA was isolated does not appear to correlate to the level of its expression in wounded leaves. *ANS* was most frequently sequenced (n=22) and most robustly induced in wounded tissues. However, the singleton *SST* was less represented than *THT* (n=5), but *SST* was more strongly expressed in wounded tissues than *THT*. Moreover, *CYT* (n=3) was not even detectable in wounded leaves. This absence of association between extent of representation in the DP3 and expression level may be predictable since representation is based on expression levels of genes in the tester relative to the driver.

Expression of wound-specific *ANS*, *SST*, and *THT* is JA-dependent

The wound response in *A. thaliana* integrates JA-dependent and -independent signaling pathways (Farmer et al., 1992; Howe, 2004). Three DP3s (*ANS*, *SST*, and *THT*) were examined by northern blot analysis for sensitivity to exogenous JA. All three were coordinately induced by MeJA treatment and their rate of induction mirrored their

activation by wounding (Fig. 3). Thus, application of JA to the vegetative tissues of *A. thaliana* was sufficient to trigger the wound-responsive expression of *ANS*, *SST* and *THT* suggesting that JA signaling is critical for their induction. However, the MeJA application did not emulate the extent of induction by wounding, demonstrating that JA-independent factors assist in amplifying DP3 expression in response to tissue damage. The essential role of the JA pathway was investigated by northern blot analysis with mutants of the JA signaling pathway (Fig. 4, Fig. 5). The *jar1* mutant failed to affect the wound induction of *ANS*, *SST* and *THT* (Fig 4). Therefore, wound induction is primarily regulated in a *JAR1*-independent manner (Fig. 4A). Though DP3 transcripts were detected, *JAR1* may partially contribute to induction of *ANS*, *SST* and *THT* in wild-type plants, as MeJA treatment could only partially rescue their expression in *jar1* plants (Fig. 4B). These results are comparable to other gene expression studies with the *jar1* mutant (Staswick et al., 1992). Definitive evidence for the role of the JA signaling pathway in the coordinate activation of *ANS*, *SST* and *THT* was obtained by studies with the *coi1-1* mutant (Feys et al., 1994; Xie et al., 1998). *ANS*, *SST* and *THT* expression was not induced in wounded *coi1-1* plants (Fig. 5A) and individually could not be rescued by application with exogenous MeJA (Fig. 5B). These results demonstrate that the JA signaling pathway is necessary and sufficient for wound-specific expression of *ANS*, *SST* and *THT*.

Systemic Expression of *ANS, SST,* and *THT*

Transcripts encoding the *ANS*, *SST* and *THT* were expressed in both locally-wounded and non-wounded, distal tissues of wounded *A. thaliana* plants (Fig. 6). In

Solanaceous plants, this expansion of the expression domain from the injured leaf to the systemic tissues is dependent upon transported JA or a JA-related molecule (Li et al., 2002c). In tomato plants, JA generated from the wounded leaf is sufficient to activate JA-dependent wound response in the undamaged leaves and *de novo* synthesis of JA in the systemic leaves is not required (Li et al., 2002c).

The systemic up-regulation of *ANS*, *LOX2*, *SST* and *THT* is weaker than their induction in the wounded leaf. The attenuated wound response in systemic leaves has been reported elsewhere and may be attributed to lower levels of active JA in systemic tissues (Howe, 2004). Systemic levels of JA in wounded plants were measured at 10% of the accumulated JA in the injured leaf. In this study, the induced level of *LOX2* expression was similarly lower in the systemic tissues relative to the wounded leaf. Consequently, the weaker activation of *LOX2* expression in the unwounded tissues may have resulted in lower JA biosynthesis in systemic leaves and hence reduced induction of JA-dependent genes like *ANS, SST* and *THT*. In tomato plants, systemic expression of wound-induced genes does not require the distal *de novo* synthesis of JA but relies on amplification of the systemic signal by the proteinaceous hormone, systemin (Li et al., 2002c). *A. thaliana* does not contain a gene encoding systemin and it is unknown whether a systemin-equivalent factor is similarly required for systemic wound signaling.

Other factors may contribute to the reduced systemic induction of *ANS*, *LOX2*, *SST* and *THT*. Accumulated JA in the systemic tissues may be sequestered into non-responsive cell types (Schilmiller and Howe, 2005). Alternatively, JA may be modified in wounded leaves such that its mobility is prohibited, or JA may be metabolized to an inactive form. Conjugated forms of JA exist in higher plants and putatively serve as

readily available reservoirs of JA (Schaller et al., 2005). Alternatively, additional components may be required to elevate JA levels in systemic tissues of wounded plants. For example, the oral secretion of herbivores can trigger the defense response in plants and may contribute to the increase of JA content (Kessler and Baldwin, 2002; Von Dahl and Baldwin, 2004).

Absence of JA-independent, wound-responsive genes in the RDA subtraction

It is striking that the four genes (*ANS*, *LOX2*, *SST*, and *THT*) characterized from DP3 are all up-regulated by JA. A single JA-independent gene was never encountered in the DP3, although it cannot be ruled out that *CYT, AtGST17*, members of the DP2 population, and unsequenced DP3 candidates are JA-independent. One reason that JA-independent genes were not isolated may have been the decision to collect tissue at one and three hours after wounding. Some JA-independent genes (e.g. *WR3*, *CK*) in *A. thaliana* are induced immediately after tissue damage but expression subsides within an hour after wounding (Titarenko et al., 1997; Rojo et al., 1999). Therefore they might not be represented in the wound-specific tester. Interestingly, these JA-independent, wound-induced genes inhibit the activation of the JA-regulated genes in locally-wounded leaves (Rojo et al., 1999).

An additional set of JA-independent genes were also characterized in a microarray analysis of the wound response in *A. thaliana* (Delessert et al., 2004). In that study, more than half (55/98) of the wound-induced genes were independent of MeJA activation and *COI* regulation. Several of these may have been eliminated in the RDA strategy applied here because of possible dual activation by SA and wounding. For

example, two WRKY transcription factors (*WRKY15*, *WRKY33*) were characterized as wound-induced and JA-independent in the microarray experiment. However, some WRKY proteins participate in SA-mediated signaling pathways (Yu et al., 2001). Thus, *WRKY* genes in the tester may have been eliminated due to possible co-induction in the SA-treated tissue. Further examination into the regulation of JA-independent, wound-responsive genes may illuminate their activation and involvement in other response pathways.

Potential role of ANS in the wound response

Plants synthesize a diversity of secondary compounds (Holton and Cornish, 1995; Pichersky and Gang, 2000; Verpoorte and Memelink, 2002). These metabolites are synthesized from branched routes of the primary metabolic biosynthetic pathways. Secondary compounds fulfill several functions. They contribute to floral scent and coloration (Holton and Cornish, 1995; Mol et al., 1998). Secondary metabolites aid in tolerance to abiotic stresses like high salinity and drought (Seki et al., 2002; Glombitza et al., 2004). Toxic secondary compounds serve to deter attack from insects and herbivores (Bennett and Wallsgrove, 1994; Halkier and Gershenzon, 2006). Moreover, anti-pest flavonoid compounds may possess bioactivity, e.g. promotion of premature molting of feeding insect larvae (Adler and Grebenok, 1995).

Anthocyanins are colored, tricyclic flavonoids, which constitute a large proportion of the secondary metabolites generated from the phenylpropanoid pathway in plants (Holton and Cornish, 1995; Mol et al., 1998). Anthocyanins are primarily known as contributing to floral coloration, but are also potent anti-oxidants, and serve multiple

stress-related roles (Dixon and Paiva, 1995; Gould, 2004). Anthocyanins, for example, are induced by wounding (Tamari et al., 1995; Ferreres et al., 1997; Gould et al., 2002; Reyes and Cisneros-Zevallos, 2003; Gould, 2004). In *Pseudowintera colorata*, leaves pierced by a needle form a ring of anthocyanin-containing cells around the damaged cells (Gould et al., 2002). Anthocyanins also accumulate in the injured midrib of processed lettuce leaves (Ferreres et al., 1997). JAs also induce the accumulation of anthocyanins in *A. thaliana* and potato tubers (Feys et al, 1994; Reyes and Cisneros-Zevallos, 2003).

ANS is a 2-oxoglutarate iron-dependent oxygenase that converts colorless leucoanthocyanidins to colored anthocyanins. The *A. thaliana* genome contains 11 genes that encode for putative 2-oxoglutarate iron-dependent oxygenases. In this study, an *ANS* (At2g38240) was shown to be transcriptionally induced by mechanical damage and JA. Activation of *ANS* in response to tissue damage and JA was previously demonstrated by microarray analysis (Devoto et al., 2005). Treatment of *A. thaliana* cell cultures with Yariv phenylglycoside triggers a wound-like response and also induces *ANS* (Guan and Nothnagel, 2004). An ortholog (At4g22880) of *ANS* was identified in wounded *Arabidopsis* (Devoto et al., 2005). It also exhibits *COI*-dependent induction by wounding and JA treatment

The function of *ANS* (At2g38240) and other anthocyanidin synthases in the wound response is still unclear. Coloration from anthocyanins functions to attract pollinators to its flowers yet may also act as a warning signal to predators (Holton and Cornish, 1995; Hamilton and Brown, 2001). It is speculated that leaf tissues containing abundant anthocyanins may be less palatable to herbivores (Hamilton and Brown, 2001). However, the inducible nature of *ANS* in response to wounding suggests that its gene

product has a responsive role in coping with tissue damage rather than function as a pre-formed defense mechanism.

Anthocyanins also protect the photosynthetic machinery from excess light and scavenge free radicals which may damage cell membranes (Bors et al., 1994; Yamasaki et al., 1996; Gould et al., 2002). The red leaves of *P. colorata* are able to eliminate the wound-induced accumulation of H_2O_2 more rapidly than its green leaves (Gould et al., 2002). Detection of H_2O_2 around the damaged area co-localized to the same area as the halo of anthocyanin accumulation. Thus the induction of *ANS* (At2g38240) could be associated with modulating levels of the free radicals or ameliorating the effects of reactive oxygen species (ROS). ROS have been shown to travel systemically in tomato and may trigger downstream wound-response genes (Orozco-Cardenas and Ryan, 1999; Orozco-Cardenas et al., 2001). Therefore the systemic activation of *ANS* may also control rising ROS levels in distal tissues.

Potential role of THT in the wound response

The phenylpropanoid pathway is also the source for multiple classes of non-flavonoid, stress-induced compounds. Cinnamic acids are metabolized by a series of enzymatic modifications that generate an assortment of phenylpropanoid-based compounds (Dixon and Paiva, 1995). Hydroxycinnamic acids are formed from the hydroxylation of cinnamic acids and are detected as conjugated forms to various compounds, including cell wall carbohydrates, organic acids and sugars (Clarke, 1982; Hohlfeld et al., 1996).

Hydroxycinnamic amides, e.g. feruloyltyramine and 4-coumaryltyramine, are secondary nitrogenous metabolites synthesized by THT through the condensation of tyramine with hydroxycinnamoyl-CoA thioesters. Tyramine is converted from tyrosine by tyrosine decarboxylase. Hydroxycinnamic amides are ubiquitous in higher plants (Martin-Tanguy et al., 1978). THT activity was initially discovered in TMV-infected *Nicotiana tabacum* but has been identified in elicitation assays, UV-exposure, and pathogen challenge (Negrel and Martin, 1984; Villegas and Brodelius, 1990; Back et al., 2001; Newman et al., 2001). THT activity was also detected in the wounded leaves of maize, tobacco, hot pepper, and potato tuber (Negrel et al., 1993; Ishihara et al., 2000; Back et al., 2001).

There are several proposed roles for hydroxycinnamic amides in response to wounding. Their association with the cell wall suggests that hydroxycinnamic amides may function to strengthen the cell wall as the amides are covalently cross-linked by peroxidation into the cell wall (Liyama et al., 1994). In damaged tissue, the accumulation of the amides may reinforce the damaged plant cell wall (Negrel et al., 1996). Given their aromatic nature, hydroxycinnamic amides may also serve as a phenolic barrier and may be cytotoxic (Clarke, 1982; Hahlbrock and Scheel, 1989).

The subtracted *THT* gene (At2g39030) is induced in wounded and JA-treated *A. thaliana* plants. The *THT* gene was expressed in both wounded and unwounded systemic tissues. As in other higher plants, increased activity of the *THT* gene may similarly function to repair and reinforce the cell walls of wounded leaves as a form of wound healing. In systemic leaves, THT activity may fortify the distal tissues to prevent possible impending damage to the remote leaves. This is the first report assigning a role

in wounding for *THT* in *A. thaliana* plants. Furthermore, regulation of *THT* by JA has not been previously documented for any other *THT* in higher plants. Because expression of *THT* in wounded *A. thaliana* was much weaker relative to the other wound-induced genes, *THT* may have escaped detection by other gene isolation methods.

The potential role of SST is discussed in detail in the next chapter.

Functions of some notable DP2 genes

ATR1, isolated in the DP2 population, encodes a MYB-like transcription factor which regulates the *ASA1* (*anthranilate synthase 1*) gene (Bender and Fink, 1998; Celenza et al., 2005). *ASA1* is the first committed step and key control point for the biosynthesis of tryptophan (Niyogi and Fink, 1992). *ASA1* transcripts are also up-regulated by tissue damage (Niyogi and Fink, 1992). The tryptophan pathway leads to the production of indole-3 acetic acid (a growth regulating cytokinin hormone) and indolic glucosinolates (Halkier and Gershenzon, 2006). Indolic glucosinolates and glucosinolates are converted by myrosinase into the active secondary metabolites thiocyanates, isothiocyanates and nitriles (Halkier and Gershenzon, 2006). Glucosinolates are induced by tissue injury and herbivore attack and function as antimicrobials and toxic anti-pest compounds (Borek et al., 1998; Tierens et al., 2001). Hence, the isolation of *ATR1* indicates the critical role of glucosinolate production in response to wounding.

Two *GST*s were isolated from the DP2 and DP3 population. GSTs mediate the transfer of glutathione (Glu-Cys-Gly) to a substrate (Dixon et al., 2002). Glutathione

transferases were first identified in animals as enzymes which detoxified drugs (Wilce and Parker, 1994). Similar activity has been documented for a GST isolated in maize which conjugated a glutathione to detoxify an herbicide (Edwards and Dixon, 2000). Plants also manufacture endogenous toxic compounds which must be sequestered for survival, but no such activity has been attributed to GSTs. However, a GST encoded by the *An9* gene in *Petunia* was shown to compartmentalize anthocyanins into the vacuole (Mueller et al., 2000). *GST*s are also activated by multiple biotic and abiotic stresses including salinity, osmotic and temperature (Marrs, 1996). These GSTs may also possess glutathione peroxidase activity and purportedly scavenge for stress-induced reactive oxygen species (ROS) (Roxas et al., 1997; Cummins et al., 1999). GST peroxidases consequently protect tissues from damaging ROS by converting the organic hydroperoxides of fatty acids and nucleic acids into inert monohydroxyalcohols. Without GST peroxide-mediated activity, the hydroperoxides could spontaneously degrade into toxic aldehydes. In response to wounding, the *GST*s isolated in this present study may play a similar function as peroxidases to neutralize accumulating wound-released ROS or a detoxification role to regulate endogenous chemotoxins.

Relationship between wounding and abiotic stress response

Several of the genes isolated from the DP2 round of subtraction also participate in abiotic stress (salinity, drought, cold, heat, ozone) responses (Table 1). Wounding may be linked to abiotic stresses due to the ensuing trauma of water escaping from wounded leaves (Reymond et al., 2000). Thus, the injured plants must compensate for the loss of water or water pressure by activating abiotic stress response.

Three (including *AtGSTF10*) of the DP2 genes are associated with responses to water deprivation. The *RD19A* gene was initially isolated as responding to dehydration but also is sensitive to other osmotic stresses like high salinity (Yamaguchi-Shinozaki et al., 1992; Koizumi et al., 1993). The *RD19A* gene encodes a cysteine proteinase. Cysteine proteinases serve several functions in water-stressed tissues and, perhaps, injured leaves (Koizumi et al., 1993; Linthorst et al., 1993; Williams et al., 1994). These genes are up-regulated by oxidative stress and function in programmed cell death (Solomon et al., 1999; Beers et al., 2000). They may also catabolize amino acids from storage proteins to supply the increased demand for protein synthesis. The *COR15A* gene enhances freeze tolerance in *A. thaliana* but also functions in response to dehydration (Lin and Thomashow, 1992b; Lin and Thomashow, 1992a). Freezing temperatures indirectly desiccate plant cells because ice crystals in the extracellular space draw water away from the cell (Yoon et al., 2003; McCully et al., 2004). The COR15A protein is targeted to the inner membrane of the chloroplast and appears to encode a non-enzymatic protein (Artus et al., 1996; Steponkus et al., 1998).

CHAPTER FOUR

CHARACTERIZATION OF A WOUND- AND JA-INDUCIBLE STEROID SULFOTRANSFERASE (SST) FROM *ARABIDOPSIS THALIANA*

Introduction

In the previous study (Chapter 3), a subtracted cDNA (*SST*) fragment, encoding a predicted steroid sulfotransferase, was isolated from wounded *A. thaliana* plants. Its activation was regulated by the JA signaling pathway. *SST* was expressed in both locally-wounded leaves and the unwounded systemic tissues. Steroid sulfotransferases are enzymes that catalyze the conjugation of a sulfonate to a steroid substrate (R-OH + PAPS $\rightarrow$ R-OSO_3^- + PAP, where R is a steroid acceptor) (Luu-The et al., 1996; Strott, 1996; Falany, 1997). The sulfonate group (SO_3^{-1}) is donated from an activated cofactor, 3'-phosphoadenosine 5'-phosphosulfate, (PAPS) (Farooqui, 1980). Although plant steroid sulfotransferases are poorly understood, these enzymes in mammals perform critical roles as regulators of hormone activity and drug detoxification (Roy, 1981; Adams et al., 1988; Mulder, 1990; Pasqualini et al., 1992). We were similarly curious as to the function of *SST* in wounded tissues and interested in exploring the possibility that it might modulate a steroid-based signal in the plant wound response. In this chapter, sequence-specific evidence that SST may encode a functional steroid sulfotransferase is introduced. A functional approach to understanding *SST* was conducted through the use of RNAi-directed silencing of the endogenous *SST* gene. Transgenic plants were analyzed for changes in steroid profiles during the wound response. However, no apparent changes in

the steroid content of wounded plants could be detected. Reasons for this result and the possible biological roles of SST are discussed.

Results

Sequence analysis of SST (At5g07010)

The gene identity of *SST* (At5g07010) was determined by searching GenBANK (http://www.ncbi.nlm.nih.gov/BLAST/) with the subtracted 329-nucleotide (nt) cDNA fragment (Fig. 1). The corresponding full-length *SST* transcript measures 1.4 kb in length and is functionally annotated as a steroid sulfotransferase. The subtracted segment corresponds to nucleotides 103-431 of the mature mRNA and is situated 35 nt distal to the predicted translational start (MET) site. Untranslated regions (UTR) flank the 5'- and 3'-ends of the open reading frame. The *SST* gene is located on chromosome five of the *A. thaliana* genome and is intronless.

The full-length *SST* sequence was used to identify related genes from higher plants. The full-length *SST* transcript is most highly related to an endogenous gene, At5g07000 (Fig 1), which is situated next to *SST* (At5g07010) on the upper arm of chromosome five. The nucleotide sequence of At5g07000 is characterized by 90% similarity to *SST*. The At5g07000 transcript is slightly larger at 1.5 kb, intronless and flanked by UTRs. The most striking difference between *SST* and At5g07000 occurs at the 5'-end of the *SST* coding region, which is extended by 30 nt relative to the putative start codon for At5g07000. In the region represented by the subtracted *SST* cDNA, an 11-nt insertion specific for *SST* disrupts any colinearity between *SST* and At5g07000. The overall sequence similarity between *SST* and At5g07000 along with thei

Figure 4.1 Pair-wise alignment of the DNA sequence for At5g07010 (*SST*) and putative paralog, At5g07000.
The nucleotide sequences depict the corresponding coding regions of *SST* and At5g07000. The sequences were collected from NCBI and compared using ClustalW. The red bar represents the region of the *SST* sequence isolated by RDA. The predicted start codon in represented by the **Met** and termination codon by **Ter**.

neighboring positions on chromosome five suggested that they are duplicates (paralogs) from a common ancestral *A. thaliana* gene.

Protein analysis of SST

Evidence that *SST* may encode a functional steroid sulfotransferase was based on the similarity of its protein sequence previously to a characterized plant steroid sulfotransferase, steroid sulfotransferase 3 (BnST3) from *Brassica napus* (Rouleau et al., 1999). Based on *in vitro* studies, the BnST3 protein preferentially sulfonates brassinosteroids. A comparison of the BnST3 and SST (AAP68286.1) reveals a 41% similarity (E-value 5e-69) based on the primary amino acid sequence (Fig. 2). The gene product of At5g07000 (AAP12890.1) also is highly similar to the BnST3 sequence (E-value 2e-67). Moreover, At2g03760 (RaR047), the *A. thaliana* ortholog of BnST3, is also highly related (E-value 2e-62) to the SST protein.

The substrate for At2g03760 is unknown. However, the crystal structure of At2g03760 most closely resembles the 3-D conformation of the human estrogen sulfotransferase (hEST), suggesting that At2g03760 may potentially sulfonate a plant steroid substrate (Smith et al., 2004). The protein similarity of the SST protein with a putative brassinosteroid sulfotransferase (BnST3) and its *A. thaliana* ortholog (At2g03760) coupled with the confirmed steroid sulfotransferase-like 3-D crystal structure of At2g03760 implied that SST may also function as a steroid sulfotransferase.

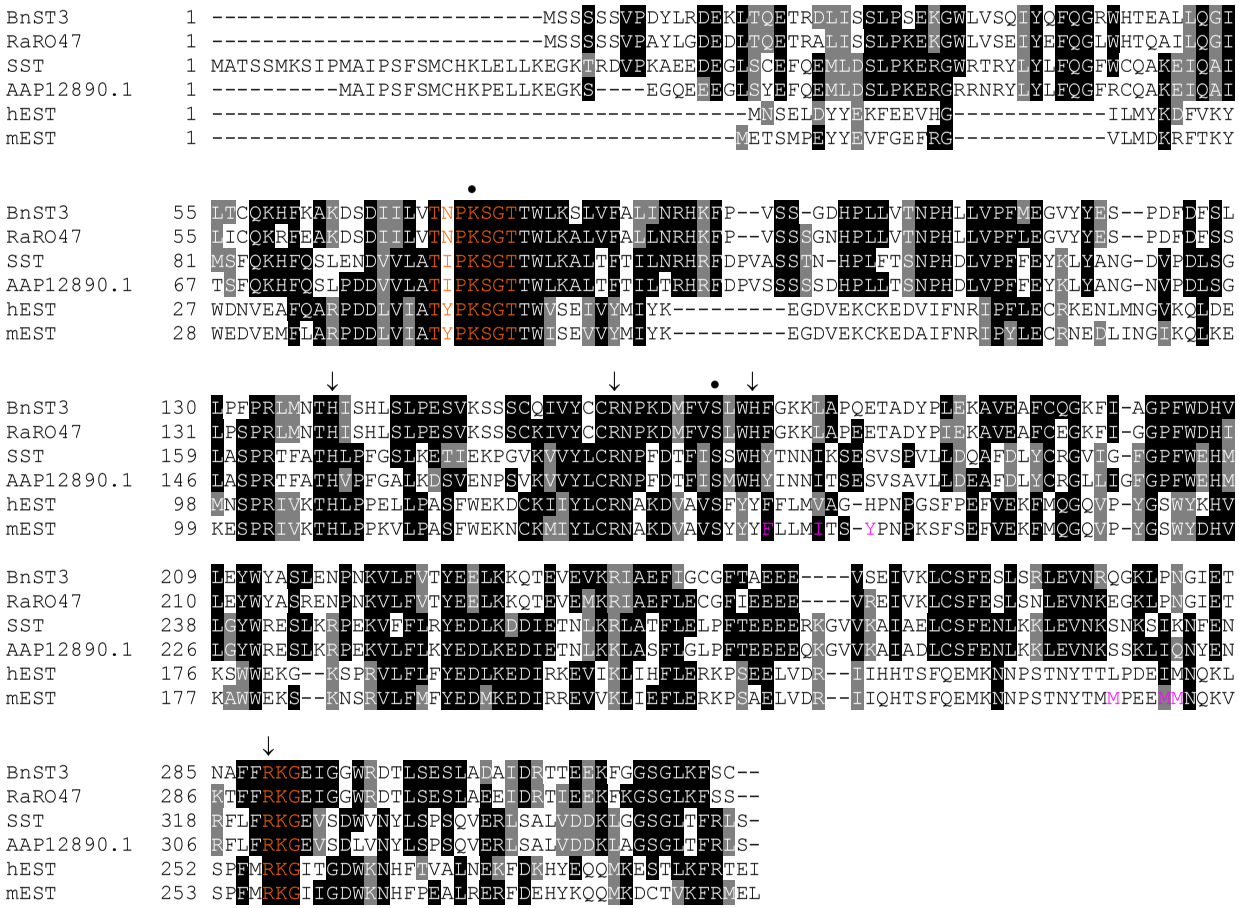


Figure 4.2 Amino acid alignment of steroid sulfotransferase proteins.
Comparison SST of with protein sequences representing human estrogen sulfotransferase (hEST), murine estrogen sulfotransferase (mEST), brassinosteroid steroid sulfotransferase 3 (BnST3), *Arabidopsis* At2g03760 (RaR047)and At5g07000 (AAP12890.1). PAPS binding domains are highlighted in orange. Conserved residues required for PAPS binding are designated by •. Catalytic residues required for steroid sulfotransferase activity are designated by ↓. The designated Arg residues may also play a role in PAPS binding. Residues shaping the steroid binding domain in mEST are indicated in purple.

PAPS and steroid-binding motifs in the amino acid sequence of SST

A comparison of protein sequences of SST with known mammalian steroid sulfotransferases also pointed to SST as a functional steroid sulfotransferase (Fig. 2). Steroid sulfotransferases contain sequence signatures that are involved with PAPS binding (Kakuta et al., 1998a). Binding is partially mediated by a P-loop motif (TYPKSGT) (Muller-Dieckmann and Schulz, 1994), which is represented in hEST by residues 44-50 and murine estrogen sulfotransferase (mEST) by residues 45-51 (Kakuta et al., 1997). Demonstrated by 3-D crystallography to physically interact with the activated PAPS molecule (Fig. 3), the Lys residue of the P-loop motif is critical for PAPS binding (Fig. 3B) (Kakuta et al., 1998b). The Lys residue is conserved in all putative plant steroid sulfotransferases but in *A. thaliana* the P-loop motif exhibits some variation of this signature (data not shown). For example, the Thr residue of the motif is represented in only a minority of *A. thaliana* steroid sulfotransferases and the second position (Tyr) of the motif is not conserved in any of the plant sulfotransferases. In SST, the Tyr residue is replaced by Ile. A second sequence hallmark for PAPS binding is derived from composite regions which form the 3'-binding site (3'-PBS) of mEST (Driscoll et al., 1995). The RKG (residues 257-259) motif, Arg 130, and Ser 138 all participate in binding the PAPS molecule (Fig. 3B) (Kakuta et al., 1997). The Ser residue resides in the PAPS binding site and is critical for PAPS binding. These residues are conserved in SST (RKG 322-324, Arg 180, Ser 198). Two Arg residues (Arg 161, Arg 289) of BnST3 were shown to be important for the bioactivity (Rouleau et al, 1999) and are similarly conserved in SST (Arg 190, Arg 322).

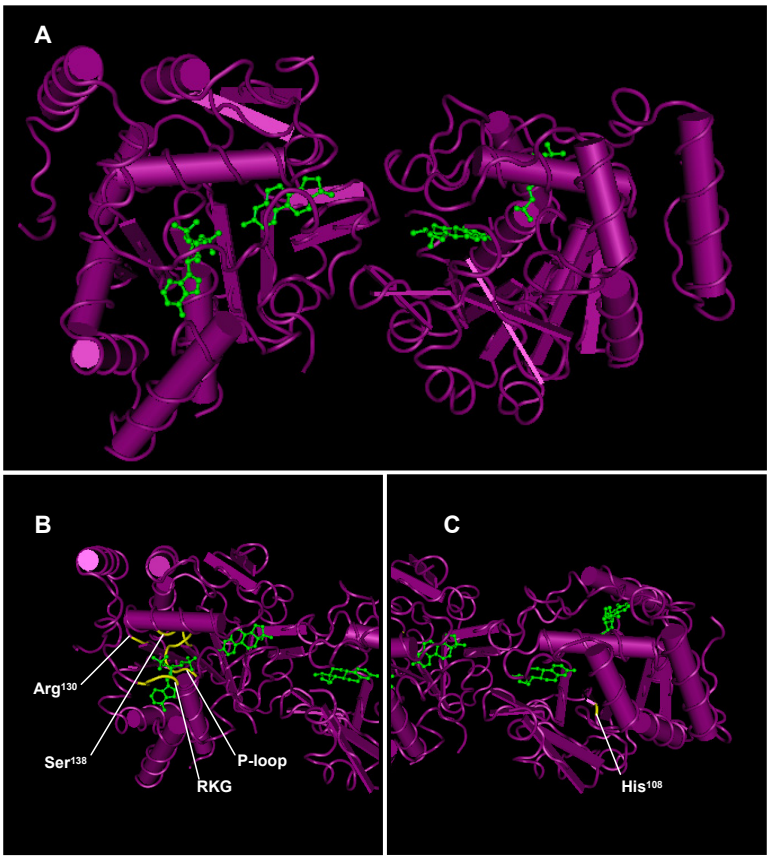


Figure 4.3 Three-dimensional crystal structure of murine estrogen sulfotransferase (mEST) bound with the activated PAPS cofactor and estradiol substrate. **A**. The mEST protein purportedly exists as a dimer in the cytosol and is represented here as such. Estradiol and PAPs are highlighted in green. **B**. Critical residues involved in PAPs binding are identified (yellow). **C**. The catalytic His108 (yellow) is involved in the sulfonate transfer from PAPS to estradiol. [The crystal structure of mEST (PDB: 1AQY) was determined by Kakuta *et al* (1994) and available at NCBI. (MMDB 8673) at http://structure.ncbi.nlm.nih.gov/Structure/mmdb/mmdbsrv.cgi?form=6&db=t&Dopt=s&uid=8673]

The steroid binding pocket of mEST is shaped by hydrophobic residues (Phe 142, Ile 146, Tyr 149 and Met 243, Met 247, Met 248), which reside mainly in two regions of the primary sequence (Kakuta et al., 1997). With exception to the Phe residue, these residues are not well conserved among steroid sulfotransferases. The Phe residue is represented in most plant steroid sulfotransferases, but in SST the Phe replaced with a Tyr. A His residue, which based on the crystal structure, resides near the steroid binding site and is essential for steroid sulfotransferases because it mediates the transfer of the sulfonate group from PAPS to the steroid substrate (Kakuta et al., 1998b). In SST, the His sits at position 169 corresponding to His 108 in mEST and His 107 in hEST (Fig. 3C).

The PAPS binding motifs and steroid binding residues are equally conserved in the SST paralog, At5g07000 (AAP12890.1) (Fig. 2). The two critical residues responsible for PAPS binding are present in At5g07000 (Lys 75, Ser 169). The His residue, which transfers the sulfonated group from PAPS to the steroid substrate (Kakuta et al., 1998b), is also conserved in AAP12890.1 (His 155). The conserved Phe that resides in the steroid binding cleft of mEST is present in BnST3 (Phe 173) but is replaced by Tyr (Tyr 190) in AAP12890.1. This substitution with Tyr is also observed in SST. The two Arg residues of BnST3 involved in either catalysis or binding of PAPS are as well represented in AAP12890.1 (Arg 87, Arg 177) (Rouleau et al., 1999). Moreover, the Arg residues are also conserved in mammalian and all plant steroid sulfotransferases. Thus, the paralog of SST (At5g07000) may similarly function as a steroid sulfotransferase based on the presence of motifs responsible for PAPS and steroid binding.

Evolutionary relationship of plant steroid sulfotransferases and mammalian ESTs

Further evidence supporting the view that SST encodes a steroid sulfotransferase comes from its phylogenetic relationship with mammalian steroid sulfotransferases. An unrooted, circular dendrogram was constructed in order to analyze the evolutionary relationships among all annotated *A. thaliana* steroid sulfotransferases, BnST3 and hEST (Fig. 4). The dendrogram depicted SST and At5g07000 (AAP12890.1) as members forming a single cluster with hEST. The association of SST and AAP12890.1 with hEST is especially striking since BnST3 and RaR047 are excluded from the clade, but BnST3 and RaR047 do appear more intimately related to hEST and SST than the remaining plant steroid sulfotransferases. Thus, phylogenetic evidence indicated that SST and At5g07000 may function as steroid sulfotransferases.

RNA-mediated gene silencing of SST expression

Given the function of the mammalian steroid sulfotransferases in regulating hormone activity, SST may also regulate the activity of a steroid-based signal involved in the plant wound response. One method to elucidate the function of a gene is to knock out or down-regulate its expression. At the time these studies were initiated, T-DNA insertional mutants were unavailable for the *SST* locus. Consequently, an alternative technique based on RNAi-mediated gene silencing of *SST* was adopted (Fire et al., 1998). Double-stranded RNA triggers a post-transcriptional mRNA degradation pathway in plants, fungi, yeast and animals (Susi et al., 2004). Plants are equally able to recognize the double-stranded region within a hairpin structure, formed from a self-annealing,

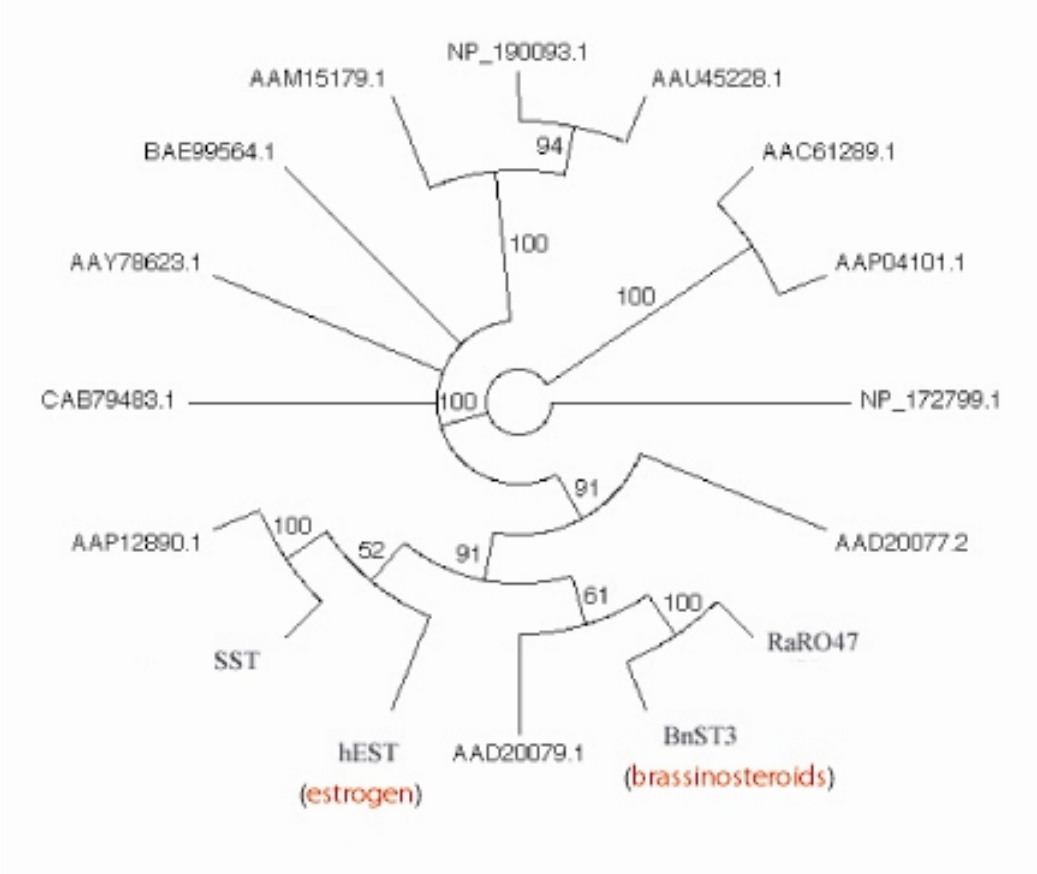


Figure 4.4 Amino acid sequence relationships of steroid sulfotransferases.
The dendrogram was constructed from annotated steroid sulfotransferases from *A. thaliana*, BnST3, and hEST. Protein sequences were collected from Genbank and TAIR. Designations for *A. thaliana* steroid sulfotranferases represent accession numbers for following loci: SST (At5g07010), AAD20079.1 (At2g03770), RaRO47 (At2g03760), AAD20077.2 (At3g03750), NP 172799.1 (At1g13420), AAP04101.1 (At1g13430), AAC61289.1 (At2g14920), AAU45228.1 (At3g45080), NP 190093.1 (At3g45070), AAM15179.1 (At2g27570), BAE99564.1 (At5g43690), AAY78623.1 (At1g28170), CAB79483.1 (At4g26280), AAP12890.1 (At5g07000). hEST-human estrogen steroid sulfotransferase, BnST3-brassinosteroid sulfotransferase 3 (*Brassica napus*). Known substrates for indicated in red. See Material and Methods (Chapter Two) for description of the phylogenetic analysis and tree construction.

single-stranded RNA molecule (Hamilton and Baulcombe, 1999). The hairpin is efficiently cleaved into 21-25 nt fragments, called small-interfering RNAs (siRNAs), executed by an endonuclease called Dicer (Tang et al., 2003). One strand of the duplex is recruited as a component of a large ribonucleoprotein complex called the RNA-induced silencing complex (RISC) (Vaucheret et al., 2004). The single-stranded siRNA serves as a template for directing the RISC complex to eliminate any complementary RNA. Thus, sequence-specific removal of transcripts by RNAi silencing is a useful technique for precise gene suppression (Waterhouse et al., 1998; Smith et al., 2000; Wesley et al., 2001). Targeted silencing by RNAi is achieved by designing an artificial hairpin molecule defined from a portion of the coding region of a gene of interest (Waterhouse et al., 1998). The efficacy of RNAi silencing is enhanced by the introduction of an intron between the sense and anti-sense strands of the hairpin (Smith et al., 2000).

The entire subtracted cDNA encoding the *SST* gene was amplified with PCR primers, whose sequences were flanked by asymmetrically orientated recognition sites for specific restriction endonucleases. This allowed the construction of the sense and antisense strands of *SST* hairpin in one PCR reaction by utilizing different combinations of the restriction endonucleases. The arms of the hairpin loop were constructed by sequentially ligating the PCR product into pHANNIBAL (Smith et al., 2000). The cassette encoding the *SST*::RNAi hairpin structure was inserted into a Ti plasmid (pART27) as a *Not*I fragment. *A. thaliana* plants were transformed with *Agrobacterium tumefaciencs* (strain GV3101), harboring the *SST*-silencing (*SST*::RNAi) vector. Transgenic plants (T1) were selected by resistance to kanamycin. Seven independent transgenic (T2) lines were examined for wound-inducible *SST* expression by northern

blot analysis. All seven lines demonstrated by northern blot analysis RNAi-mediated suppression of *SST* induction in wounded plants (data not shown). The T2 and T3 generations of one transgenic line (RNAiWig9-2) was used for subsequent analysis.

Putative transgenic plants (T2 and T3) harboring the RNAi-silencing vector appeared morphologically and developmentally similar to wild-type and vector control plants. The plants were fertile and bolted at approximately an equal age to wild-type and vector control plants. Confirmation of the integration of the T-DNA containing the *SST*::RNAi construct was confirmed by Southern blotting (Fig. 5A). The genomic DNA of a kanamycin-resistant T2 transgenic line was digested with *Bam*HI and fractionated on a TBE-containing agarose gel. The radiolabeled probe was generated from a purified DNA fragment, enzymatically released from the silencing vector by digestion with *Cla*I and *Bam*HI. The excised fragment contained the silence-enhancing intron and one arm of the *SST*-hairpin loop. Southern blotting confirmed the presence and genomic integration of the *SST*::RNAi T-DNA as a predicted 3-kb DNA fragment. The hairpin construct was absent from the transgenic line containing the vector control. A signal, representing a significantly larger genomic DNA fragment was observed in both the vector and the *SST*::RNAi silencing lines (Fig. 5A). This band may correspond to the *Bam*HI-digested *A. thaliana* genomic fragment containing the full-length endogenous *SST* gene, which is partially homologous to the *SST* arm of the hairpin structure.

The efficacy of *SST* silencing mediated by RNAi in the transgenic plants was assessed by northern blot analysis (Fig. 5B). The putative *SST*-silenced line and control *A. thaliana* plants were damaged by crushing three leaves per plant. The aerial tissues from the unwounded and wounded plants were collected in a time-course assay and

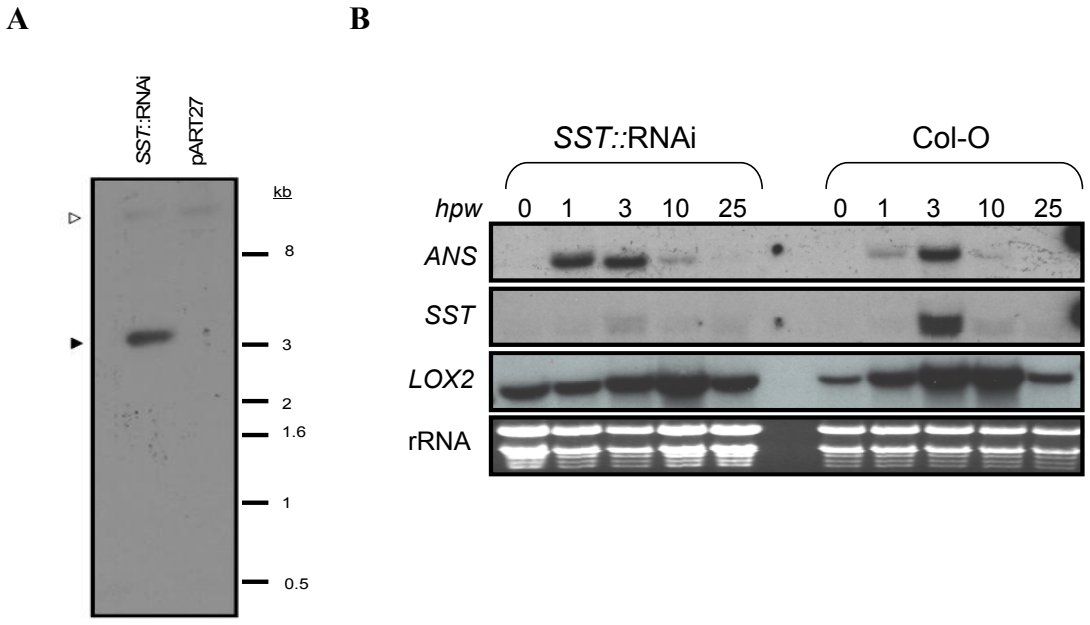


Figure 4.5 Analysis of *SST*-silenced transgenic *A. thaliana* plants.
A. Southern blot of the genomic DNA from putative transgenic plants (*SST*::RNAi) expressing a construct that targets gene-specific silencing of *SST*. The blot was analyzed with a probe derived from a fragment of the T-DNA cassette containing a portion of the *SST* cDNA. Plants transformed from an empty Ti plasmid (pART27) lack the T-DNA construct. The *Bam*HI-digested genomic DNA released the predicted 3.0 kb T-DNA (designated by closed arrow). Partial hybridization of the probe to the endogenous *SST* gene is predicted by a larger genomic fragment (represented by the empty arrow) in the *SST*::RNAi and vector control. **B**. Wound expression of the *SST* gene was tested in a time-course experiment by northern blot hybridization in putative *SST*-silenced and wild-type plants. Wounded plants were probed for expression of genes representing *SST, ANS* (anthocyanidin synthase), and *LOX2* (lipoxygenase 2). Loading was monitored by ethidium bromide staining of ribosomal RNA (rRNA).

examined for *SST* expression. Based on northern blot analysis, transcripts encoding the *SST* gene were absent in the wounded *SST*::RNAi transgenic plants, whereas *SST* induction was robust in injured wild-type plants peaking at three hours after wounding. In the putative *SST*-silenced plants, the *SST* transcript was not detected at any point during the extended time-course experiment. These later time points were performed as a precaution for possible postponed activation of *SST* in the putative RNAi-silenced plants. Thus, *SST* induction by wounding was successfully suppressed by RNAi silencing.

The silenced *SST* plants were also examined for changes in gene expression of other DP3 transcripts (Fig. 5B). The silencing of the *SST* expression did not disrupt wound-induced *LOX2* accumulation. Furthermore, the timing and the amplitude of *LOX2* induction were not affected by the absence of *SST* expression. The silencing of *SST* also failed to derail the wound induction of *ANS*. Its up-regulation appeared unaffected in both timing and extent by the depletion of *SST* transcripts in the transgenic plants. Therefore, northern blot analysis suggested that silencing of *SST* expression in wounded plants was specific for the expression of *SST*. Its absence did not directly affect the transcription of two other wound-induced genes. This also indicated that the *SST* protein is not normally involved in the transcriptional regulation of wound- and JA-induced signaling, since the levels of *LOX2* were unchanged in *SST*-silenced plants.

Sterol extraction from wounded SST-silenced plants

The sequence similarity of SST to known steroid sulfo-conjugating enzymes prompted a study of changes in steroid and steroid sulfonate content during the wound response in *A. thaliana*. Steroid levels in injured plants were monitored by comparing the

metabolic profiles of wounded *SST*–silenced plants with the profiles of unwounded and wounded controls. We predicted that if SST converts a specific steroid substrate into its sulfonated form, then levels of a specific steroid substrate in wounded wild-type plants should progressively decline. A corresponding accumulation of a sulfonated steroid compound should be detectable in the extracts of wounded wild-type plants. If SST can specifically modulate the level of a specific steroid, then in the wounded *SST*::RNAi transgenic plants the absence of *SST* expression should result in abnormally high levels of the steroid substrate and the emergence of a sulfonated steroid should be absent in the wounded *SST*-silenced plants. To investigate this, we profiled wound-induced changes in steroid and steroid sulfonate in wounded wild-type *A. thaliana* and transgenic *SST*::RNAi plants.

Transgenic and control plants were mechanically damaged by wounding three rosette leaves per plant. After six hours, the wounded plants were collected and flash frozen. Extraction of steroids was based upon protocols published describing methods for brassinosteroid isolation (Fujioka et al., 1996) with some modification. The extracts were submitted for analysis by liquid chromatography/mass spectrometry (LC/MS).

From the chromatograms, one prominent peak with a retention time at approximately 41 minutes was observed in both wounded samples of the control and the *SST*-silenced line (Fig. 6). It was annotated as methyl ester of linolenate, the major fatty acid component of plant cell membranes that is liberated by phospholipase A_2 and a substrate for lipoxygenase. (Conconi et al., 1996). Its presence in only the wounded samples confirmed that tissue damage was detected in both the transgenic and wild-type plants. A complex mixture, present in later fractions, was identified as a steroidal mix

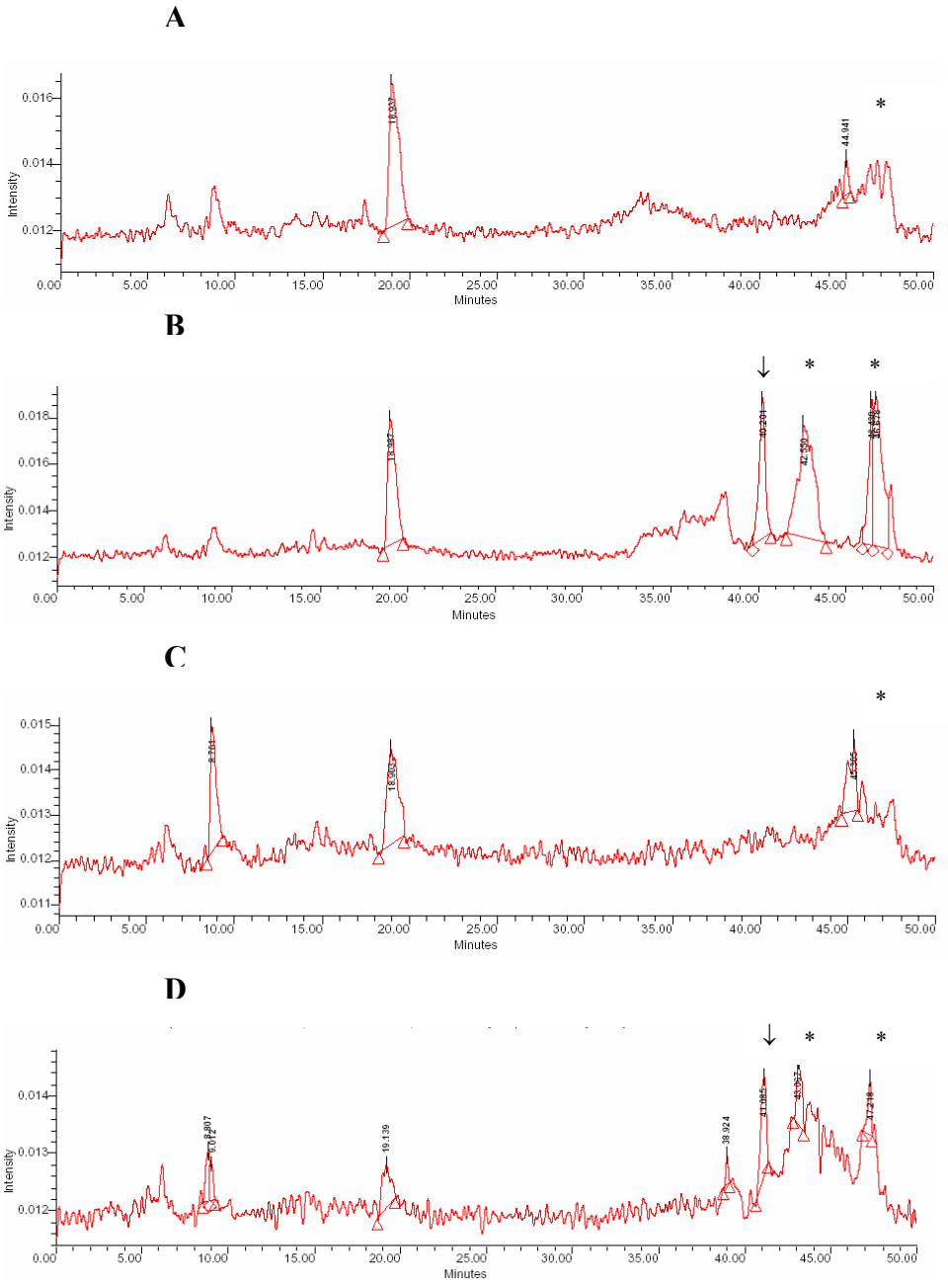


Figure 4.6 Metabolic profiling of the changes occurring in steroid content of wounded plants. Three leaves per plant were crushed with forceps. The aerial tissues were harvested six hours after imparting damage and then extracted for steroids. The extracts were analyzed LC-MS. A-unwounded vector control (pART27), B-wounded vector control plants, C-unwounded *SST*-silenced plants, D-wounded *SS*-silenced plants. The peak representing the steroid mix is represented with an asterisk (*) and the methyl linolenate by an arrow (↓).

(indicated by * in Fig. 6) by the LC/MS software (Waters Millennium®, v. 3.2; Wiley's Registry of Spectral Data, 6th edition). The compounds composing the steroidal mixture were tightly clustered, making detection and identification of the individual steroidal compounds too difficult to resolve. Further efforts to fractionate the mixture greatly reduced the efficiency of steroid isolation, as verified by the loss of injected estradiol in the spiked extracts. Other problems experienced during the profiling process include inconsistencies in the yield and repeatability. For example, the methyl linolenate peak characteristic of wounding was not reliably observed in all extracts from wounded tissues.

The metabolic profiles of wounded *A. thaliana* plants were inconclusive in determining whether *SST* regulates the availability of an active steroid in response to wounding. A hydroxylated form of JA (12-OH JA) was demonstrated to be sulfonated by SST in response to JA treatment (Gidda et al., 2003). However, the 12-OH JA and its sulfonated form were not publicly available. Using the published isolation method, we were unable to detect 12-OH JA or its sulfonated form in unwounded and wounded *A. thaliana* plants (data not shown). Therefore, we could not confirm that the function of SST is involved in hydroxyjasmonate metabolism.

Residual SST expression in SST-*silencing plants and wound-induced At5g07000*

Although complications with the analysis of steroid content in wounded *A. thaliana* may have played a role in not identifying an endogenous steroid for SST, other factors may have contributed. To determine whether this could be attributed to residual

SST expression that evaded targeted degradation in the *SST*::RNAi plants, wounded transgenic plants were tested more rigorously by RT-PCR for the presence of the *SST* transcript (Fig 7A). Both wounded wild-type and transgenic *SST*::RNAi plants demonstrated the presence of *SST* expression. The expression of SST in the transgenic silenced line was lower than wild-type levels. Its appearance, however, contradicts the northern blot analysis, which showed that *SST* expression was silenced.

An alternative explanation for the absence of a phenotype in the *SST*-silenced plants may have been partial compensation by a related steroid sulfotransferase. Expression of the most highly-related steroid sulfotransferase (At5g07000) in the *A. thaliana* genome was examined in wounded wild-type and transgenic plants by RT-PCR (Fig. 7A, B). The transcript encoding At5g07000 was slightly activated by wounding in wild-type plants. However, the expression of At5g07000 was noticeably weaker than the expression of *SST*. Moreover, targeted silencing of the *SST* gene did not affect the wound-induced accumulation of the At5g07000 transcript.

Discussion

In the previous study (Chapter 3), *SST* was isolated by subtractive hybridization from wounded *A. thaliana* plants and found to encode a putative steroid sulfotransferase. Expression of *SST* was induced by treatment with MeJA but absent in the wounded *coi1-1* mutant. Hence, *SST* relies upon the JA-dependent signal transduction pathway for its activation. The activation of *SST* also spreads to the distal uninjured leaves of wounded plants. Moreover, *SST* is not induced by SA. Microarray analyses have also demonstrated that *SST* is induced in response to herbivore feeding (Reymond et al., 2004)

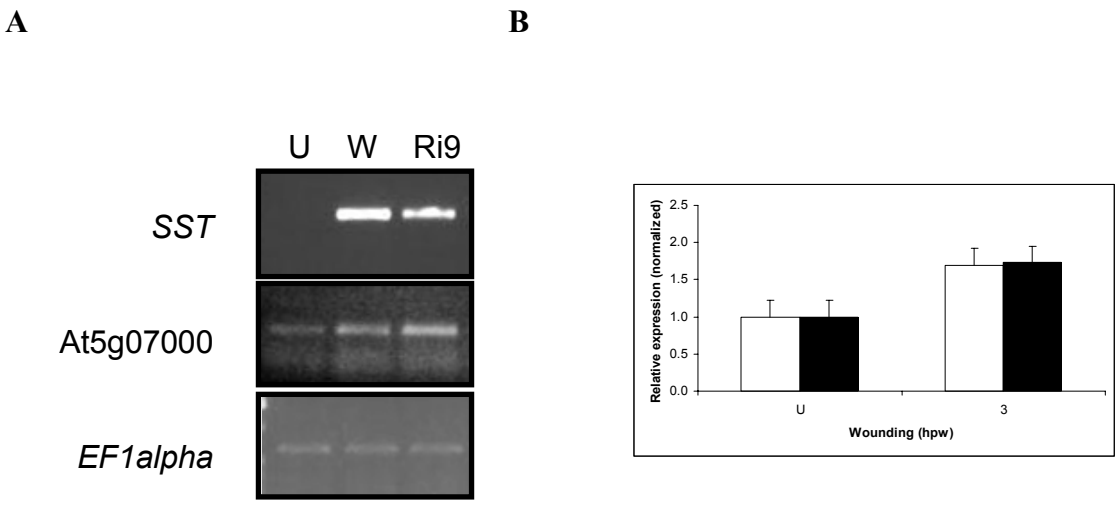


Figure 4.7 Expression of *SST* and At5g07000 in wild-type and *SST*-silencing plants. **A**. Wound-induced expression of *SST* and At5g07000 was examined by RT-PCR in wild-type and transgenic plants. Three leaves per plant were wounded and aerial tissues were harvested six hours later for analysis. Loading for each sample was normalized by the expression level of EF1alpha. U: Unwounded wild-type plants, W: Wounded wild-type plants, *Ri9*: wounded *SST*-silenced plants **B**. Quantitation of the wound-induced expression of At5g07000. Open bars: wildtype, Black bars: RNAi-silenced transgenic plants.

and exposure to Yariv reagent, which triggers a wound-like response, in tissue culture cells (Guan and Nothnagel, 2004).

The annotation of SST and At5g07000 as a steroid sulfotransferase and insight into a possible functional role in the wound response was examined by using protein sequence databases and sequence analysis tools. The *A. thaliana* genome contains 14 annotated steroid sulfotransferases. SST is most highly related to At5g07000 (Fig. 1), whose locus is located next to *SST* on chromosome five. Both SST and At5g07000 contain signature sequences of PAPS-binding proteins and residues critical for steroid binding and sulfonate transfer (Fig. 2). Both are more evolutionarily related to hEST than to BnST3 and any other *A. thaliana* sulfotransferase gene (Fig. 4). Therefore, it is possible that SST functions as a wound-specific steroid sulfotransferase in *A. thaliana*. Possibilities exist that SST may not represent a steroid sulfotransferase. PAPS binding sites are a common motif found in other types of sulfotransferases. Moreover, substrate prediction by phylogenetic methods may be inconclusive as very few of the substrates for steroid sulfotransferase in plants are known. Consequently, a pattern connecting the individual phylogenetic clades with specific substrates may be presumptive.

An *in vivo* study of SST was carried out in an effort to confirm its bioactivity as a steroid sulfotransferase and to identify its putative steroid substrate and sulfonated product. Wild-type plants and transgenic plants, engineered to suppress *SST* expression (Fig. 5B), were analyzed for changes in the steroid profile of wounded *A. thaliana* plants by metabolic profiling (Fig. 6). However, we were unsuccessful in generating coherent steroid profiles from the plant tissues. Specifically, interpretation of the chromatograms of the steroid extractions was hindered by the inability to reliably resolve the steroid

fraction into its individual components and changes in steroid content could not be detected due to this tight clustering. The cluster of co-eluted steroid compounds could have obstructed the SST-mediated depletion of the steroid substrate during the wound response, especially if the substrate represents a negligible portion of the total steroid content in plant tissues. No decisive conclusion can be generated from the steroid profiling.

In addition, other complications may have exacerbated the steroid analysis. One possibility is that the silenced line was producing minor amounts of the SST protein due to the incomplete silencing of the *SST* gene (Fig. 6A). Consequently, in the *SST*-silencing line, the leaky expression of the endogenous *SST* gene may have maintained partial SST bioactivity. Confirmation whether SST was silenced must be determined by measuring protein levels of SST by western blot analysis. The residual expression of *SST* may have been sufficient in furnishing sufficient SST activity for the wound response in the silenced line. Consequently, phenotypic differences between the transgenic line and control plants might be indistinguishable. The absence of SST activity may have also been partially compensated by a closely related steroid sulfotransferase. Sequence analysis demonstrated the presence of a possible paralog (At5g07000) to *SST* in the *A. thaliana* genome (Fig. 1). Given their relationship, they may also share similar substrates. The At5g07000 is modestly wound-induced suggesting that it may also play a role in wound response (Fig. 7A, B). It is possible that At5g07000 protein could have accepted and sulfonated the natural steroid substrate for SST and compensated for the absence of SST activity. Based on *in vitro* evidence, some steroid sulfotransferases are promiscuous enzymes which exhibit relaxed substrate specificity (Glatt et al., 2001).

Thus differences between the control and the transgenic silencing plants may have been indistinguishable as evaluated by the steroid profiles.

Alternatively, the absence of any identifiable role of *SST* in wounded plants may reflect that it does not function specifically in the wound response. Perhaps SST normally performs a role in growth and development but in wounded leaves, its activation may be an indirect consequence of the release of *de novo* JA. A second reason may involve the multiple contributions of other signaling pathways. Since only SA-treated plants were used as a reference for RDA, SST may be involved in an ancillary pathway, like abiotic stress response, metabolic reconfiguration, or secondary metabolism. Thus, SST and the other DP3 genes (*ANS*, *THT*) may in fact not truly represent wound-specific genes.

Role of steroid sulfotransferases

In animals, sulfotransferases conjugate a sulfonate group (SO_3^{-1}) to a variety of acceptor molecules including hormones, neurotransmitters, and drugs (Hobkirk, 1985; Strott, 1996). Sulfoconjugation of the acceptor steroid occurs by esterification at an alcohol group (*O*-sulfonation) and subsequently alters the physicochemical nature of the substrate. For example, sulfonation increases the water solubility of the acceptor, alters its conformation, and transforms the lipophilic character of the steroid to amphilic. The presence of the sulfonate also ensures that in most biological conditions the sulfonated steroid maintains its fully ionized form at all pHs due to its pK_a <1.5 (Huxtable and Lafranconi, 1986).

Mammalian studies have shown that sulfonation is an integral process for several different aspects of growth and development. Glycosaminoglycans and proteoglycans are sulfonated macromolecules that compose the connective tissue and cell surface of mammalian tissues and affect cation composition, tissue elasticity and hydration (Kjellen and Lindahl, 1991). They bind cell surface receptors, growth factors, enzymes and proteins in the extracellular matrix and participate in transmembrane signaling (David, 1993). Sulfonation of the sugar residues of glycoprotein hormones can affect their bioactivity (Baenziger, 2003). Sulfoglycolipids are components of several tissues and function by interacting with proteins of the extracellular matrix (Farooqui and Horrocks, 1985). Post-translational sulfonation of Tyr residues can affect the functionality of membrane and secreted proteins (Moore, 2003). Sulfonation is also an important process that modifies cholesterol and its derivatives (Strott and Higashi, 2003). Lastly, it also performs a critical task in the biotransformation of certain low-weight molecules (Falany, 1997).

The transformation of a steroid by sulfonation can have two possible effects. In some cases, bioactivity is provoked by sulfonation of a steroid substrate. For example, the sulfonation of pregnenolone causes novel neuroexcitatory activity that antagonizes the γ–aminobutyric acid receptor $(GABA)_A$ receptor, which controls chloride channels (Lambert et al., 1995; Rupprecht, 1997). Sulfonation can also inhibit the bioactivity of a steroid by preventing the binding of the steroid to its cognate nuclear receptor. The human estrogen sulfotransferase (hEST) in tandem with its sulfatase regulate levels of free 17β-estradiol in normal breast tissue (Adams et al., 1988; Pasqualini et al., 1992). Absence of hEST activity has been correlated with enhanced cancer risk due to an

overabundance of unconjugated estrogens (Pasqualini et al., 1992; Anderson and Howell, 1995). Therefore, hEST is essential for preventing unmitigated hormone-dependent cell growth.

Our understanding of steroid sulfotransferases in plants is limited. BnST3 from *Brassica napus* is an SA-inducible steroid sulfotranferase, which preferentially (but not exclusively) sulfonates *in vitro* 24-epibrassinolide (Rouleau et al., 1999). While exogenous 24-epibrassinolide promotes elongation of bean nodes, the sulfoconjugated form prevents the growth-promoting activity of its desulfonated form. This demonstrates that sulfonation in plants can regulate bioactivity of a plant hormone. The induction of *BnST3* by SA suggests that BnST3 may play a role in response to pathogen attack. The authors of this study reason that during pathogen attack sulfonated epibrassinosteroid reduces the vulnerability of the healthy tissues to subsequent attack by preventing the loosening of the cellulose microfibrils which occurs during cell growth. Thus, sulfonation serves as a critical mechanism for signaling in biotic stress responses. The ortholog (At2g03760) of *BnST3* in *A. thaliana* is also involved in pathogen defense (Lacomme and Roby, 1996) but the identity of the ligand for At2g03760 is still unknown (Lacomme and Roby, 1996; Smith et al., 2004). However, the evidence with BnST3 shows that in higher plants, steroid sulfotransferases act to regulate bioactive molecules similarly to steroid sulfotransferases in mammalian systems.

Recently a sulfotransferase (*AtST5a*) was found to respond to coronatine and wounding in *A. thaliana* (Piotrowski et al., 2004). *AtST5a* is annotated as belonging to the flavonol-type class of sulfotransferases. *AtST5a* is expressed in both systemic and locally-wounded leaves. Treatment with octadecanoids and ethylene to *A. thaliana*

leaves activate *AtST5a* expression whereas SA does not. *AtST5a* functions as a desulfoglucosinolate sulfotransferase (Piotrowski et al., 2004), which catalyzes the last biosynthetic step in the production of glucosinolates. Glucosinolates are secondary nitrogenous compounds that possess antimicrobial properties and may be toxic to herbivores (Brader et al., 2001; Tierens et al., 2001; Halkier and Gershenzon, 2006). Therefore, sulfotransferases may also perform biosynthetic function in higher plants. This result also demonstrates that sulfotransferases may be involved in the wound response in *A. thaliana*.

Hydroxyjasmonates as substrates for SST

SST reportedly sulfonates *in vitro* 11- and 12-OH JA (Fig. 8) (Gidda et al., 2003). The levels of 12-OH JA and 12-OH JA sulfonate increase in 12-OH JA- and JA-treated *A. thaliana* plants. Moreover, the *SST* transcript is induced by JA and 12-OH JA. Addition of 12-OH JA fails to induce the JA-, wound- and pathogen-inducible thionin (*Thi2.1*) transcript. The effect of wounding on 12-OH JA sulfonate levels was not tested. The assertion that the natural ligand for SST is a metabolite of JA is still unsettled as *in vivo* data are lacking. Moreover, the *in vitro* characterization of SST may not reflect its true activity in plants. Second, data were not provided in this report regarding the acceptability of other substrates for SST, especially since some sulfotransferases are known to accommodate multiple substrates. Attempts to confirm the presence of 12-OH JA and 12-OH JA sulfonate in MeJA- and wound-treated plants were not successful (data not shown).

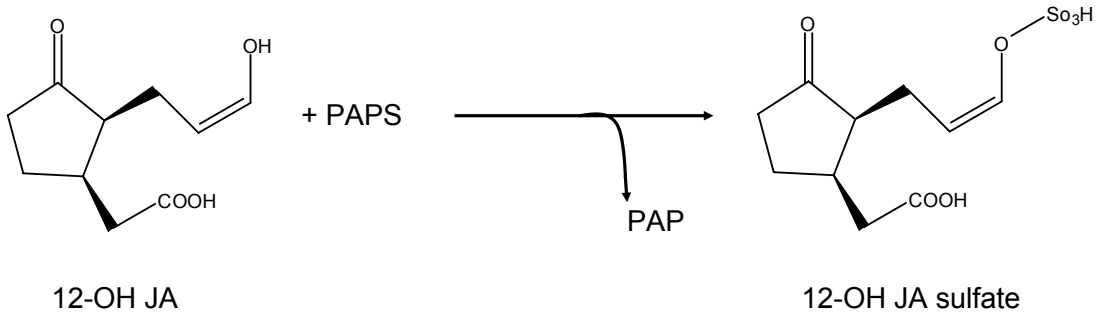


Figure 4.8 Proposed substrate for *SST* (*AtST2a*) is 12-OH JA.
Gidda et al (2003) demonstrated that *AtST2a* catalyzes the sulfonation of 12-OH JA. PAPS acts as an activated donor for the sulfonate.

Though the functional role of 12-OH JA in *A. thaliana* has not been determined, hydroxyjasmonates have been detected in other higher plants. Hydroxylated forms of JA are secreted into the liquid media of tobacco BY-2 cells (Swiatek et al., 2004). The absence of 11- and 12-OH JA in the cell extracts implies that they may function in long-distance signaling or represent modified JAs that are eliminated from the cell. In tubering plants, 12-OH JA and its glucoside (tuberonic acid) stimulate the *in vitro* tuberization of potato stem nodes (Koda et al., 1988; Yoshihara et al., 1989). Tuberonic acid is detected in the leaves of potato and proposed to function as a photoperiod-dependent signal that is mobilized from the foliar tissues to induce tuberization at the stolon (Koda et al., 1988; Yoshihara et al., 1989). However, the tuber-inducing effects of 12-OH JA and its glucoside have yet to be demonstrated in soil-grown plants and the functional role of 12-OH JA in non-tuber forming plants is still unknown. If hydroxyjasmonates are indeed the genuine substrate for SST, then perhaps in non-tubering plants, sulfonation is a mechanism to create a reservoir of inactive jasmonates (Schilmiller and Howe, 2005). Although the mobility of hydroxyjasmonate is not known in plants, they could also function as mobile sources of jasmonates for systemic tissues.

Absence of At5g07000 silencing

The suppression of *SST* accumulation in wounded transgenic plants failed to affect the weakly-expressed paralog, At5g07000 (Fig. 7). Gene-specific suppression among highly-related gene family members using a single RNAi construct has been demonstrated in the Os*RAC* genes of rice where the 3' ends of the coding region and 3' UTRs with only 44-52% in sequence identity were used as templates for the RNAi

constructs (Miki et al., 2005). The template used for the *SST*::RNAi construct was generated from the subtracted RDA fragment. The region is nearly identical to the corresponding region in At5g07000 except for an insertion that is absent in the corresponding region in At5g07000. However, the similarity is not sufficient in silencing the A5g07000 transcript.

In plants and nematodes, siRNAs can be generated from sequences upstream of the targeted region of a transcript (Sijen et al., 2001; Braunstein et al., 2002; Alder et al., 2003). Plants also produce siRNAs downstream of the targeted region (Braunstein et al., 2002; Vaistij et al., 2002). These secondary siRNAs have been shown to silence genes non-homologous to the silencing templates. Amplification of silencing mediated by these secondary siRNAs is called transitive silencing. Transitive silencing has been demonstrated with transgene sequences but not with endogenous genes. Silencing of non-targeted genes may depend upon the extremely robust levels of expression of the targeted gene (Vaistij et al., 2002; Himber et al., 2003; Van Houdt et al., 2003; Miki et al., 2005). Thus, the low basal expression of At5g07000 may have prevented its indirect silencing in the transgenic *SST*::RNAi plants.

Substrate Plasticity of Sulfotransferases

Unlike the paradigm of "lock and key" enzyme selectivity for a unique substrate, steroid sulfotransferases exhibit substrate plasticity (Falany, 1997). Some sulfotransferases are capable of transferring sulfonates to a variety of structurally unrelated molecules. For example, SULT1A1 is a human-derived sulfotransferase that metabolizes a variety of xenobiotics (eg. *p*-nitrophenol) but also displays substrate

flexibility in the metabolism of iodothronines and estrogens (Young Jr et al., 1988; Falany and Kerl, 1990; Walle and Walle, 1992; Falany et al., 1994). The L-shaped active site of SULT1A1 can accommodate disparate structures ranging from small planar structures to the fused ring compounds of steroids (Gamage et al., 2003; Gamage et al., 2005). The plasticity of SULT1A1 is witnessed in the crystal structure of SULT1A1 complexed with PAPS and 17β-estradiol (Gamage et al., 2005). The active site of SULT1A1 contorts from its native L-shaped pocket to accept the fused ring structure.

The substrate promiscuity of the sulfotransferases complicates the pursuit to definitively identify their cognate *in vivo* substrate. Adaptability of plant sulfotransferases to metabolize several candidate substrates has also been documented (Rouleau et al., 1999) demonstrating versatility similar to other eukaryotic sulfotransferases. Thus, identification has been limited to their study based on *in vitro* conditions. For example, the BnST3 enzyme sulfonates several different candidates with varying efficiencies (Rouleau et al., 1999). Confirmation that the putative substrates are the natural ligands for the steroid sulfotransferases in plants is lacking and the functional role of steroid sulfotransferases is unknown.

The difficulty of identifying an endogenous substrate for SST may be partially attributed to the tolerance of steroid sulfotransferases to accept multiple substrates. This may have been the case for the analyses with the wounded *SST*-suppressed transgenic line. The putative paralog, At5g07000, or other sulfotransferases could have assumed SST function by sulfonating the *in vivo* substrate for SST. As demonstrated with hSULT1A1, even non-steroid sulfotransferases can accept steroids into their active site.

Role of Brassinosteroids

Knowledge of steroid synthesis and signaling in higher plants is limited to the study of brassinosteroids. Brassinosteroids are polyhydroxylated steroid compounds that regulate growth and development (Fujioka and Yokota, 2003; Vert et al., 2005; Belkhadir and Chory, 2006). Brassinosteroids are perceived by the plasma membrane-bound *BRI* receptor, a serine/threonine-type receptor kinase (Li and Chory, 1997). Activation of BRI is triggered by binding with the brassinolide ligand, the most active of the known brassinosteroids and sets in motion a downstream phosphorylation cascade (Li and Chory, 1997; He et al., 2002a; Li and Nam, 2002; Li et al., 2002b; Nam and Li, 2002; Li, 2005).

Whether the ligand for SST could be a brassinosteroid is still undetermined. One prediction is that SST may modulate brassinosteroid homeostasis during the wound response. Inactivation of brassinosteroids is demonstrated in the *bas1D* mutant in *A. thaliana* (Neff et al., 1999). The *BAS1* locus encodes a steroid hydroxylase (CYP72B1). Mutant *bas-1D* plants hyperaccumulate 26-hydroxybrassinosteroid and are deficient of bioactive brassinolide. Consequently, the *bas1-D* mutant phenotypically resembles brassinosteroid-deficient plants. In addition, inactivation of brassinosteroid by BnST3 occurs in plants responding to biotic stress (Rouleau et al., 1999). Perhaps the growth prohibitive effect by brassinosteroid sulfonation is relevant in wound response. The sulfonation of a brassinosteroid could function to suppress its activity and, as a consequence, prevent cell growth during wound recovery. Though knocked down expression of *SST* did not appear to affect gross morphology or developments relative to wild-type or vector controls, subtle differences in these transgenic plants may exist,

affecting brassinosteroid-dependent processes (e.g. cell expansion, germination, height, root growth, seed viability).

CHAPTER FIVE

ISOLATION AND CHARACTERIZATION OF WOUND-DEPLETED TRANSCRIPTS IN *ARABIDOPSIS THALIANA*

Introduction

Response to tissue damage in higher plants is not solely restricted to the wounded leaf. Early work showed that the unwounded remote (systemic) leaves could generate wound-responses similar to those occurring in the injured (local) leaf (Green and Ryan, 1972; Leon et al., 2001; Schilmiller and Howe, 2005). Several different mechanisms (ABA, hydraulic, electrical) have been proposed to be involved in long-distance wound signaling (Wildon et al., 1992; Pena-Cortes et al., 1995; Malone, 1996; Birkenmeier and Ryan, 1998). However, the likely systemic wound signal is a JA-related molecule (Schilmiller and Howe, 2005). To date, known systemically-induced wound-responsive genes are concomitantly activated in local leaves. This chapter describes the search for a novel class of systemically-induced wound genes (*SYST*s) whose activation is expressed exclusively in the systemic leaves of wounded *A. thaliana*. *SYST* genes were isolated by RDA. Unexpectedly, these genes corresponded not to systemically-induced genes but to genes that are instead wound-depleted in injured leaves. The reduction of one of the *SYST*s (*SYST1-3*) occurred within five minutes of injury while systemic levels were unaffected by wounding. The reduced expression of *SYST1-3* was maintained for at least four days after wounding. Known regulators of wound response (JA, ethylene, oligogalacturonans) and other plant hormones were ineffective in triggering *SYST1-3* depletion. Expression of *SYST1-3* was detected in three-week old plants and the younger

tissues of mature *A. thaliana* plants but not in six-week old vegetative leaves indicating that SYST1-3 may play a role in growth and development. The At5g16250 gene product is highly related to *SYST1-3* and its transcript is similarly depleted in wounded leaves suggesting that a *SYST1-3*-like gene family may be coordinately regulated by wounding. *KAT5*, which encodes a 3-ketoacyl-CoA thiolase, was reported to be a systemic-specific wound-induced gene (Cruz Castillo et al., 2004), but we found that the wound-induction of *KAT5* was expressed in locally-wounded leaves. The depletion of SYST1-3 and similarly regulated genes may represent a still uncharacterized wound-regulated pathway which operates in locally-wounded tissues.

Results

Isolation of SYST *candidates*

For the isolation of wound-responsive, systemic-specific genes, mechanically-wounded *A. thaliana* leaves and their corresponding unwounded distal leaves were harvested at one, three, and six hours after wounding and collected into separate local and systemic pools. Poly-$(A)^+$ RNA was purified from the local and systemic tissues. Three rounds of RDA were performed (Hubank and Schatz, 1994) using tester:driver ratios as described (Chapter 2) with the cDNAs from systemic tissues as the tester library and wounded leaves as the driver representation. The enriched DP3 cDNAs were released from the tester-specific adaptors by *Bam*HI digestion and then ligated into the vector pZero-1. Plasmids were harvested from overnight cultures of zeocin-resistant DH5α colonies. Inserts from these plasmids were manually sequenced using T7 and T3 primers.

Screening of 26 total candidates from the DP3 round of subtraction, collectively called *SYST*s, resulted in a highly diverse population composed of fifteen different genes (Table 1). The identities of the subtracted genes were determined by BLAST searches through NCBI (http://www.ncbi.nlm.nih.gov/) or TAIR (http://www.arabidopsis.org/). The most highly represented gene was a putative xyloglucan endotransglycosylase (*XTH9*, At4g03210) which was encountered five times (5/26). Two genes encoding ribosomal proteins (6S ribosomal (*L27*) protein, At3g22230; *RPP2C*, At3g28500) were represented in the DP3 population. The *L27* ribosomal gene was the second most represented candidate (4/26) and the acidic ribosomal (*RPP2C*) was represented twice. A peroxidase (*PER64*, At5g42180) appeared three times (3/26). An unknown expressed protein (At3g09860) was also isolated twice.

The remaining genes were singletons in the DP3 population. Two subtracted genes (At3g02640, At5g46020) were not functionally assigned by either NCBI or TAIR and their functions remain unknown. An SMC domain-containing protein (*ATSMC3*, At5g48600) and a small nuclear ribonucleoprotein E (At2g18740) were detected in the third subtraction (Lam et al., 2005). Both are predicted to function in the nucleus. A cysteine proteinase inhibitor (At2g31980) was identified as an insoluble cystatin. Two genes were isolated that are involved in light response and photosynthesis. One (At4g37590) is a member of the phototrophic NPH3 family of proteins whose expression is light stimulated. The other (At2g25060) encodes a plastocyanin-containing domain involved in electron transport (Borner et al., 2003). Three genes are involved in signal transduction. A putative GTPase involved in tRNA modification was isolated (At1g78010). A protein product for At5g20150 containing an SPX domain was isolated.

SPX domain-containing proteins are involved in phosphate transport and sensing. Finally, a calmodulin binding protein (At3g15050) was represented in the DP3 population (Abel et al., 2005).

Relative to the wound *versus* SA subtraction previously characterized (Chapter 3, Table 1), the *SYST* collection derived from the systemic tissues was much more diverse as 15 different genes were isolated and singly-represented genes (10/26) accounted for the majority of the heterogeneity. The large number of singletons encountered indicated that only a small portion of the total number of subtracted genes present in the DP3 was identified. Also no clear *SYST* was clearly overrepresented relative to the other *SYST*s suggesting that the DP3 collection was still highly complex. Remarkably absent from this systemic DP3 population were any known wound-responsive genes (e.g. JA biosynthetic enzymes, *VSP)*. Moreover, the microarray studies of the wound response did not identify any of the *SYST* genes as wound-induced. For these reasons, further sequencing of the cloned cDNAs was ceased in order to determine the molecular basis for these unexpected results.

Subtracted cDNAs represent wound-depleted transcripts specific to damaged leaves

The *SYST* genes were predicted to represent transcripts that are induced exclusively in the systemic tissues of wounded plants. To test this, tissues from wounded leaves and the uninjured distal tissues were harvested in a time-course experiment and analyzed by northern blot hybridization. Radiolabeled probes were generated from DP3 cDNAs. Based on northern blot analysis using both total and purified poly-$(A)^+$ RNA, wound-specific induction of the *SYST* genes could not be detected in systemic tissues

Subtracted cDNA	Gene	Locus	*n*	Purported Function
Xyloglucan endotransglycosylase	*XTH9*	At4g03210	5	Cell wall rearrangement
60S ribosomal protein L27		At3g22230	4	Translation
Peroxidase 64 (PER64)	*PER64*	At5g42180	3	Responds to oxidative stress
Expressed Protein		At3g09860	2	
60S acidic ribosomal protein P2	*RPP2C*	At3g28500	2	Translation
Cystein proteinase inhibitor	Cystatin	At2g31980	1	Inhibits peptidases
Calmodulin-binding family protein		At3g15050	1	Ca^{2+}-mediated signaling
Plastocyanin-like domain-containing protein		At2g25060	1	Electron transport
SPX domain-containing protein		At5g20150	1	G-protein associated signaling
Small nuclear ribonucleoprotein E	*snRNP-E*	At2g18740	1	RNA processing
SMC family protein	*ATSMC3*	At5g48600	1	Chromosome separation
GTPase		At1g78010	1	tRNA modification
NPH3 family domain protein		At4g37590	1	Light-responsive signaling
Expressed Protein	*SYST1-3*	At3g02640	1	
Expressed Protein		At5g46020	1	

Table 5.1 List of subtracted cDNAs isolated from the unwounded systemic tissues of wounded *Arabidopsis* leaves. *n*-number of times a cDNA was recovered from the DP3 pool.

(data not shown). Expression of the *SYST*s was also not detected in these northern blot analyses in either damaged leaves or unwounded plants.

Because expression of the DP3 clones was not detected by northern blot analysis in locally-wounded or systemic tissues, an alternative approach using RT-PCR was adopted. Primers were designed for selected *SYST*s (see Appendix A). A control gene (*EF1*α) was used to normalize for discrepancies between samples in cDNA synthesis, PCR amplification and gel loading. The results from RT-PCR yielded an unexpected finding (Fig. 1). Rather than exhibiting up-regulated expression in systemic tissues, the *SYST* transcripts were instead down-regulated in wounded leaves. The level of the *SYST* transcripts in the systemic leaves did not change. Transcript levels of cDNAs encoding *PER64*, cystatin, expressed protein (*SYST1-3*), and *XTH9* were reduced within one hour of injury. The diminished transcript levels were sustained for at least six hours after wounding in the damaged leaves. In the corresponding unwounded systemic leaves, expression of these transcripts remained unchanged. Therefore, the *SYST* genes do not represent the systemically-expressed wounding genes. On the contrary, *SYST*s are weakly expressed genes whose expression is rapidly reduced in the wounded leaves.

Reduction of SYST1-3 *is rapid and sustained in injured leaves*

A time-course experiment was conducted to measure the rate and duration of wound-depletion for the *SYST* transcripts. Levels of *SYST* expression were determined by semi-quantitative RT-PCR (sqRTPCR). The linear phase of PCR amplification for each *SYST* primer pair was ascertained by sampling amplification every four cycles (until 36 cycles) for each primer pair. After observing consistent wound-specific reduction in

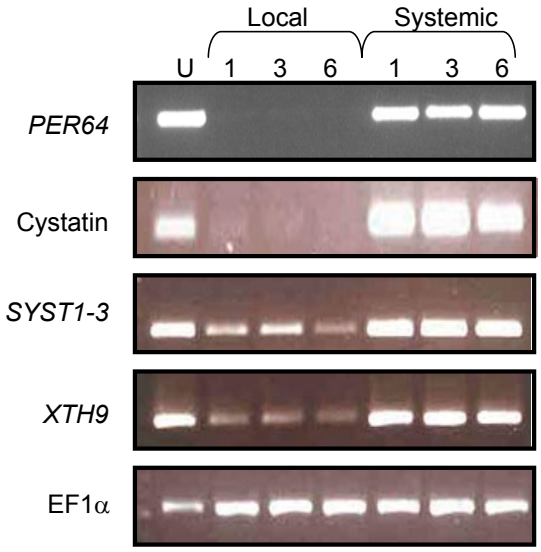


Figure 5.1 Expression of select DP3 genes in locally-wounded and unwounded distal *Arabidopsis* leaves. Wounded leaves and the distal tissues were separately harvested and processed for RNA. Expression of the DP3 genes was determined by RT-PCR. Amplified fragments were detected on a TBE-containing (1.2%)–agarose gel. The level of EF1α transcript in each sample was used to compare for loading among the samples. U-unwounded leaves, L-wounded leaves, S-systemic leaves, with corresponding number representing hours post-wounding.

the expression of these *SYST* transcripts in three independent experiments, the depletion of several DP3 genes (e.g. *XTH9*, cystatin, *PER64*) could not be verified in subsequent trials. However, these studies showed that *SYST1-3* expression was the most consistently reduced by wounding. Consequently, *SYST1-3* was used for further characterization of this wound-regulated depletion. Relative amounts of *SYST1-3* were first determined by normalizing expression with respect to the *EF1α* transcript and then expressed as a ratio relative to the basal level in the unwounded sample. The normalized *SYST1-3* expression in unwounded controls was set to a relative value of one.

A time-course experiment investigating the immediate effects of wounding showed that the reduction of the *SYST1-3* transcript in the injured leaf was extremely rapid (Fig 2A). Within five minutes of tissue damage, the level of *SYST1-3* expression dropped to approximately 20% of unwounded levels and remained at this level for the duration of the experiment. Systemic expression levels measured one hour after wounding did not change relative to the unwounded control from the tissue damage. Thus, the depletion of the *SYST1-3* transcript was a rapid response restricted to the damaged leaf.

To determine the duration of this response, the level of *SYST1-3* expression was monitored by sqRTPCR in mechanically-damaged leaves of *A. thaliana* plants for four days following the initial wounding (Fig. 2B). In these damaged leaves, the down-regulated expression of *SYST1-3* never recovered during the four days of monitoring. The reduced level of *SYST1-3* in the wounded leaves contained approximately 20-30% of the amount detected in unwounded leaves. Thus, the wound-triggered reduction of the *SYST1-3* transcript was a rapid and sustained response in the wounded leaf.

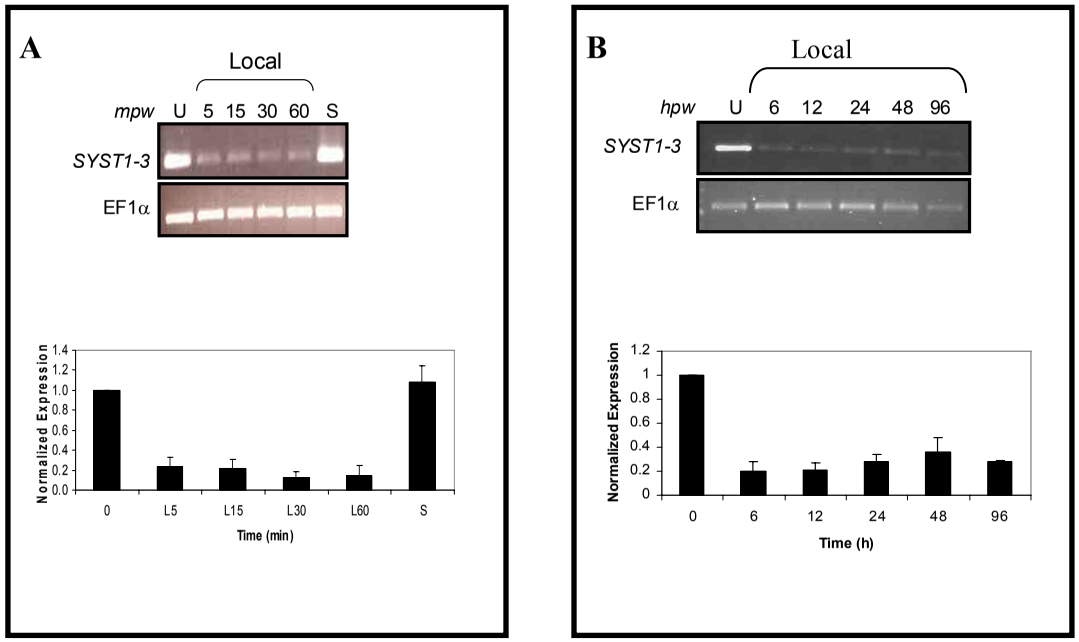


Figure 5.2 Quantitation of *SYST1*-3 transcripts in wounded *Arabidopsis* leaves by semi-quantitative RT-PCR (sqRTPCR). Wounded leaves were harvested in two separate time-courses to determine the duration of the wound-reduction transcript of *SYST1-3*. Level of transcripts were analyzed by gel electrophoresis and measured by Scion Image. Quantitation is depicted in bar graph below the autoradiogram. EF1α was used to normalize discrepancies among samples. Relative amount of *SYST1-3* was calculated based on the expression in unwounded controls, which was set at a value of 1. The standard deviation is indicated by the error bars. **A**. Early response time-course, *mpw*-minutes post-wounding, U-unwounded, S-systemic **B**. Extended time course in wounded leaves, *hpw*-hours post-wounding

Depletion of the SYST1-3 *is independent of JA and ethylene signaling*

Response to wounding in the injured leaf involves the interaction of competing signaling pathways (Chapter 1, Fig. 3) (Knight and Knight, 2001; Pieterse et al., 2001; Kunkel and Brooks, 2002; Reymond et al., 2004). To determine whether the JA (Farmer et al., 1992; Farmer and Ryan, 1992) or ethylene (O'Donnell et al., 1996) signaling pathway contributes to the reduction of the *SYST1-3* gene in mechanically wounded leaves, sqRTPCR was conducted to determine levels of *SYST1-3* in the *jar1* (Staswick et al., 1992) and *coi1-1* (Feys et al., 1994) mutants, which regulate JA signaling, and the *ein2-1* mutant (Alonso et al., 1999), which is a signal transducer of ethylene perception.

Leaves from *jar1, ein2-1* and wild-type plants were wounded and tissue was collected from local and systemic parts of the plants (Fig. 3A). Transcript levels of *SYST1*-3 were measured in the local and systemic tissues of wild-type and mutant plants by sqRTPCR. The levels of *SYST1-3* transcript in local leaves three hours after wounding showed no differences in *SYST1-3* depletion among the *jar1*, *ein2-1* and control plants. In each mutant background, the amount of *SYST1-3* transcript in locally-wounded tissues dropped to about 25% of unwounded levels. Systemic levels of the *SYST1-3*, sampled at three hours after tissue damage were unaffected in the mutants and wild-type plants and were present at levels similar to unwounded controls. Therefore, lesions in the *JAR1* and *EIN2* loci did not affect wound-depletion of the *SYST1-3* in injured leaves. The expression of *SYST1-3* was also monitored in another JA-signaling mutant, *coi1-1* (Fig. 3B) (Xie et al., 1998). At three hours after wounding, the *SYST1-3*

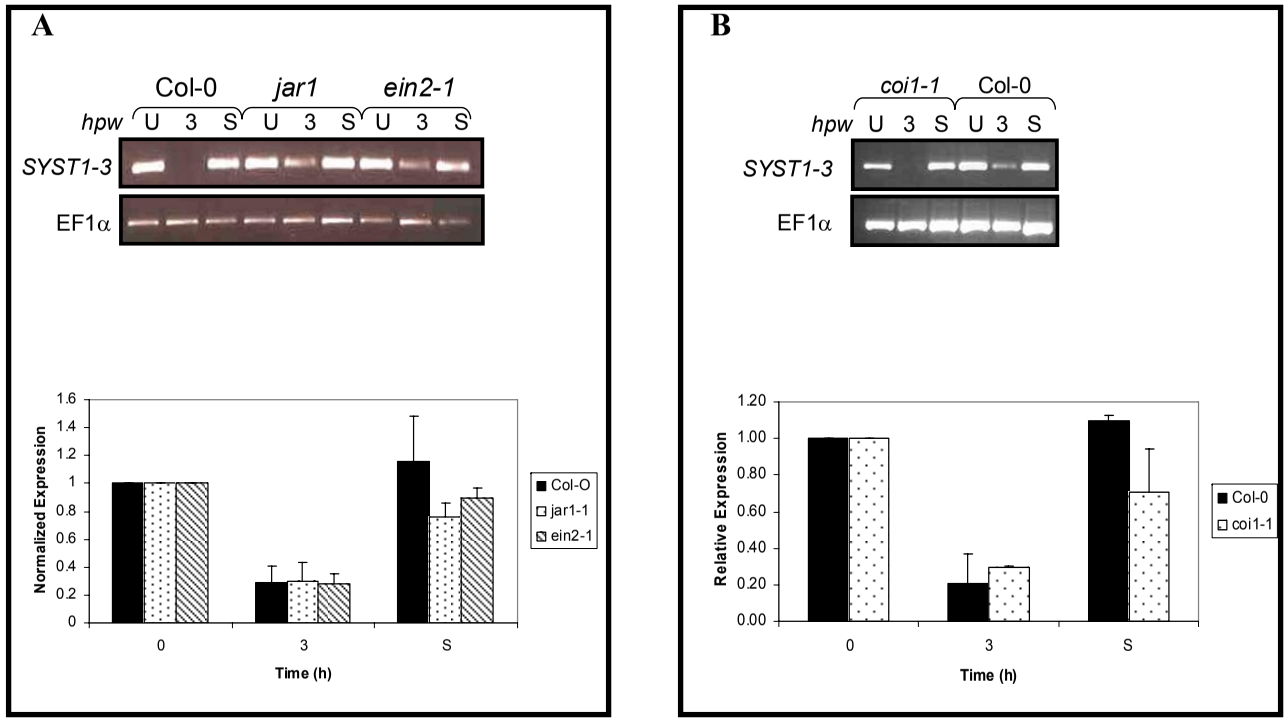


Figure 5.3 Expression of *SYST1*-3 in the *jar1*, *ein2-1* and *coi1-1* mutant backgrounds.
Wounded leaves and unwounded systemic leaves from mutant lines and wild-type plants were collected three hours after wounding. Levels of *SYST1*-3 were measured relative to unwounded plants by sqRTPCR. Quantitation is depicted by a bar graph. EF1α was used for normalization among the samples. The standard deviation is indicated by the error bars. **A**. Expression of *SYST1*-3 in the *jar1* and *ein2*-1 mutant background. **B**. Expression of *SYST1*-3 in *coi1-1* mutant. U-unwounded leaves, S-systemic leaves, *hpw*-hours post wounding

transcript levels in injured levels declined to ~25% of the levels present in unwounded tissues in both the *coi1-1* and wild-type plants. This reduction was comparable to those observed in *jar1* and *ein2-1* mutants. Systemic accumulation of *SYST1-3* was not affected by the *coi1-1* mutation. Thus the reduction of *SYST1-3* gene expression in wounded leaves was neither regulated by the JA nor ethylene signaling pathways. These pathways also did not regulate the basal levels of these transcripts in unwounded plants or the systemic levels of *SYST1-3* in injured plants.

Effects of elicitors and plant hormones on SYST1-3 *expression*

Since JA or ethylene was not involved in the reduction of *SYST1-3* transcripts in wounded leaves, other signaling pathways were examined. Some wound-induced genes are also triggered when treated with plant- or fungally-derived elicitors (Choi et al., 1992; Choi et al., 1994; Doares et al., 1995a; Ellard-Ivey and Douglas, 1996; Rojo et al., 1999). Elicitor-triggered gene expression in *A. thaliana* delays the induction of JA-and wound-dependent transcripts through the activity of ethylene (Chapter 1, Fig. 4A) (Rojo et al., 1999). Moreover, liberated fragments of the cell wall possess limited mobility and consequently elicitor-dependent gene responses are typically restricted to the injured leaf (Baydoun and Fry, 1985). Oligogalacturonans (OGA), an elicitor derived from the cell wall of plants, was previously shown to repress JA-induced wounding genes in the wounded leaf (Rojo et al., 1999). Therefore, we investigated whether OGA or fungally-derived chitosan (CS) could elicit a decline in *SYST1-3* levels in injured leaves. CS is equally effective in triggering wound responses in other higher plants (Doares et al.,

1995a). In *A. thaliana* seedlings treated with elicitors, levels of *SYST1-3* transcripts were unaffected (Fig. 4). This suggested that OGA and CS did not mediate the *SYST1-3* reduction in wounded leaves. However, the possibility exists that other elicitors may trigger the wound-induced reduction of steady-state *SYST1-3* expression.

In a similar assay, plant hormones were tested for their effect on *SYST1-3* expression in liquid grown seedlings (Fig. 4B). Abscisic acid (ABA); 1-aminocyclopropane-1-carboxylic acid (ACC), a bioactive precursor of ethylene; the cytokinin benzylaminopurine (BA); gibberellic acid (GA_3), and the auxin indole-3- acetic acid (IAA) were separately added into the plant growth media. Concentrations used for the hormone treatments were based on previous reports. After one hour, mRNA levels from the seedlings were analyzed by sqRTPCR. Examination of *SYST1-3* expression demonstrated that all of the plant hormones tested failed to reduce the levels of *SYST1-3* compared to untreated controls. Thus, elicitors and plant hormones were not responsible for the wound-related depletion of *SYST1-3* levels in injured *A. thaliana* leaves.

Expression of SYST1-3 *in aerial tissues*

One clue to elucidating gene function for *SYST1*-3 was to determine whether expression of a gene is confined to certain organ types or age-dependent. Various organs from *A. thaliana* were collected from three- and six-week old plants and tested for *SYST1-3* expression by RT-PCR (Fig. 5). *SYST1-3* expression was observed in the cauline leaves, stems and flowers of six-week old plants. However, its expression was absent in the rosette leaves from six-week old *A. thaliana* plants, but present in the rosette leaves of three-week old *A. thaliana* plants. Thus, the expression of *SYST1-3* is age-

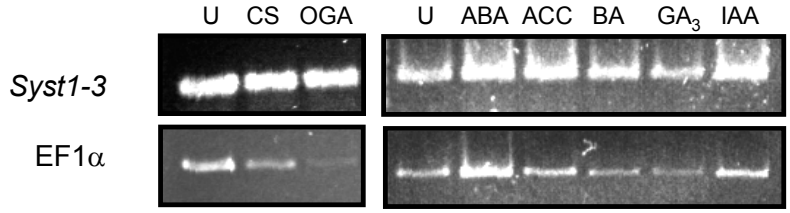


Figure 5.4 Effect of elicitors and plant hormones on the expression of *SYST1-3*. Ten-day old *Arabidopsis* plantlets were germinated in liquid Murashige-Skoog media supplemented with sucrose. Elicitors (30μg/μl) or plant hormones (1 μM) were added with fresh media and then incubated for three hours. Plantlets were processed for RNA and then analyzed by RT-PCR. U-untreated, CS-chitosan, OGA-oligogalacturonan, ABA-abscisic acid, ACC-aminocyclopropane-1-carboxylic acid, BA-benzylamino purine, GA_3-gibberellic acid, IAA-indole-3-acetic acid. EF1α was used to calibrate for discrepancies in loading.

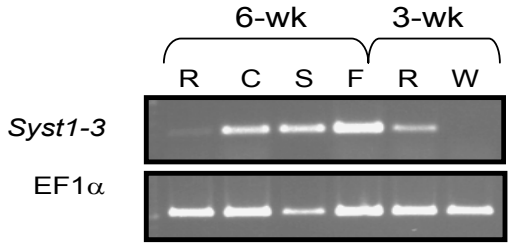


Figure 5.5 Age- and tissue-specific expression of SYST1-3 in adult *Arabidopsis* plants. Aerial organs from a six-week old *Arabidopsis* plant were collected. Three-week old leaves were wounded and harvested three hours later for analysis. Expression for *SYST1*-3 was evaluated by RT-PCR. EFα was used for detecting unequal loading. R-unwounded rosette leaves, C-cauline leaves, S-stems, F-flowers, W-wounded rosette leaves

dependent, as mature leaves failed to express *SYST1-3*. The presence of *SYST1-3* transcripts in the developing tissues suggests that its expression is important for growth and development. These results also demonstrated that plants as young as three weeks of age were competent to carry out wound-activated depletion of *SYST1-3*. This is distinct from other wound-induced genes whose expression is increased in senescing tissues.

Features of the SYST1-3 *nucleotide and amino acid sequence*

The full-length *SYST1-3* gene (At3g02640) is 825 nt in length, intronless and the coding sequence is flanked by 5'- and 3'-untranslated regions. The *Dpn*II-delimited DNA fragment of the *SYST1-3* gene, representing the subtracted fragment, is positioned proximal to the 3' end of the coding sequence between nucleotides 506-709 of the full-length transcript. At the amino acid level (Fig. 6), three *A. thaliana* genes (At5g16250, At3g6710, and At3g36800) encode proteins similar to the SYST1-3 (At3g02640) gene product. The At5g16250 gene product was most highly related to the SYST1-3 protein (E-value of 1e-67) while At3g6710 and At3g36800 were less related (E-value of 2e-40). These three gene products have no assigned function. The SYST1-3 protein also bears similarities to proteins in other higher plants. Two proteins (AAZ32985, ABE78001) are present in *Medicago trunculata*, of which one was annotated as a putative adenosylhomocysteine hydrolase. SYST1-3 related proteins are also found in *Brassica rapa* (ABL97950) and rice (NP_001053199).

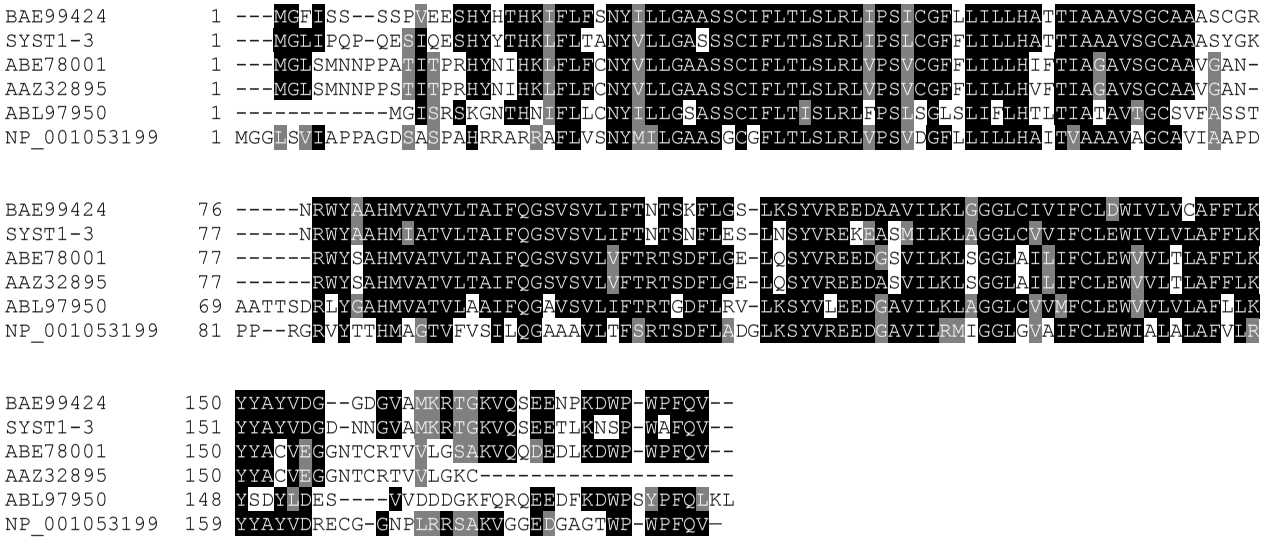


Figure 5.6 Comparison of SYST1-3-like proteins.
Highly-related proteins to SYST1-3 were aligned using ClustalW(ver1.8) and then formatted with Boxshade (ver3.21). Both ABE78001 and AAZ32985 originate from *Medicago trunculata*, NP_001053199 (*Oryza sativa*), ABL97950 (*Brassica rapa*), and the remaining proteins derive from *Arabidopsis*: BAE99424(At5g16250), SYST1-3 (NP_566179).

Transcripts of At5g16250 *are reduced in mechanically wounded leaves*

Based on the high similarity at the level of amino acid sequence of *SYST1-3* to At5g16250, we speculated that the expression of At5g16250 might also be repressed in injured leaves (Fig. 7). To examine if At5g16250 was perhaps co-regulated with *SYST1-3* in response to wounding, RT-PCR was performed to analyze At5g16250 expression in locally-wounded leaves and systemic tissues. Based on RT-PCR, the transcript encoding At5g16250 was depleted in wounded leaves relative to the unwounded plants. This response was evident within the first hour after mechanical injury and sustained for at least six hours. The systemic expression of the At5g16250 transcript remained unchanged and approximately equivalent to unwounded levels. Both *SYST1-3* and At5g16250 are members of a gene family whose transcripts are coordinately reduced by wounding. Further experiments with related proteins encoded by At3g6710, and At3g36800 may determine if the *SYST1-3* family of genes is entirely co-regulated by wounding. This also emphasized that additional wound-depleted transcripts exist and that the RDA strategy may have only uncovered a small proportion of the wound-depleted transcripts.

KAT5 *expression in wounded leaves*

In a survey of β-oxidation genes involved in wound response and dehydration, Cruz Castillo (2004) noted that *KAT5*, encoding a 3-ketoacyl-CoA thiolase, was expressed specifically in the systemic tissues of wounded *A. thaliana* plants. The strategy for subtraction hybridization applied in the present studies did not isolate any

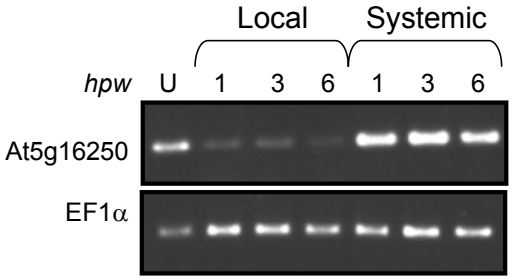


Figure 5.7 Wound-regulated depletion of *SYST1*-3-like gene, At5g16250, in injured leaves. Expression of At5g16250 was analyzed in wounded leaves and unwounded distal tissues in a time-course assay. EF1α was used to normalize loading differences between samples. U-unwounded plants. *hpw*-hour post-wounding.

systemically-specific wounding genes. To corroborate that the expression of *KAT5* is restricted to the systemic tissues of wounded plants, experiments were performed to characterize its expression in injured *A. thaliana* plants (Fig. 8). Our results conflicted with the authors' assertion that *KAT5* expression is restricted to systemic tissues of wounded plants. On the contrary, the *KAT5* transcript accumulated in the injured leaves. Its expression was transiently induced in wounded leaves and subsequently receded to basal levels within three hours of wounding. The systemic level of *KAT5* did not change from the level observed in unwounded plants.

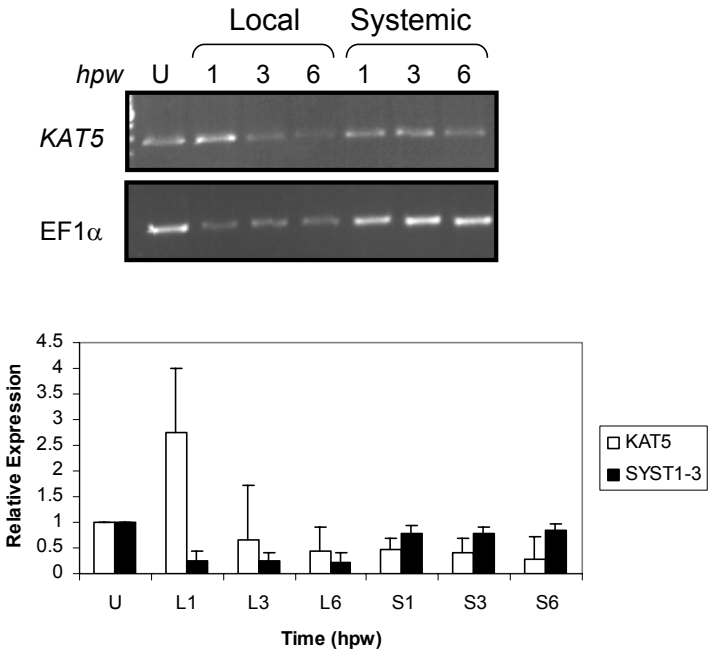


Figure 5.8 Expression of *KAT5* in wounded *Arabidopsis* plants.
Levels of *KAT5* in wounded leaves and systemic tissues were determined in a time-course experiment. EF1α was used to normalize the samples. Representative bar graph depicts quantitated amounts determined by sqRTPCR. Standard deviation is indicated by error bars. *hpw*-hours post-wounding.

Discussion

Systemic expression of wound-responsive genes in plants has been documented for a multitude of genes (Bergey et al., 1999; Hara et al., 2000; van de Ven et al., 2000; LeBrasseur et al., 2002; Szczegielniak et al., 2005). However, the expression of all systemic-responsive genes characterized to date is also accompanied by corresponding expression in wounded tissues, usually earlier and at higher levels than in systemic tissues. RDA was used to isolate and identify wound-induced genes that function explicitly in the undamaged tissues of wounded *A. thaliana* plants. A novel collection of previously uncharacterized wounding genes was isolated (Table 1). However, the anticipated systemic-specific expression of the DP3 genes (*SYST1-3*, *PER64*, *XTH9*, and CYSTATIN) failed to comply with predictions (Fig. 1). On the contrary, the change in expression levels of these *SYST* genes was delimited to the wounded leaf, where they were collectively down-regulated in response to tissue damage. Expression of the *SYST*s in systemic leaves was unaffected by wounding. Thus, the subtraction strategy in fact isolated a class of wound-responsive genes that were coordinately depleted in injured leaves.

The absence of systemic-exclusive wounding genes does not imply that they do not exist. Systemic-restricted wound-responsive genes may represent rare transcripts that were masked in the DP3 by the abundance and diversity of locally wound-depleted transcripts. Moreover, the choice of not including unwounded tissue in the driver representation may have hindered the isolation of systemically-induced genes. As determined by sqRTPCR, the *SYST*s are expressed in unwounded plants. The presence of unwounded tissue in the driver may have prevented the isolation of these wound-depleted

transcripts. Consequently, the subtraction mixture would have been less enriched for this class of wound-depleted *SYST* transcript. Another modification to improve the probability of isolating systemically-induced genes is to use an extended time course for tissue collection since the tissue harvested for RDA was biased towards early systemic responses. For example, a late responding wound pathway exists (Yamada et al., 2004) that is activated by SA. Systemic-specific genes may be activated during this delayed response.

The effort to characterize systemically-expressed wounding genes was attempted previously in *A. thaliana* and poplar (Parsons et al., 1989; Delessert et al., 2004). In both studies, activation of specific systemic expression was also observed in the locally-wounded leaf. This suggests that local expression is critical for the expansion of wound-induced transcripts into the undamaged sections of the wounded plant. *KAT5,* a gene involved in the β-oxidation of fatty acids in peroxisomes, was allegedly expressed in the systemic tissues of wounded *A. thaliana* plants (Cruz Castillo et al., 2004), but our experiments contradicted this claim as *KAT5* expression was induced in injured leaves (Fig. 8).

The assumption of the existence of systemic-restricted expression of wounding genes may also be naïve. Wound signaling is initiated in the injured leaf, where *de novo* JA is synthesized and transported to systemic parts of the wounded plant (Howe and Schilmiller, 2002; Li et al., 2002c). Thus, both wounded and unwounded tissues are exposed to bioactive JA and consequently activate similar JA-regulated wounding profiles. Local effects in the wounded leaf could alter some aspects of JA-regulation in the wounded leaf. Nonetheless, most systemically-expressed wounding genes exhibit

similar induction kinetics as local expression (Delessert et al., 2004). Activation of systemic-specific expression though may require factors additional to the signals emanating from the damaged plant cells. For example, herbivory can elicit a gene expression profile that is distinctive from mechanical wounding attributed to saliva, oral regurgitants and feeding patterns of insects (Von Dahl and Baldwin, 2004) and it is possible that factors contained in the saliva can also activate systemic-specific genes.

Characterization of *SYST1-3* down-regulation

The steady-state levels of *SYST1-3* transcription in unwounded plants are low. The levels of *SYST1-3* transcripts, monitored by sqRTPCR, fell below unwounded levels in mechanically damaged leaves within five minutes of wounding (Fig. 2A) and repression was sustained as *SYST1-3* did not recover for at least four days after the initial wounding (Fig. 2B). Known hormone signaling pathways involved in the wound response, such as JA and ethylene, played no role in the wound repression of *SYST1-3* (Fig. 3), even though local production of ethylene in the wounded leaf inhibited the early activation of JA-responsive genes. Moreover, treatments of *A. thaliana* seedlings with elicitors, OGAs and chitosan, failed to mimic the wound-repression of *SYST1-3* in *A. thaliana* seedlings (Fig. 4). None of the other plant hormones tested could replicate the wound-induced reduction of *SYST1-3* expression. Absence of *SYST1-3* depletion may have been a consequence of using liquid grown plants. *SYST1-3* expression may be regulated differently in the plantlets relative to the soil-grown plants used for wounding experiments. In addition, the entire plantlet was used for analysis. Regulation of *SYST1-*

3 expression in leaf, root and stem tissues may behave differently in response to wounding, elicitors or phytohormone.

Though wounding affected only a minor (~20%) portion of the leaf surface, the impact of the injury appeared to down-regulate the *SYST* genes and the *SYST1-3*-like At5g16250 to about 80% of initial levels. The effect of the injury does not appear to be confined to the damaged cells. A local wound signal must emanate from the wound site into the surrounding healthy tissues of the wounded leaf to trigger depletion in these areas. The residual expression of the locally-depleted genes in wounded leaves may be restricted to certain tissue types not wound-competent or cells outside the range of the locally-dispersed signal.

Transcript depletion in response to tuber wounding was previously described in potato with patatin, a storage protein (Logemann et al., 1988). Both the level of soluble patatin protein and its mRNA decreased in response to wounding. The levels of mRNA encoding patatin remained suppressed for at least 24 hours after damage but extended timepoints were not investigated. Unlike *SYST1*-3, patatin mRNA is not expressed in the leaves of potato. Thus, wound-triggered transcript reduction is not unique to the vegetative tissues and occurs in other higher plants.

A separate report identified a class of genes that were down-regulated upon wounding (Delessert et al., 2004). They were categorized as late-responding (four to eight hours after wounding) depleted genes with commensurate reduction in transcript levels in the systemic tissues. Most of these repressed genes were either involved in photosynthesis or metabolism. Reduction for some of these transcripts was dependent upon ethylene signaling. The *SYST* genes isolated by our RDA approach were not

identified in the latter study because of the limited array of 3,500 cDNAs that was used. However, differences exist between the two classes of wound-depleted genes. The expression of *SYST* genes is rapidly reduced and down-regulation is restricted to the wounded leaf while systemic levels are unchanged. Ethylene signaling is also not responsible for the wound-depletion of *SYST1-3*. Thus, at least two independent signaling pathways in *A. thaliana* must mediate the depletion of these wounding genes.

Transcriptional regulation of *SYST1-3*

The SYST1-3 protein may perform a role in developing or photosynthetically active tissue since *SYST1-3* expression was detected in all aerial tissues except for the older leaves (Fig. 5). Analysis of the wound-repressed genes showed the presence of I-box motifs in the promoters of these genes (Delessert et al., 2004). I-box motifs were first identified within the promoter of the small subunit of the rubisco (*rbcS-1A*) gene (Donald and Cashmore, 1990). Similarly, the four subtracted genes isolated in this project also contain these signatures in the promoters. This may indicate the regulation by light and wound-reduction of these transcripts may be linked to diminished photosynthetic activity in the damaged leaf. Reports have shown that wounded plants operate at lower rates of photosynthesis (Herde et al., 1999; Chang et al., 2004; Quilliam et al., 2006). After wounding, photosynthetic activity is disrupted within five minutes (Chang et al., 2004). The immediate response of photosynthesis to wounding parallels the rapidity of the reduction response in *SYST1*-3 level. However, photosynthetic activities are partially revived within six hours, whereas the level of the *SYST1-3* transcript does not return to unwounded levels (Chang et al., 2004). Therefore,

compromised photosynthetic activity cannot fully account for the sustained absence of *SYST1*-3 expression. Additional factors may be needed to prolong *SYST1-3* suppression in wounded leaves.

Further evidence suggests wound-induced depletion is regulated at the level of transcription. Transcriptional nuclear run-on assays demonstrated that the mode of repression for patatin in damaged potato tubers was exerted at the level of transcription (Logemann et al., 1988). Recently, a report described a transcription factor (Fig. 9), called ATAF2, that was rapidly induced in wounded *A. thaliana* leaves and functions as a negative regulator by repressing the accumulation of several transcripts in the wounded leaf, including genes associated with pathogen resistance (*PR-1* and *PR-4*) (Delessert et al., 2005).

The depletion of *SYST1-3* expression may also be regulated post-transcriptionally (Gutierrez et al., 1999). Transcript stability may be determined by sequence-specific motifs found in the 5'- and 3'-UTRs, such as downstream (DST) elements, AUUUA-repeats, or by the presence of premature stop codons (non-sense mediated decay). The UTRs of *SYST1-3* did not possess any of these sequence elements, nor was *SYST1-3* reduction in wounded leaves affected in *dst* mutant plants (data not shown). However, decay rates of mRNA in plants can be regulated by external stimuli including light, sucrose, phytohormones and elicitation (Gutierrez et al., 1999). Whether mRNA stability regulates the expression of some wound-responsive genes is still unknown.

The importance of down-regulation

The response to biotic and abiotic stress is metabolically expensive for the plant as activation of defense responses expends stored energy and resources (Baldwin, 1998; Mauricio, 1998; Boege and Marquis, 2005; Schwachtje et al., 2006). The cost of generating a defense response is partially reflected in the lower seed production in wild *Nicotiana attenuata* plants pre-treated with MeJA (Baldwin, 1998). Wounding of *N. sylvestris* also resulted in diminished seed yield (Baldwin et al., 1990). Thus, the benefits from activating the inducible defenses are partially mitigated by a negative effect on fitness. Moreover, stringent management of carbon reserves and energy allocation is pivotal for effective response to stress and reproductive fitness.

The wounded leaf experiences an increase in metabolism, supplying much needed carbon skeletons and energy sources to subsidize the cost of responding to tissue damage (Sturm and Chrispeels, 1990; Quilliam et al., 2006). Photosynthetic imaging and ^{14}C-labeled photosynthate show that phloem unloading intensifies in a wounded *A. thaliana* leaf indicating increased sink strength. By boosting the availability of carbon and energy sources, the wounded plant can sustain the high metabolic demands required for responding to tissue damage.

Partial contribution of resources to supply the wound response may be furnished by deactivating certain cellular processes. Damaged leaves may then instead divert these available resources to fuel wound repair. This is also reinforced in the identities of the subtracted genes. For example, *XTH9* was depleted in the wounded leaf. XTHs mediate cell expansion by loosening and rearranging the xyloglucan chains composing the cell wall (Rose et al., 2002). In a wounded leaf, leaf expansion is not a critical process during

wound healing. By salvaging from deactivated processes in the wounded leaf along with the influx of phloem from active tissues to provide carbon and energy, the injured plant can marshal the resources to recuperate.

CHAPTER SIX

CHARACTERIZATION OF THE WOUND RESPONSE IN *PHYSCOMITRELLA PATENS*

Introduction

Bryophytes, which include the mosses, hornworts and liverworts, are ancient land plants that split from the angiosperms about 450 million years ago (Kenrick and Crane, 1997). Unlike higher plants, mosses are seedless, do not possess a true vascular system and the sporophyte is epiphytic to the gametophore. Interestingly, the cell membrane of mosses (Table 1) is composed of polyunsaturated fatty acids found in the cell membranes of both plant (linolenic acid) and animals (arachidonic acid) (Grimsley et al., 1981; Qi et al., 2004). Propagation methods for *in vitro* culture for the moss *Physcomitrella patens*, genetics and sophisticated molecular tools are well-established (Reski, 1998; Frank et al., 2005). In addition, the genome for the moss, *P. patens*, was recently sequenced in 2006 (http://genome.jgi-psf.org//Phypa1_1/Phypa1_1.home.html). Functional genomics in *P. patens* benefits from the high efficiency with which targeted gene knockouts can be constructed (Schaefer and Zryd, 1997). For these reasons, *P. patens* has emerged as a model organism for the study of mosses and investigation for the differences that exist between bryophytes and higher plants (Cove et al., 1997; Nishiyama et al., 2003).

One piece of evidence suggests that mosses are capable of mounting a wound response. Volatile oxylipins were synthesized in wounded protonema, the filamentous juvenile phase of mosses (Wichard et al., 2004). A lipoxygenase (PpLOX1) from protonema was

Fatty Acid	P.patens (% mol)	A.thaliana (% mol)
16:0 (PA)	11.7	17.5
16:3 ω3 (HDT)	14.5	12.3
18:2 ω6 (LA)	10.9	13.7
18:3 ω6 (γLA)	3.7	
18:3 ω3 (αLA)	23.6	48.0
18:4 ω3 (STA)	0.4	
20:4 ω6 (AA)	17.0	ND

Table 6.1 Comparison of fatty acid content between *Arabidopsis thaliana* and *Physcomitrella patens*. Fatty acid levels were measured as percent molar. Values were compiled from two reports (Grimsley et al., 1981; Qi et al., 2004). PA-palmitic acid, HDT-hexadecatrienoic acid, LA-linoleic acid, γLA-(6,9,12) linolenic acid, αLA-(9,12,15) linolenic acid, STA-stearidonic acid, AA-arachidonic acid. ND-not detectable

isolated which *in vitro* generates a variety of volatile oxylipins (Grimsley et al., 1981; Qi et al., 2004). Nonetheless, the wound response in *P. patens* from the molecular perspective is poorly explored and little is known about the wound response in the gametophore. Therefore, we investigated the physiological and molecular responses to wounding in the adult-phased vegetative gametophore of *P. patens*. The gametophore was found to mount a wound response similar to the wound response in higher plants. Wounding of the gametophore caused minimal damage to the leaf as one to two cell layers bordering the cut site do not survive. Some of the intact cells along the wound developed putative callose-based structures. Three putative lipoxygenase ESTs (*PpLEST4*, *PpLEST6*, *PpLEST7*) were induced in the wounded gametophore. Predicted full-length proteins encoded by *PpLEST6* (PpLOX6) and *PpLEST7* (PpLOX7) bear motifs specific to lipoxygenases and are most highly related to the lipoxygenases from higher plants. However, phylogenetic analysis showed that only PpLOX7 clustered with the type-2 13-lipoxygenases. In preliminary experiments, *PpLOX7* was induced by MeJA suggesting that a JA-dependent wound response pathway may exist in *P. patens*. Putative ESTs for JA biosynthetic enzymes, allene oxide cyclase (AOC) and allene oxide synthase (AOS) were also induced by wounding, but 12-oxophytodienoic acid 3 (OPR3) was not activated.

Results

Impact of wounding on intact gametophore cells assessed by FDA staining

The impact of wounding on intact gametophore cells was examined after a portion of the leaf was removed with a razor blade. Under bright-field illumination, the cells of

unwounded leaves were packed with chloroplasts (Fig. 1A). The cells which were directly wounded lost their cytoplasmic contents and appeared as empty shells (Fig. 1C). The cytoplasm of the intact cells adjacent to the severed cells appeared condensed and less translucent than cells in wounded leaves. Moreover, the chloroplasts in these abnormal-looking cells were no longer individually distinguishable. This phenotype was observed immediately after wounding. To determine whether these bordering cells had died, cell viability was monitored in wounded gametophore leaves using the vital stain, fluoroscein diacetate (FDA), which detects cytoplasmic esterase activity that is associated with living cells. In this assay, the cells in undamaged gametophore leaves fluoresced green, indicating that they were living (Fig. 1B). Upon removal of the tip of the gametophore leaf, the cell layers (usually one or two) proximal to the edge of the cut immediately failed to fluoresce, indicating the presence of dead cells (Fig. 1D). The absence of fluorescence in these presumed dead layers correlated with the condensed cell content phenotype observed under white light illumination. After 24 hours, the region of death never progressed beyond the first two layers of cells (data not shown). Therefore, the impact of wounding on cell survival affected only the cells bordering close to the immediate wound site.

Callose deposition in response to wounding

Callose is a β-1,3-glucan that is ubiquitiously found in higher plants (Stone et al., 1985). In response to biotic and abiotic stresses, including pathogen attack, wounding and dessication, callose accumulates between the plasma membrane and the cell wall

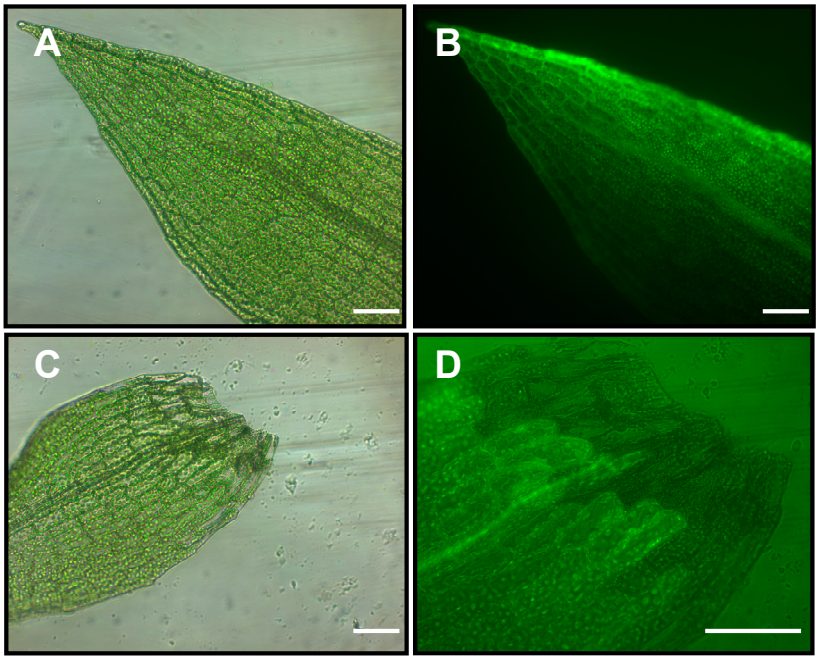


Figure 6.1 Effect of wounding on cell viability in a *P. patens* gametophore leaf. Leaves were cut and then stained for living cells by FDA. Live cells fluoresce green under UV illumination Panels **A**, **B**: unwounded leaves; **C**,**D**: leaf immediately after wounding. Leaves under bright field light microscopy (**A**, **C**) and UV illumination (**B**, **D**). The (—) represents 10 μm. See Methods (Chapter 2) for details on staining and microscopy.

(Stone and Clarke, 1992; Donofrio and Delaney, 2001; Jacobs et al., 2003). The deposition of callose is speculated to enhance resistance by delaying infection while the alerted plant mounts a concerted defense response (Brown et al., 1998). To determine whether the moss *P. patens* is capable of activating a physiological response to wounding, damaged leaves of the gametophore were analyzed for the *de novo* deposition of callose.

The presence of deposited callose is detectable with an aniline blue stain under fluorescence microscopy (Stone et al., 1985). Gametophore leaves were damaged with a razor, fixed in a formaldehyde solution, and then stained for the presence of callose (Fig. 2). Under UV light, no aniline blue-positive fluorescent signals were detected (Fig. 2B), but intensely fluorescent specks were observed in wounded leaves within 12 hours of incurring the injury (Fig. 2D). The aniline-blue stained spots were deposited along the cell layers abutting cells walls facing the wound site. In wounded gametophore plantlets, the fluorescent spots were localized specifically to the injured leaf and were not detected in unwounded systemic gametophore leaves. Three and seven days after wounding, there was no detected increase in presumed callose deposition (data not shown). Thus, *P. patens* is capable of mobilizing a directed physiological response to wounding in the injured gametophore leaf.

Callose is synthesized by callose synthase (glucan synthase-like gene, *GSL*), a membrane-bound, multimeric complex composed of six to nine polypeptides (Hong et al., 2001; Østergaard et al., 2002; Jacobs et al., 2003). In response to wounding, callose synthesis may be regulated post-translationally (Verma and Hong, 2001). However, regulation of callose synthase in *P. patens* is unexplored. Using a putative EST encoding

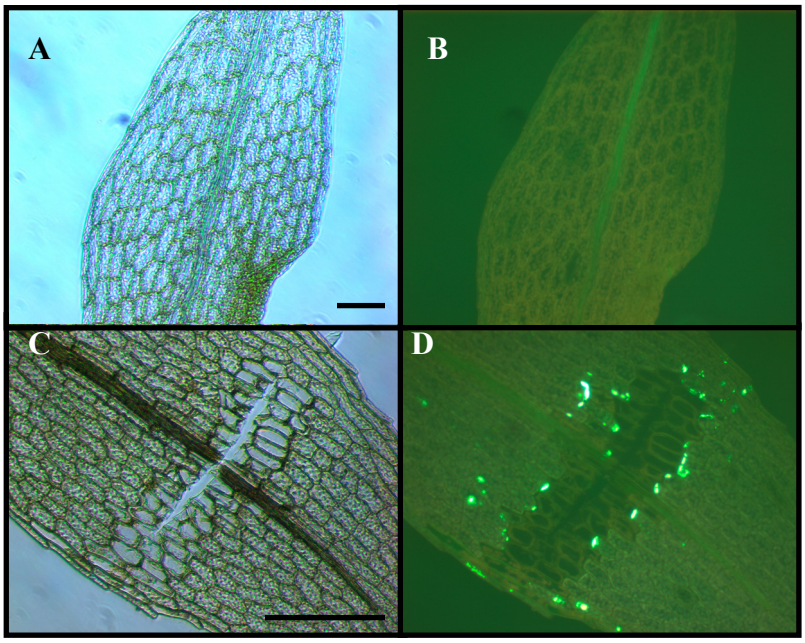


Figure 6.2 Callose is deposited in response to wounding of leaves from *Physcomitrella* gametophore. Leaves cut with a razor were fixed in FAA solution, stained with aniline blue, and then examined by fluorescence microscopy. (See Methods for description) Panels **A**,**B**: unwounded leaf; **C**,**D**: wounded leaf (12 hours post-wounding). Leaves were illuminated by white light (**A**,**C**) or UV (**B**, **D**). The bar (—) represents 10 μm. See Methods (Chapter 2) for details on staining and microscopy.

the *GSL* previously identified from *P. patens* (http://cellwall.stanford.edu/), wounded gametophores were examined for the transcriptional activation of callose synthase by RT-PCR (Fig. 3). The results show that tissue injury in *P. patens* does not trigger the transcriptional up-regulation of *GSL*. The absence of *GSL* induction coupled with the accumulation of aniline blue-positive specks in wounded gametophore leaves suggests that callose deposition in *P. patens* is regulated post-transcriptionally, as it in higher plants.

Identification of novel wound-induced lipoxygenase genes in Physcomitrella

The identification and characterization of a lipoxygenase gene (*PpLOX1*) in the *P. patens* protonema tissues (Senger et al., 2005) prompted us to investigate the expression of wound-induced lipoxygenases in the adult gametophore plants. To identify novel lipoxygenases from *P. patens*, the PpLOX1 protein was used to query the translated *Physcomitrella* EST database (PHYSCObase, http://moss.nibb.ac.jp) for lipoxygenase-related genes since at the time these studies were initiated, the *Physcomitrella* genome had yet to be completely sequenced and annotated. The search identified six highly-similar ESTs (designated *PpLEST2-7*) to *PpLOX1* (Fig. 4), representing the highest scoring ESTs (E-values ranging from 1e-170 to 1e-48), and were used for further analysis.

Each of the *PpLEST*s was used to examine the capacity of the gametophore to activate a lipoxygenase-mediated wound response. Expression of the *PpLEST*s in wounded gametophores in a time-course assay was analyzed by semi-quantitative RT-PCR (sqRTPCR) analysis (Fig. 5). The expression of actin was used to normalize

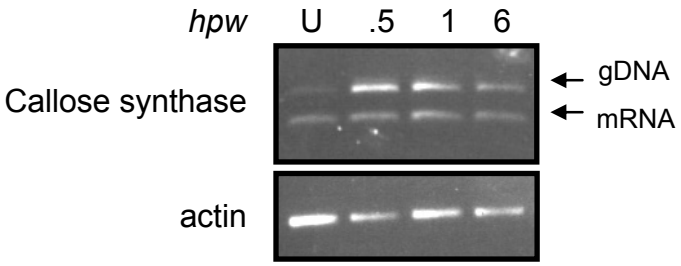
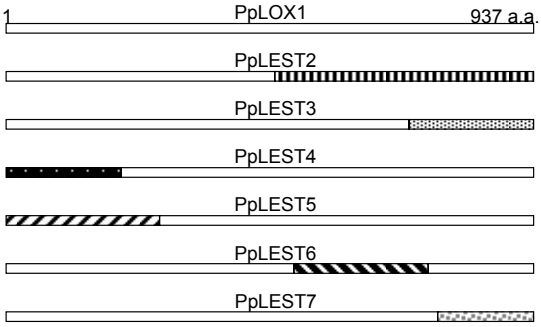


Figure. 6.3 Callose synthase activation in *P. patens* is not regulated by wounding at the level of transcription. Wounded leaves of *Physcomitrella* gametophore were harvested in a time-course experiment and extracted for RNA. Expression of callose synthase was examined by RT-PCR. The upper band amplified by the primers designed against the callose synthase EST represents genomic DNA. The level of actin mRNA was used to normalize for any discrepancy between samples. *hpw*-hours post-wounding



EST name (PpLEST)	Contig	Region of *PpLOX1*	e-value
PpLOX1	4549	1-937	0.0
2	3033	444 - 937	e-170
3	606	715 - 937	e-125
4	607	1 - 182	e-92
5	3032	1 – 254	e-63
6	pphf32d19	524 – 717	e-50
7	7348	738 – 937	e-48

Figure 6.4 Schematic illustrating the six *PpLOX1*-like ESTs extracted from the translated *P. patens* database (PHYSCOBASE). The highlighted regions of the proteins (left) correspond to the translated EST represented in the database with similarities to PpLOX1. The relatedness is described in the table (right) with designation for the purported lipoxygenase-like genes.

inconsistencies in loading and cDNA synthesis. Expression of *PpLOX1*, whose activity was originally detected in the juvenile stage of the protonema, was also examined in wounded adult-phase (gametophore) tissues. The results from sqRTPCR demonstrated the induction of three (*PpLEST4*, *PpLEST6*, *PpLEST7*) of the six *PpLEST*s in wounded gametophore plants. Induction occurred within one hour of leaf damage. The increases of *PpLEST6* and *PpLEST7* transcripts were more marked, while *PpLEST4* was only modestly induced. No activation of *PpLEST2*, *PpLEST3*, and *PpLEST5*) was observed. Because of their marked induction, additional molecular analyses focused on *PpLEST6* and *PpLEST7*. Interestingly, the *PpLOX1* gene was not triggered by wounding in the gametophore and its steady-state levels were very low, suggesting possible stage-specific regulation of *PpLOX1*.

The Physcomitrella *genome contains multiple chloroplast-targeted lipoxygenase genes*

The completion of the genome sequence and its annotation facilitated further analyses of lipoxygenases in *P. patens* (JGI, http://shake.jgi-psf.org/Phypa1/Phypa1.home.html). The genome of *Physcomitrella* contains at least ten lipoxygenase-like genes (Table 2). The *PpLEST* sequences represent five of the annotated *Physcomitrella* lipoxygenase genes including *PpLOX1*. Two *PpLEST*s (*PpLEST2*, *PpLEST5*) correspond to the same lipoxygenase gene (estEXT-gwp-gw1.C_4190009) while *PpLEST3* encodes *PpLOX1*. The wound-induced and gametophore-expressed *PpLEST4*, *PpLEST6*, and *PpLEST7* represent individual lipoxygenase-like genes. Henceforth, they will be subsequently designated as *PpLOX4* (*PpLEST4*), *PpLOX6* (*PpLEST6*), and *PpLOX7* (*PpLEST7*).

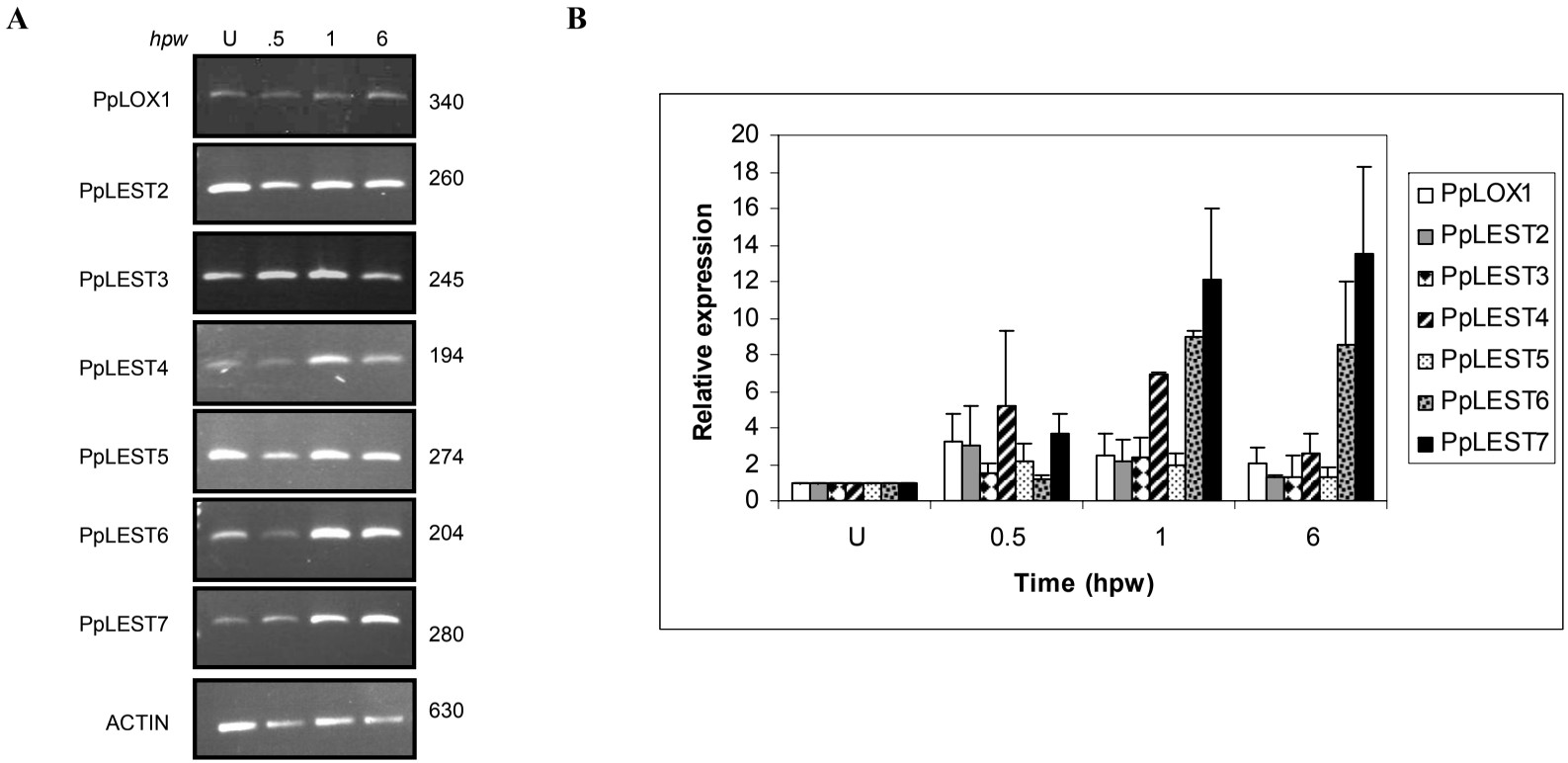


Figure 6.5 Putative lipoxygenase genes are induced by wounding of the vegetative tissues from *Physcomitrella* gametophore. Approximately one-third of the leaves from individual gametophores were cut with a razor and plantlets were harvested in a time-course study. The tissues were evaluated for the up-regulation of predicted lipoxygenases ESTs by sqRT-PCR. **A**. RT-PCR detailing the expression of the lipoxygenase genes. Numbers adjacent to the individuals ESTs represent calculated size of the amplified product. Actin was used for normalization. **B**. Graph depicting the changes in expression of the putative lipoxygenases genes triggered by wounding in A. Relative induction was calculated from the normalized values of the expression and compared with the intensity of the unwounded control (See Methods-Chapter 2 for details). Graph is representative of three independent experiments. Standard error is represented by error bars. *hpw*-hours post wounding, U-Unwounded gametophore

Some wound-induced lipoxygenases (type 2) in higher plants are targeted to the chloroplast (Bell et al., 1995; Heitz et al., 1997; Farmaki et al., 2007) and PpLOX1 is predicted to be localized to the chloroplast (Senger et al., 2005). The *P. patens* lipoxygenases were examined for putative chloroplast localization motifs with the prediction software TargetP1.1 (Emanuelsson et al., 2000). Four of the *P. patens* lipoxygenases (*PpLOX1*, *PpLEST2*/*5*, *PpLOX4*, *PpLOX7*) were predicted to be targeted to the chloroplast, but the subcellular localization of *PpLOX6* was not designated (Table 1). Interestingly, one of the annotated lipoxygenases may be shuttled to the mitochondria. Evidence of mitochondrial lipoxygenases in pea plants was reported (Braidot et al., 2004) and may be involved in mitochondrial degradation during xylem tracheary formation. Other lipoxygenases do not possess identifiable signal peptides.

Some lipoxygenases for *P. patens* are more related to known lipoxygenases from higher plants. Both PpLOX6 and PpLOX7 are most similar to the lipoxygenase proteins from *Corylus avellana* (European filbert, Family Betulaceae) and *Sesbania rostrata* (a legume, Family Fabaceae), respectively. The PpLOX6 lipoxygenase also has highest similarities to lipoxygenases from bean (*Phaseolus vulgaris*), AtLOX1 from *Arabidopsis thaliana*, and a soybean seed lipoxygenase (Fig. 6). The PpLOX7 lipoxygenase is related to a separate family of lipoxygenases from higher plants, including potato (*Solanum tuberosum*), tobacco (*Nicotiana attenuata*) and AtLOX3 from *A. thaliana (*Fig. 7). The PpLOX4 lipoxygenase is almost identical (99% in amino acid sequence) to the protein sequence of *PpLOX1* (Fig. 8). In contrast, the PpLOX6 and PpLOX7 lipoxygenases are significantly less related (each at 42%) to PpLOX1.

JGI designation	Size (aa)	EST	Predicted Plastid Target	Highest Scoring Relative	
				Accession	Organism
estExt_gwp_gw1.C_4190017	937	PpLOX1/PpLEST3	Chloroplast	AAB18970	Phaseolus vulgaris
estExt_gwp_gw1.C_4190009	922	PpLEST2/PpLEST5	Chloroplast	CAE47464	Physcomitrella patens
e_gw1.434.22.1	825	PpLEST6		CAD10740	Corylus avellana
estExt_fgenesh1_pg.C_4190004	938	PpLEST4	Chloroplast	CAE47464	Physcomitrella patens
estExt_gwp_gw1.C_700115	936	PpLEST7	Chloroplast	CAC43237	Sesbania rostrata
fgenesh1_pg.scaffold_190000062	819		Mitochondria	AAG18376	Zantedeschia aethiopica
e_gw1.97.30.1	746			CAD10740	Corylus avellana
estExt_gwp_gw1.C_2320058	859			CAD10740	Corylus avellana
e_gw1.21.1.1	914			CAE47464	Physcomitrella patens
e_gw1.171.1.1	914			CAE47464	Physcomitrella patens

Table 6.2 List of all annotated lipoxygenase genes contained in the genome of *P. patens*.
Predicted *P. patens* lipoxygenases proteins were identified by JGI (http://genome.jgi-psf.org//Phypa1_1/Phypa1_1.home.html). Possible representation for these putative lipoxygenases by any of the PpLESTs is noted. Predictions for subcellular localization was performed with TargetP1.1 (Emanuelsson et al., 2000).

Figure 6.6 Alignment of PpLOX6 with highly-related lipoxygenases from higher plants. Protein sequences were aligned with ClustalW ((Thompson et al., 1994) and formatted with Boxshade. CAD10740.1 (*Corylus avellana*), AAB18970.1 (*Phaseolus vulgaris*), AtLOX1 (*Arabidopsis thaliana* lipoxygenase1), and P24095 (*Glycine max* seed).

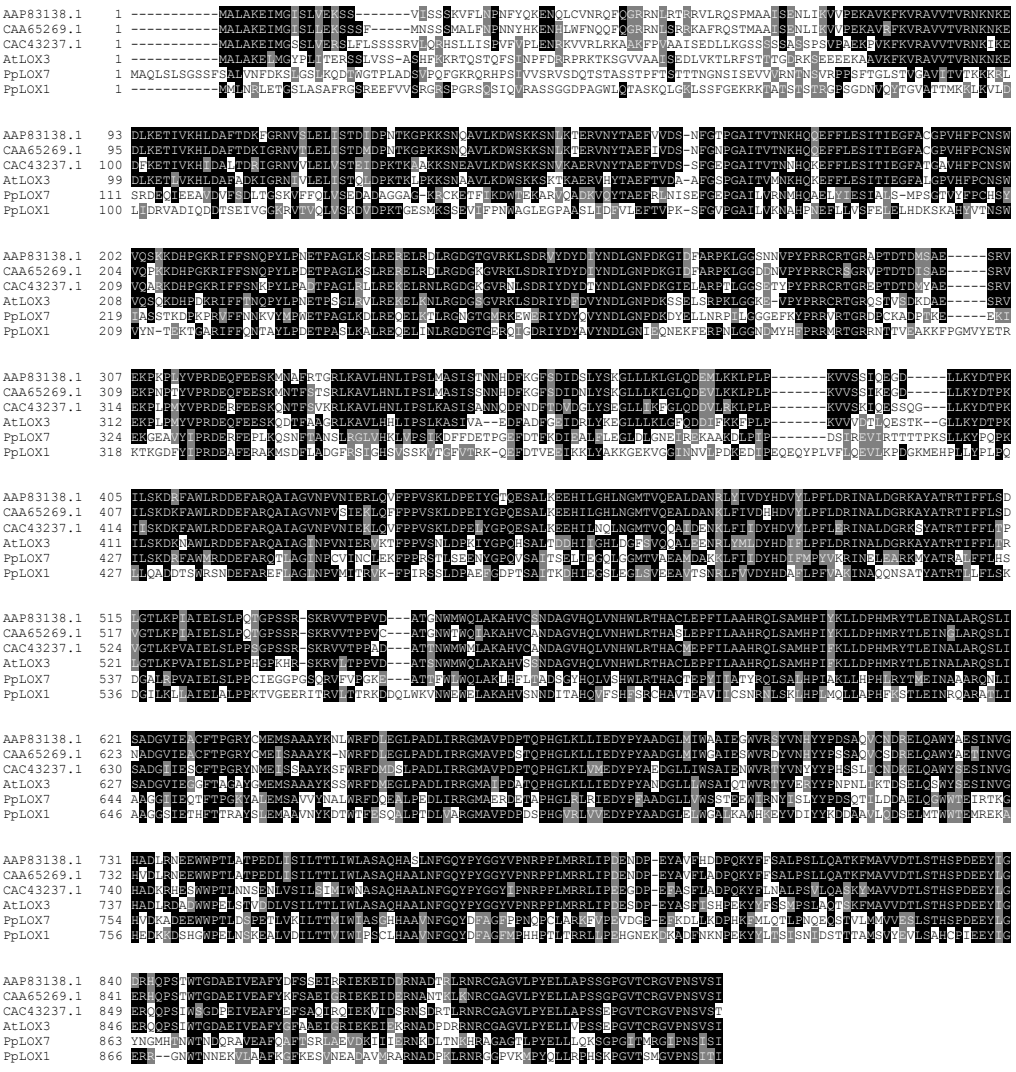


Figure 6.7 Alignment of lipoxygenases from higher plants with the protein sequence of PpLOX7. AAP83138.1 (*Nicotiana attenuata*), CAA65269.1 (*Solanum tuberosum*), AtLOX3 (*Arabidopsis thaliana* lipoxygenase3) and CAC43237.1 (*Sesbania rostrata*). See legend of Fig. 6 for alignment details.

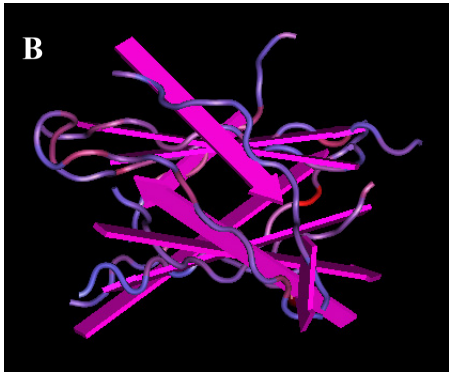
Structural Features of PpLOX6 *and* PpLOX7 *lipoxygenases*

Lipoxygenases from higher plants are composed of two identifying domains (Fig. 8A). The PLAT/LH2 (Polycystin-1, Lipoxygenase, Alpha Toxin/Lipoxygenase Homology2) domain is situated at the N-terminus of plant lipoxygenases (Minor et al., 1996). The three-dimensional crystal structure of soybean lipoxygenase L-1 demonstrates that the PLAT/LH2 domain forms a β-pleated sheet sandwich, constructed from two sheets with each composed of four strands (Fig. 8B) (Minor et al., 1996). The PLAT/LH2 domain may facilitate binding with lipids and membrane-bound proteins, positioning the enzyme in the vicinity of its substrate (Bateman and Sandford, 1999). Both PpLOX6 and PpLOX7 are predicted by the Conserved Domain Database (CDD) to possess the PLAT/LH2 domain (Fig. 8C) (Marchler-Bauer et al., 2007).

A second domain contains multiple conserved His residues, clustered within a span of 40 amino acids, that are involved in binding the non-heme iron (Fig. 9) (Steczko et al., 1992; Boyington et al., 1993). Three of the His residues bind the iron ligand (ref). The remaining His residues are involved in lipoxygenase activity. The iron-binding domain is a composite of two signatures. The first consensus pattern [**H**-(E/Q)-xxx-**H**-x-(L/M/A)-(N/E/Q/H/R/C/S)-(G/S/T/A)-**H**-(L/I/V/S/T/A/C)-(L/I/V/S/T/A/C)-x-E] contains three invariant His residues. The second signature [(L/I/V/M/A/C/S/T)-**H**-P-(L/I/V/M)-x-(K/R/Q/V)-(L/I/V/M/F)-(L/I/V/M/F)-x-(A/P)-**H**] contains two conserved His residues. A comparative analysis of the PpLOX6 and PpLOX7 with known lipoxygenases showed that the amino acid sequences of the wound-inducible *Physcomitrella* lipoxygenases also possess the invariant His residues involved with both Fe-binding and catalysis. Thus the

A

B



C

```
PpLOX6       3   IKTSVTLVRSNfrGLDVGNV-FSDiLELGQELIGNQVTLQLVSNDVDPATGSGKLSGKVTFEHW---TKPRnllrSTPFEFNMD  82
PpLOX7      97  TVGAVITVTKKK--RLSRDEQiEEA-VDVFSDLTGSKVFFQLVSEDAD-AGGAGKRCKETFIKDWtekARVQ----ADKVQYTAE  173
1YGE         6 HKIKGTVVLMPKN--ELEVNPD-GSA-VDNLNAFLGRSVSLQLISATKADAHGKGKVGKDTFLEGI---NTSLptlgAGESAFNIH  79
Consensus    1 VKVKATVTVMKKN--KLDLNDD-GSG-LDDLDDLLGRSLLLELVSSELDPKTGKGKKGKAAFLEGW---GKSL----AGESAYEAE  75

PpLOX6      83 FYVPKN-FGTPGAILVFNGHNnldvpilstitTEFKITDAHVV-MPDKNVIGFFCNSWVFASDMDKDGRLFFAN 154
PpLOX7     174 FRLNISeFGEPGAILVRNMHQ-----------AELYIESIALSMP--SGTVYFPCHSYIASSTKDPKPRVFFNN 234
1YGE        80 FEWDGS-MGIPGAFYIKNYMQ-----------VEFFLKSLTLEAISNQGTIRFVCNSWVYNTKLYKSVRIFFAN 146
Consensus   76 FEVPAS-FGPPGAVLVKNEHH-----------SEFFLKSITLEGFGGSGTITFVCNSWVYPKKDYPDKRIFFPN 137
```

Figure 6.8 Conserved domains of lipoxygenases in higher plants is onserved in PpLOX6 and PpLOX7 lipoxygenases.
A. Schematic of the conserved domains of a plant-derived lipoxygenases. SP: signal peptide, PLAT/LH2 (polycystin-1, lipoxygenase, alpha toxin/lipoxygenase Homology2) domain. **B**. Three-dimensional crystal structure of the PLAT/LH2 domain from soybean lipoxygenase L-1 (1YGE). **C**. Alignment of the consensus PLAT/LH2 domain sequence with the respective sequence from 1YGE (soybean lipoxygenase L-1), PpLOX6, and PpLOX7. Alignment was performed through the Conserved Domain Database (CDD). Red residues represent amino acids conserved in all three proteins and the consensus sequence. Blue residues represent three of the four residues are identical.

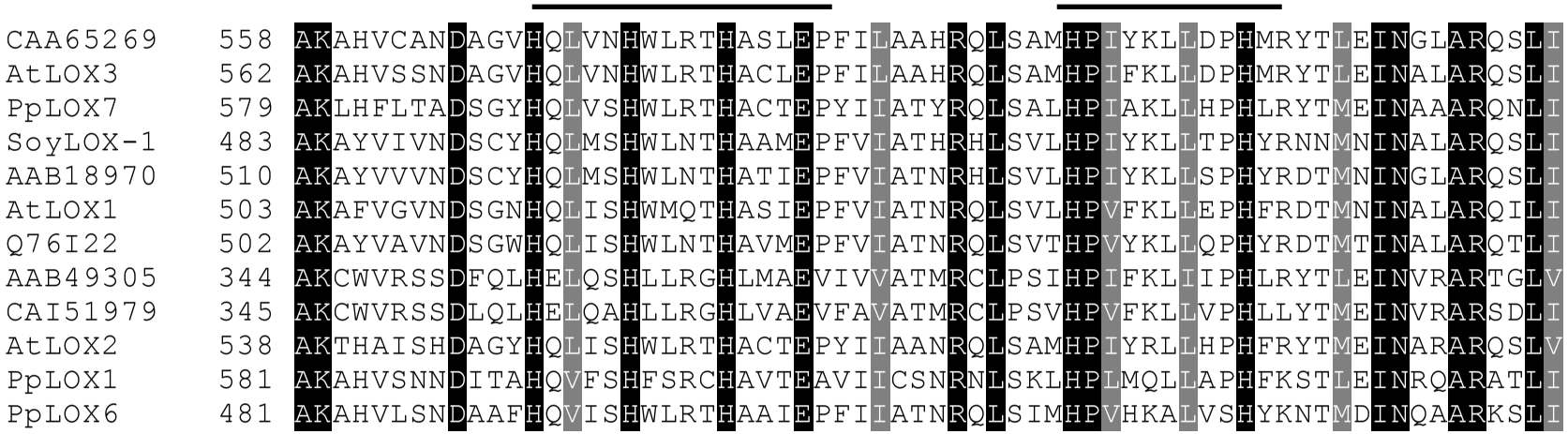


Figure 6.9 Lineup of the His-rich region involved in binding the non-heme iron and catalysis from mammalian and plant lipoxygenases. The lined region represents the two consensus motifs containing the invariant His. CAA65269.1 (*Solanum tuberosum*), AtLOX3 (*Arabidopsis thaliana* lipoxyugenase3), SoyLOX-1 (Soybean lipoxygenase 1), AAB18970.1 (*Phaseolus vulgaris*), AtLOX1 (*Arabidopsis thaliana* lipoxygenase1), Q76I22.1 (*Oryza sativa*), AAB49305.1 (*Homo sapiens*), CAI51979 (*Mus musculus*), AtLOX2 (*Arabidopsis thaliana* lipoxygenase 2). See legend for Fig. 6 of the details for alignment and presentation.

PpLOX6 and PpLOX7 proteins contain the hallmarks of a catalytically-active lipoxygenase.

Phylogenetic analysis of wound-induced Physcomitrella lipoxygenases

Plant lipoxygenases introduce molecular oxygen into their polyunsaturated fatty acid substrate at either the C_9 or C_{13} positions (Feussner and Wasternack, 2002; Porta and Rocha-Sosa, 2002). The primary amino acid sequences of the *P. patens* lipoxygenases were compared with the published sequences of the 9- and 13-lipoxygenases from higher plants and the arachidonate lipoxygenases from animals. A dendrogram (Fig. 10) was constructed to determine the evolutionary relationships among the lipoxygenases. For the most part, the seed and 9-lipoxygenases, 13-lipoxygenases, arachidonic-based lipoxygenases and the moss-derived lipoxygenases formed separate clusters. The dendrogram demonstrated that the wound-inducible *PpLOX7* lipoxygenase is most related to the 13-lipoxygenases from higher plants. The characterized lipoxygenases in this clade includes the chloroplast-localized (type 2) lipoxygenases that are induced by JA and may be responsible for the wound-induced accumulation of JA (Bell et al., 1995; Royo et al., 1996; Heitz et al., 1997). Some of these enzymes (potato lipoxygenases CAA65268.1/CAA65269.1 and *A. thaliana* LOX2) have been demonstrated to use α-linolenic acid as their natural substrate (Vick and Zimmerman, 1983; Bell et al., 1995; Royo et al., 1996; Heitz et al., 1997). The *P. patens* lipoxygenases, *PpLOX1*, *PpLOX4* and *PpLOX6*, were not identified with any of the remaining lipoxygenase clusters.

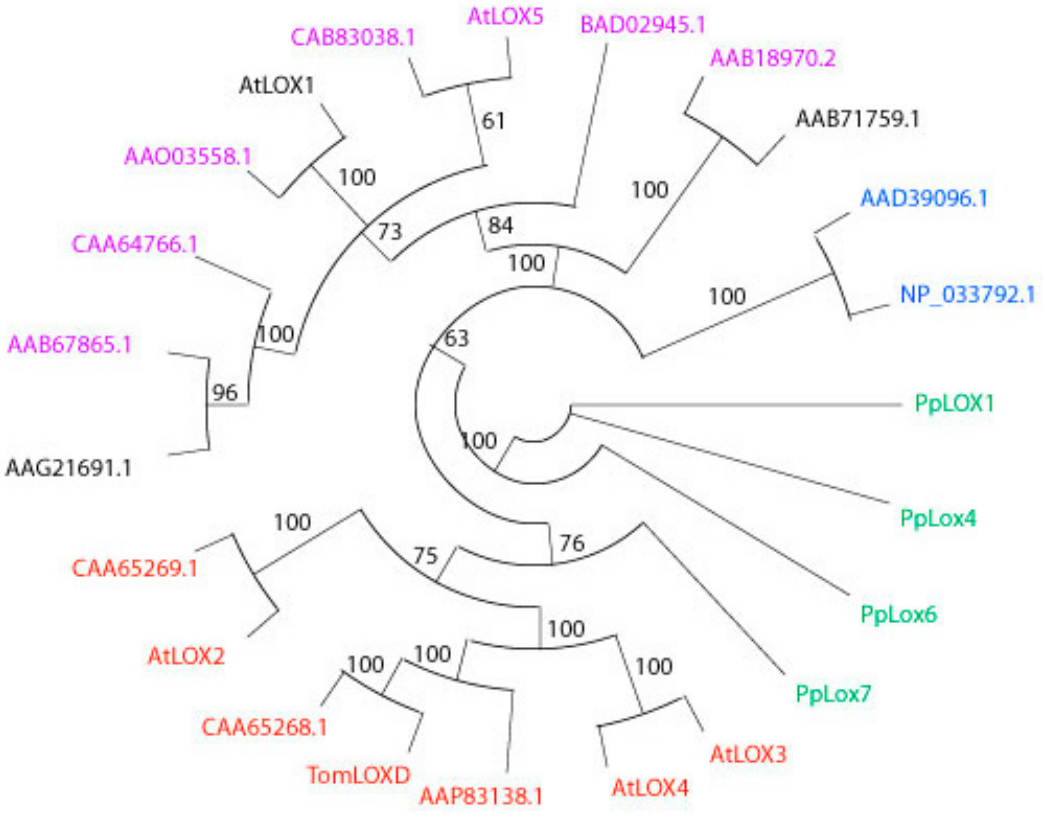


Figure 6.10 Dendrogram indicating phylogenetic relationships between lipoxygenases from mammals and plants. The moss lipoxygenases are shaded in green. Type-2 13-lipoxygenases are shaded in red. The 9-lipoxygenases or seed lipoxygenases are colored in purple while the arachidonic lipoxygenases from mammals are in blue. AtLOX1 (*Arabidopsis thaliana*, NP_175900), AtLOX2 (*Arabidopsis thaliana*, P38418), AtLOX3 (*Arabidopsis thaliana*, NP_564021), AtLOX4 (*Arabidopsis thaliana*, NP_177396.1), AtLOX5 (*Arabidopsis thaliana*, NP_188879.2), AAD39096.1 (*Bos taurus*), AAO3558.1 (*Brassica napus*), CAB83038.1 (*Cucumis sativus*), AAG21691.1 (*Lycopersicon esculentum*), TomLOXD (*Lycopersicon esculutum*, AAB65767.1), NP_033792.1 (*Mus musculus*), AAP83138.1 (*Nicotiana attenuata*), AAB18970.2 (*Phaseolus vulgaris*), AAB71759.1 (*Pisum sativum*), BAD02945.1 (*Oryza sativa*), AAB67865.1 (*Solanum tuberosum*), CAA65268.1 (*Solanum tuberosum*, clone H1), AAB67865.1(*Solanum tuberosum*), CAA65269.1 (*Solanum tuberosum*, clone H3), CAA64766.1 (*Solanum tuberosum*, clone T8).

Effects of JA and wounding on PpLOX6, PpLOX7 and JA biosynthesis

Some aspects of the wound response in higher plants are regulated by the jasmonate (JA) signaling pathway (Farmer et al., 1992; Farmer and Ryan, 1992; Howe, 2004; Wasternack et al., 2006). The expression of *PpLOX6*, *PpLOX7* and activation of biosynthetic enzymes of JA in gametophore was tested in preliminary experiments for induction by MeJA. After three hours of exposure, wounded and MeJa-treated *P. patens* gametophores were analyzed by RT-PCR for up-regulation of *PpLOX6*, *PpLOX7*, and expression of putative orthologs for the JA biosynthetic enzymes *allene oxide cyclase* (*AOC*), *allene oxide synthase* (*AOS*) and *12-oxophytodienoate reductase3* (*OPR3*) (Fig. 11). The samples were normalized by comparison with the expression of the putative *EF1α* transcript. These results demonstrated that *PpLOX7* is both wound-inducible and JA-responsive. However, there was an observable induction by the dilute ethanol control with the other genes tested, thereby masking possible activating effects of JA. Wounding did up-regulate *AOC* and *AOS* but did not induce *OPR3*. These results suggest that the wound induction of *PpLOX7* is partially consistent with the paradigm of JA-mediated wound response in higher plants.

Discussion

Injury of plant tissue resulting from abiotic or biotic events can heighten the risk of desiccation, salt stress, and enhances susceptibility to opportunistic microbes for the wounded plant (Reymond et al., 2000; Cheong et al., 2002). Hence, higher plants have evolved a variety of responses to counter and compensate for the trauma of incurring

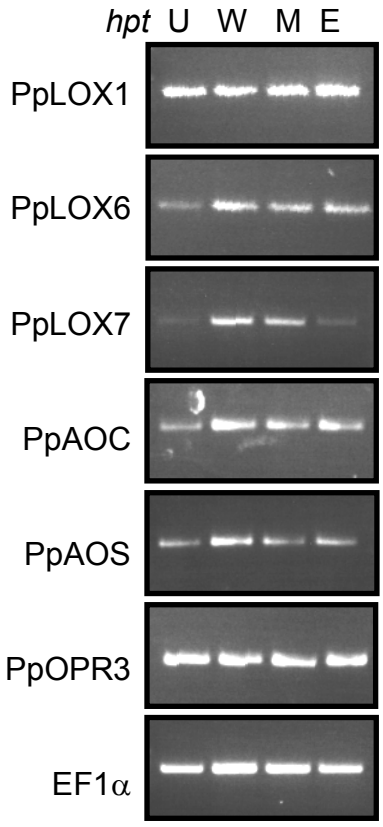


Figure 6.11 Effect of exogenous MeJA (30 μM) on the expression of *PpLOX6*, *PpLOX7*, and putative biosynthetic enzymes for JA. Gametophore were treated by either tissue damage (W), MeJA treatment (M), or a diluted ethanol control (E). Expression of *PpLOX1*, *PpLOX6*, *PpLOX7*, *PpAOC* (allene oxide synthase), *PpAOS* (allene oxide synthase), and *PpOPR3* (12-oxophytodienoate reductase) by RT-PCR. Expression of *EF1α* was used to normalize expression. *hpt*-hours post-treatment.

tissue damage and loss of photosynthetically-active tissues (Meyer et al., 2004; Quilliam et al., 2006; Schwachtje et al., 2006). The activation of the wound response has been widely documented in a diversity of higher plants, including *Arabidopsis* (Bell and Mullet, 1991), tobacco (Walker-Simmons and Ryan, 1977), petunia (Vogt et al., 1994), and woody species like poplar (Clarke et al., 1994).

Bryophytes are considered lower land plants, as they do not produce seed, lack a vascular system and whose gametophyte generation dominates its lifespan. To date, very little is known regarding the wound response in bryophytes although scarring (browning along the wounded edge) is absent in moss (Reski, 1998). We have shown that at the cellular level *Physcomitrella* can mount a physiological response to wounding (Figs. 1, 2). In wounded gametophore leaves, one or two layers of dead cells border the edge of the cut site (Fig. 1C, D). The basis of the cell death may be attributed to either indirect damage due to the physical tearing and torsion during the act of wounding. Alternatively, the cell death may be directly caused by wound-triggered release of apoptotic signals from the injured cells. Evidence of programmed cell death activated by wounding is lacking. However, induction of the *Bax Inhibitor-1* (*AtBI-1*) gene is observed in wounded *A. thaliana* leaves (Sanchez et al., 2000). The Bax protein, a member of the Bcl-2 family, promotes cell death and is functionally conserved in a variety of organisms including nematodes, insects, plants and mammals (Hückelhoven, 2004). The expression of *AtBI-1* is induced by a variety of abiotic and biotic stresses implying that it functions as a general stress response gene (Sanchez et al., 2000). Although the exact function of *AtBI-1* in plants is still unknown, perhaps *AtBI-1* is recruited in wounded leaves to suppress escalation of cell death to the injured leaf. The expression of the lone *BI-1*

ortholog in *P. patens* (*PpBI-1*) has not been characterized with regard to the wound response and requires further exploration. However, *PpBI-1* has been demonstrated to function as an inhibitor of programmed cell death in *P. patens* in response to pathogen attack and abiotic stress (unpublished results, H. Saidasan and M. Lawton)

Callose is deposited along the cell wall in response to pathogenic threats and abiotic stresses (Stone and Clarke, 1992; Delaney et al., 1994). Callose is speculated to obstruct secondary infection by opportunistic pathogens (Brown et al., 1998). Some of the surviving *Physcomitrella* cells along the border of the wound site respond to tissue damage by depositing presumed callose (Fig. 2B). The appearance of callose deposition in moss mirrors the pattern of wound-triggered accumulation in higher plants. Moreover, wound-induced callose deposition is observed in the unicellular, branched and multi-cellular green alga (Scherp et al., 2001), suggesting that wound-induced callose formation is a primitive response that was retained by modern land plants. Mosses may emulate the regulation of callose synthesis in higher plants as deposition may be regulated post-transcriptionally in *P. patens*. Verma and Hong (2001) speculate that the activation of callose synthase (AtCalS*)* in *A. thaliana* may require the additional interaction of G-proteins or the presence of Ca^{2+} with the callose synthase complex. This is the first report of wound-induced deposition of callose in *P. patens*. However, callose was observed in the moss *Funaria* (Scherp et al., 2001).

Wound-induced lipoxygenases of *P. patens*

Lipoxygenases are non-heme iron-containing enzymes that introduce molecular oxygen into polyunsaturated fatty acids possessing a (1*Z*, 4*Z*)-pentadiene system to form

hydroperoxides (Feussner and Wasternack, 2002; Porta and Rocha-Sosa, 2002). In higher plants, α-linolenic acid primarily serves as the fatty acid substrate for lipoxygenases, whereas in animals, arachidonic acid is preferred. The oxylipin, JA, is synthesized from linolenic acid by the octadecanoic pathway and is the key signal for wound response in higher plants (Leon et al., 2001; Wasternack et al., 2006). The presence of both arachidonic acid and linolenic acid in their cell membranes (Table 1) make mosses unique among the land plants and potentially moss lipoxygenases can use both as subtrates (Grimsley et al., 1981; Qi et al., 2004).

To determine if lipoxygenases might play a role in the wound response in *P. patens*, six putative lipoxygenase-like ESTs were isolated using the PpLOX1 sequence to mine the *Physcomitrella* EST database (Fig. 4). Three (*PpLEST4*, *PpLEST6*, *PpLEST7*) were up-regulated by wounding of the gametophore, while the remaining ESTs (*PpLEST2*, *PpLEST3*, *PpLEST5*) along with *PpLOX1* were not activated by wounding. Wound induction of *PpLEST6* and *PpLEST7* in the gametophore was triggered within one hour of incurring tissue damage (Fig. 5). Activation of *PpLEST4* was less pronounced. The timing of *PpLEST6* and *PpLEST7* up-regulation coincides with the immediate induction of some wound-triggered lipoxygenases in higher plants (Bell and Mullet, 1993; Royo et al., 1996). This suggests activation of lipoxygenases in response to wounding is conserved in mosses. The activation of at least three *PpLOXs* suggests that mosses may rely on multiple lipoxygenases for the wound response in the gametophore. In potato, at least two chloroplastidic 13-lipoxygenases are activated by wounding (Royo et al., 1996). The wound-induced potato lipoxygenases are tissue specific and temporally regulated. Recruitment of multiple lipoxygenases may fine tune

responses to a variety of responses, including pathogen defense, growth and development (Feussner and Wasternack, 2002). Additional analyses of the putative *P. patens* lipoxygenases may further detail possible differential regulation of individual lipoxygenases.

Our results showed that levels of *PpLOX1* were not significantly induced by wounding in adult gametophore leaves. The lack of activation in the gametophore may represent that PpLOX1 is not involved in the wound response or it may indicate phase-dependent regulation of certain lipoxygenases. The *PpLOX1* sequence was originally isolated from a cDNA library generated from unwounded protonemal tissue (Senger et al., 2005). Tissue-specific regulation for isoforms of wound-induced lipoxygenases has been documented in bean and potato (Eiben and Slusarenko, 1994; Royo et al., 1996). Additional investigation of *PpLOX6* and *PpLOX7* expression in the protonema may determine if expression of these lipoxygenases is stage-dependent. Alternatively, the *PpLOX1* transcript may effectively function at low steady-state levels or activation of PpLOX1 may occur post-transcriptionally in wounded gametophore leaves, although evidence of this form of regulation of lipoxygenases is not documented in higher plants. The nature of *PpLOX1* activation in the protonema is still unknown as the level of its mRNA expression was never determined.

Genome analysis of *Physcomitrella* lipoxygenases

At least ten putative lipoxygenase-like genes exist in the *Physcomitrella* genome (Table 2). A search of lipoxygenases related to these putative lipoxygenases identified two basic classifications. One group is formed by lipoxygenases that most resemble the

lipoxygenases from higher plants. The second group consists of PpLOX1-like lipoxygenases. Thus during the evolution of *P. patens*, duplication of a progenitor lipoxygenase may have bifurcated into two paralogous groups, one (including the higher plants) retained features of its ancestor while the other evolved moss-specific characteristics.

Phylogenetic tree construction is useful for the prediction of plastid localization, regio- and stereo-specificity of lipoxygenases, but can be inadequate for the substrate predictions (Feussner and Wasternack, 2002). A dendrogram depicted that PpLOX7 was most related to the lipoxygenases of higher plants (Fig. 10). The characterized lipoxygenases in this clade are targeted to the chloroplast and transform the α-linolenic substrate to its 13-hydroperoxy fatty acid (Vick, 1993; Royo et al., 1996). Some of the lipoxygenases in this clade are also responsible for the wound-induced JA production (Bell et al., 1995; Royo et al., 1996; Heitz et al., 1997). PpLOX7 may behave similarly but molecular and biochemical data are needed to substantiate the phylogenetic predictions (e.g. plastid localization, preference for α-linolenic acid, role is JA biosynthesis). The PpLOX4 clusters with PpLOX1. PpLOX1 accepts C_{18} to C_{22} substrates but prefers the C_{20} fatty acid substrates (arachidonic and eicosapentanenoic acids) (Senger et al., 2005). PpLOX4 may perform similar role to PpLOX1 but instead functions in the gametophore. Interestingly, wound-induced PpLOX6 is neither associated with PpLOX7 nor the PpLOX1-like lipoxygenases and is not predicted to be trafficked to any sub-cellular organelle. Cytoplasmic lipoxygenases have been implicated in a variety of functions including senescence, root nodule development, seed

germination and wounding (Matsui et al., 1999; Hause et al., 2000a; He et al., 2002b; Fortes et al., 2004).

Role of JA in *Physcomitrella* wound response

The wound-induced formation of JA in higher plants is critical for resistance to insect herbivory and necrotrophic pathogens (Staswick et al., 1998; Ellis et al., 2002a; Thaler et al., 2004). In preliminary experiments, only *PpLOX7* was discernibly activated by MeJa (Fig. 11). Therefore, wound-dependent and JA-induced activation of *PpLOX7* may be a conserved wound response pathway in *P. patens*. Analysis of JA content in wounded gametophore would demonstrate a capacity to manufacture *de novo* JA. Although the protonema produces volatile oxylipins derived from arachidonic acid, the presence of JA in wounded protonema was not determined (Wichard et al., 2004; Senger et al., 2005).

The up-regulation of *AOS* and *AOC* by wounding in *P. patens* is consistent with the JA-mediated wound response in higher plants (Ziegler et al., 2000; Park et al., 2002; Stenzel et al., 2003a). However, *OPR3* was not transcriptionally activated in wounded or MeJA-treated-gametophore. In tomato and *A. thaliana*, *OPR3* is induced by wounding (Stintzi and Browse, 2000; Strassner et al., 2002). In higher plants, the activity of OPR3 shifts the oxylipin pathway toward JA biosynthesis. The absence of *OPR3* induction in wounded gametophore may suggest that JA biosynthesis may not occur in *P. patens*. Alternatively, up-regulation of *OPR3* expression may not be required for *de novo* JA biosynthesis in wounded moss or *OPR3* may be post-transcriptionally activated. Also,

isoforms of *OPR* (*OPR1*, *OPR2* in *A. thaliana*) which exist in higher plants may instead be critical for JA biosynthesis in *P. patens*.

P. patens as a model organism for dissecting the wound response in land plants

Some aspects of the wound response in higher plants appear conserved in mosses. Further characterization of the wound response in *P. patens* may uncover additional similarities and differences between bryophytes and higher land plants. These variations may illustrate the evolution of the wound response during the transition of plants as they emerged from their aquatic origins. Anatomical difference between mosses and higher plants may have also shaped refinements of the wound response. For example, the lack of a true vascular system in moss may have precluded the evolution of systemic wound signaling. In addition, the single-celled thickness of the gametophore leaf appears more exposed to external stimuli than the protective layers of cells in the leaves of higher plants. However, the simple anatomy of the moss leaf offers an opportunity to analyze the wound response in a two-dimensional format. Visual tools can be used to examine the changes during the wound response which might be more challenging in higher plants.

The capability of generating targeted knockouts makes *P. patens* an attractive system (Schaefer, 2001). The function of a wound-induced gene in *P. patens* can be elucidated by knockout mutant analysis and then be assigned to the corresponding ortholog in higher plants. For example, the functions of two (*ANS*, *THT*) of the wound-specific genes in *A. thaliana* described in Chapter 3 are unknown. However, they are represented by highly related proteins (E-values of 2e-64, 2e-58, respectively) in *P.*

patens. If *ANS* and *THT* perform a similar role in the wound response of *P. patens*, then functional studies through the construction of targeted knockouts may lead to the elucidation of their function in other plants. This system may also be used to determine the function of *BI-1* in regulating programmed cell death during the wound response. The progression of cell death can be easily monitored through the use of vitality stains in the leaf of wild-type gametophore and a *PpBI-1* knockout mutant.

Components of the wound response in higher plants remain unknown in *P. patens*. For example, wounding triggers the synthesis of cell wall-localized or extracellular proteins, like hydroxyproline-rich glycoprotein, proline-rich proteins and hydroxycinnamic amides, which function in wound healing and re-organization of damaged plant cells walls (Jose and Puigdomenech, 1993; Negrel et al., 1993). Since mosses do not produce scar tissue, alternative methods of wound healing and cell wall repair must exist. The pathways generating secondary metabolites in moss are also not well known. In higher plants, secondary compounds are induced by wounding and serve as anti-pest compounds or as scavengers of ROS (Holton and Cornish, 1995; Gould et al., 2002). Examination of the wound-response in *P. patens* may reveal the pathways for secondary metabolism and the diversity of the secondary compounds that are produced. In addition to JA, other signals are deployed in wounded tissues. Both ethylene and ROS contribute to the wound response in higher plants (O'Donnell et al., 1996; Orozco-Cardenas and Ryan, 1999), but the roles of each are still unknown in *P. patens*. If JA is not the principle wound signal in P. patens wound response, these two may represent leading candidates. Ethylene and ROS are involved in the expression of locally-wounded genes in higher plants (Bowles, 1993; Rojo et al., 1999; Delessert et al., 2004). ROS

were shown to spread into systemic leaves in tomato plants (Orozco-Cardenas and Ryan, 1999). In moss, they could serve as a systemic wound signal.

CHAPTER SEVEN

FUTURE DIRECTIONS

The roles for *ANS* and *THT* in the wound response have not been elucidated. However, a reverse genetic and transgenic approach may be useful in identifying their putative functions. An *A. thaliana* T-DNA insertion mutant exists for *ANS*. However, no T-DNA mutant is available for *THT*, but RNAi or anti-sense technology offers an alternate route for disrupting *THT* expression. Moreover, ectopic expression of *ANS* or *THT* through the constitutive activation by the cauliflower mosaic 35S promoter may indicate their wound-related function. If ANS functions in the production of anthocyanidins that subsequently scavenge wound-induced ROS, then differences in the ability to scavenge should be detectable among the T-DNA insertion mutant, wild-type and transgenic overexpression plants. This could be tested by monitoring, in real time, levels of H_2O_2 in the unwounded and wounded leaves with the fluorochromes scopoletin and dichlorofluorescein under epifluorescent microscopy. The function of *THT* in the wound response can be initially gauged by biochemically measuring THT activity to confirm its role in conjugating tyramine to hydroxycinnamic acids. In plants pre-treated with ^{14}C-tyramine, deposition of tyramine hydroxycinnamic acid should be detected near wound sites. Combined with the *THT* knockdown and overexpression in plants, this approach can demonstrate a role for THT in wound healing. Additionally, if THT functions in wound healing, then entry of pathogens should be affected in the transgenic knock-out and overexpression lines. Introgression of GFP-labeled pathogens into a

wound opening may be affected based on the presence or absence of deposited tyramine hydroxycinnamic acids at the wound site.

Since initiating this work, a T-DNA insertion in *SST* has been made available. The insertion appears to truncate the SST protein. This mutant may prove more effective for functional analysis than the RNAi construct engineered to silence *SST*. If the putative paralog At5g07000 is capable of compensating for the loss of SST function, a double mutant may need to be generated. Several T-DNA insertion mutants exist for the At5g07000 locus. The T-DNA insertion and overexpression lines can be analyzed for metabolic profiling of wound- and JA-treated plants. A more sensitive assay would use the mutant lines and wild-type plants fed with ^{14}C-labeled campesterol and then analyzed for steroid content during the wound response. The identity of the putative substrate for SST might indicate the role of the steroid and SST in wound response.

Further experiments are needed to determine if *SYST1-3* reduction occurs during transcriptional initiation or post-transcriptionally. Treatment of transcriptional inhibitors to wounded seedlings or transcriptional run-on assays may identify the stage of *SYST1-3* wound regulation. Two pathways may trigger the reduction of *SYST1-3*. Since *SYST1-3* expression is absent in older leaves, a senescence-related pathway may regulate its expression in the wounded leaf. Senescence mutants can be used to assess for their effects on wound-reduced expression of *SYST1-3*. A second candidate is auxin signaling. The auxin pathway is turned off in wounded plants (Cheong et al., 2002) and the suppression of auxin signaling in wounded plants may be linked to the reduction of *SYST1-3*. Analysis with auxin mutants may elucidate if auxin signaling plays a role in the wound-reduction of *SYST1-3*.

Because systemically-expressed wounding genes were not discovered, several modifications for RDA can be implemented to facilitate their isolation. Inclusion of the unwounded tissue in the driver would eliminate the locally-depleted genes. This could significantly reduce the complexity of the tester cDNA population. The time points used for collection may have been too early for the emergence of a systemically-induced gene as some wounding genes are also induced much later (Yamada et al., 2004). Therefore, later time points may be more productive. Since herbivore feeding elicits a distinctive gene expression from wounding (Stotz et al., 1999), insect-infested plants or wounded plants treated with oral secretions of feeding larva may trigger systemic responses that are absent in wounded plants.

The two sets of genes isolated by the two different RDA strategies may in fact represent secondary responses to wounding. Hence characterization of these genes in response to other stresses (pathogen, drought, heat, salt) may resolve if they are wound-specific or behave as general response genes. The collection of wound-specific genes can also be elaborated through additional RDA strategies using drivers generated from other types of treated tissues (pathogen, heat, drought, salt).

Lipoxygenases were activated in wounded *P. patens* gametophore but only one (*PpLOX7*) was activated by MeJA. PpLOX7 appears most related to the type2-lipoxygenases of higher plants suggesting that PpLOX7 may also be targeted to the chloroplast. PpLOX7 tagged with GFP would demonstrate if PpLOX7 is targeted to the chloroplast. Currently, it has not been shown if *P. patens* can synthesize JA and consequently levels of JA need to be monitored in unwounded and wounded *P. patens*. To date, the JA biosynthetic and signaling pathways are still unexamined in *P. patens*.

The isolation of mutants in the JA pathway might identify genes involved in the wound response.

Biochemical experiments can confirm that PpLOX7 possesses lipoxygenase activity and consequently establish *in vitro* its preferential fatty acid substrates. The *in vivo* substrate for PpLOX7 can be identified by analyzing the changes in fatty acid content in unwounded and wounded gametophore of wild-type and transgenic plants with a disrupted PpLOX7 gene. This may reveal novel defense strategies for land plants equipped with two fatty acids potentially serving as signaling sources. An important issue is whether *P. patens* can synthesize prostaglandins from arachidonic acid. Red algae have arachidonic acid and linolenic acid in their cell membrane and can produce both prostaglandins and JA in response to pathogen infection (Bouarab et al., 2004).

Fluorescently-labeled lipoxygenases can be used to monitor the progression of the wound response in the cells of the wounded leaf and systemically. Cell-cell movement of the wound signal is poorly understood in plants and the use of moss could serve as a model for studying the local transmission of the wound signal. Mosses do not possess a true vascular system and the presence of a systemic wound response is still unclear. The transmitting tissue may act as a conduit for a systemic wound signal. The wound signal may also be deployed through plasmodesmata for cell-cell and long-distance signaling, but the existence of plasmodesmata in *P. patens* is debated. The analysis of long-distance signaling in *P patens* could establish the origin of systemic wound responses as an ancient feature of land plants or as a recently evolved trait of angiosperms.

An herbivore system for moss needs to be established in order to examine the significance of wound-related *P. patens* lipoxygenases in response to insect feeding. Tardigrates and some arthropods are natural predators of mosses and represent possible candidates for a moss-herbivore system (Gerson, 1969). The significance of the wound-induced lipoxygenases can be determined by the effects of herbivory in overexpressing or silenced lines for specific *P. patens* lipoxygenases. Moreover, targeted gene knockouts of known herbivore-induced genes in higher plants can be examined with orthologs in *P. patens*. If an herbivore-moss system can be established, screens for mutants that confer greater resistance or hyper-susceptibility can be isolated and then analyzed for the responsible loci. This may generate a variety of novel herbivore-regulated genes including signaling components and effector molecules that could prove beneficial for application in higher plants.

APPENDIX A

Arabidopsis Primers

Gene	Sequence	Tm (°C)
At5g07000	5’-GAT CCG GTT TCC TCA TCA AGT TCC G-3’	59.5
	5’-TCC GAT CAG TAA TCC CCG GCA AT-3’	60.3
At5g16250	5’-CCT CCA CGC CAC CAC AAT CG-3’	61.1
	5’-GGA ACG GCC ATG GCC AGT-3’	61.3
Cystatin	5’-TGA CTC CAT CGG CGG CGA-3’	62.4
	5’-GAG TCC AGC GAC GAC CTG TT-3’	60.2
EF1α	5’-TCA CAT CAA CAT TGT GGT CAT TGG C-3’	60.1
	5’-TTG ATC TGG TCA AGA GCC TCA AG-3’	58.2
J-12	5’-GAT CTG TTC ATG-3’	21.0
J-24	5’-ACC GAC GTC GAC TAT CCA TGA ACA-3’	52.0
KAT5	5’-GCG CCT GGT TCT CAG AGA GC-3’	60.6
	5’-GCG CGT TTG TGA GAC TCC ACC-3’	61.1
N-12	5’-GAT CTT CCC TCG-3’	28.0
N-24	5’-AGG CAA CTG TGC TAT CCG AGG GAA-3’	54.0
PER64	5’-GTC CCG GTA TCG TCT CTT GC-3’	58.1
	5’-CTT CAC CGT GTT ATG GGC CG-3’	59.3

Gene	Sequence	Tm (°C)
R-12	5’-GAT CTG CGG TGA-3’	28.0
R-24	5’-AGC ACT CTC CAG CCT CTC ACC GCA-3’	57.0
SST	5’-GAT CCG GTT GCC TCG AGT ACC-3’	59.7
	5’-CCG ATC ACT CCC CGG CAA T-3’	60.1
SYST1-3	5’-CCT CCT CGG TGC ATC GTC AA-3’	60.4
	5’-AAC ACA GAG CCC ACC AGC-3’	58.6
XTH9	5’-GGA ACA CTA CCG GCG AGC CTT-3’	61.9
	5’-GCA ACG AAA GGA GCG TGA CTC CA-3’	61.7

APPENDIX B

Physcomitrella Primers

Gene	Sequence	Tm (°C)
AOC	5’-GCA TGT CAA CAG CCT CGG GG-3’	61.3
	5’-GCT GGA GCC ACG GAC GG-3’	62.0
AOS	5’-TGA GCT TCA CCA GCG GGT AC-3’	59.6
	5’-GGG AGT GGG ACG GTG TGT AA-3’	59.1
PpLOX1	5’-CGA AGA CGG TCG GAG AGG AG-3’	59.2
	5’-TCC AAC GAG TAC GCG CGT G-3’	60.4
PpLEST2	5’-CTC GCG GGC TTC AAC CCT AT-3’	59.7
	5’-AGT GTC CTG CTG GCG TAG TTC-3’	59.3
PpLEST3	5’-GTG CTG TCG GCG CAC TCA C-3’	62.0
	5’-GCT GTT GGG CAC ACC CAT GAA TG-3’	61.3
PpLEST4	5’-GGC TCC CGC GAA GGG TTT G-3’	61.8
	5’-GTC GCC GGA TCC TTG AGT G-3’	61.8
PpLEST5	5’-CCG GCA CCG TTG CAT CGA CA-3’	63.3
	5’-GTC CGC AAC GCG ATC CAT CAC G-3’	63.0
PpLEST6	5’-CTA CCT CAC CAA GGA CGG CAC C-3’	61.8
	5’-GTG CGT CCG CAA CCA GTG AC-3’	61.6

Gene	Sequence	Tm (°C)
PpLEST7	5’-GCC CAA CCA AGA GCA ATC CAC C-3’	61.1
	5’-CCT CGC ATG GTG ATC CCG G-3’	60.9
PpOPR	5’-CGG GTG ATG GAA CGG CAG AG-3’	60.7
	5’-CGG TCT CGA TGT GGG GCG TT-3’	62.4

BIBLIOGRAPHY

Abel, S., Savchenko, T., and Levy, M. (2005). Genome-wide comparative analysis of the IQD gene families in *Arabidopsis thaliana* and *Oryza sativa*. BMC Evol Biol. **5,** 72.

Adams, J.B., Phillips, N.S., and Hall, R. (1988). Metabolic fate of estradiol in human mammary cancer cells in culture: estrogen sulfate formation and cooperativity exhibited by estrogen sulfotransferase. Mol Cell Endocrinol. **58,** 231-242.

Adler, J.H., and Grebenok, R.J. (1995). Phytoecdysteroids. Lipids **30,** 257-262.

Aist, J.R. (1976). Papillae and related wound plugs of plant cells. Annu. Rev. Phytopath. **14,** 145-163.

Alder, M.N., Dames, S., Gaudet, J., and Mango, S.E. (2003). Gene silencing in *Caenorhabditis elegans* by transitive RNA interference. RNA **9,** 25-32.

Aloni, R., Baum, S.F., and Peterson, C.A. (1990). The role of cytokinin in sieve tube regeneration and callose production in wounded *Coleus* internodes. Plant Physiol. **93,** 982-989.

Alonso, J.M., Hirayama, T., Roman, G., Nourizadeh, S., and Ecker, J.R. (1999). EIN2, a bifunctional transducer of ethylene and stress responses in *Arabidopsis*. Science **284,** 2148-2152.

Anderson, E., and Howell, A. (1995). Oestrogen sulphotransferases in malignant and normal human breast tissue. Endocr-Rel Cancer. **2,** 227-233.

Arimura, G., Ozawa, R., Shimoda, T., Nishioka, T., Boland, W., and Takabayashi, J. (2000). Herbivory-induced volatiles elicit defence genes in lima bean leaves. Nature **406,** 512-515.

Artus, N.N., Uemura, M., Steponkus, P.L., Gilmour, S.J., Lin, C., and Thomashow, M.F. (1996). Constitutive expression of the cold-regulated *Arabidopsis thaliana COR15a* gene affects both chloroplast and protoplast freezing tolerance. PNAS **93,** 13404-13409.

Ausubel, F.M. (2005). Are innate immune signaling pathways in plants and animals conserved? Nat. Immunol. **6,** 973-979.

Back, K., Jang, S.M., Lee, B.C., Schmidt, A., Strack, D., and Kim, K.M. (2001). Cloning and characterization of a hydroxycinnamoyl-CoA:tyramine *N*-(hydroxycinnamoyl)transferase induced in response to UV-C and wounding from *Capsicum annuum*. Plant Cell Physiol. **42,** 475-481.

Baenziger, J.U. (2003). Glycoprotein hormone GalNAc-4-sulphotransferase. Biochem Soc Trans **31,** 326-330.

Baker, S.S., Wilhelm, K.S., and Thomashow, M.F. (1994). The 5'-region of *Arabidopsis thaliana COR15a* has cis-acting elements that confer cold-, drought- and ABA-regulated gene expression. Plant Mol Biol. **24,** 701-713.

Baldwin, I.T. (1998). Jasmonate-induced responses are costly but benefit plants under attack in native populations. PNAS **95,** 8113-8118.

Baldwin, I.T. (1999). Functional interactions in the use of direct and indirect defences in native *Nicotiana* plants. Novartis Found Symp. **223,** 74-87.

Baldwin, I.T., Sims, C.L., and Kean, S.E. (1990). The reproductive consequences associated with inducible alkaloidal responses in wild tobacco. Ecology **71,** 252-262.

Barckhausen, R. (1978). Ultrastructural changes in wounded plant storage tissue cells. In Biochemistry of Wounded Plant Tissues, G. Kahl, ed (New York: Walter de Gruyter), pp. 1-42.

Bateman, A., and Sandford, R. (1999). The PLAT domain: a new piece in the PKD1 puzzle. Curr Biol. **9,** R588-R590.

Baydoun, E.A.H., and Fry, S.C. (1985). The immobility of pectic substances in injured tomato leaves and its bearing on the identity of the wound hormone. Planta **165,** 269-276.

Beers, E.P., Woffenden, B.J., and Zhao, C. (2000). Plant proteolytic enzymes: possible roles during programmed cell death. Plant Mol Biol. **44,** 399-415.

Beisson, F., Koo, A.J., Ruuska, S., Schwender, J., Pollard, M., Thelen, J.J., Paddock, T., Salas, J.J., Savage, L., Milcamps, A., Mhaske, V.B., Cho, Y., and Ohlrogge, J.B. (2003). Arabidopsis genes involved in acyl lipid metabolism. A 2003 census of the candidates, a study of the distribution of expressed sequence tags in organs, and a web-based database. Plant Physiol. **132,** 681-697.

Belkhadir, Y., and Chory, J. (2006). Brassinosteroid signaling: a paradigm for steroid hormone signaling from the cell surface. Science **314,** 1410-1411.

Bell, E., and Mullet, J.E. (1991). Lipoxygenase gene expression is modulated in plants by water deficit, wounding, and methyl jasmonate. Mol Gen Genet **230,** 456-462.

Bell, E., and Mullet, J.E. (1993). Characterization of an *Arabidopsis* lipoxygenase gene responsive to methyl jasmonate and wounding. Plant Physiol. **103,** 1133-1137.

Bell, E., Creelman, R.A., and Mullet, J.E. (1995). A chloroplast lipoxygenase is required for wound-induced jasmonic acid accumulation in *Arabidopsis*. PNAS **92,** 8675-8679.

Bender, J., and Fink, G.R. (1998). A Myb homologue, *ATR1*, activates tryptophan gene expression in *Arabidopsis*. PNAS **95,** 5655-5660.

Bennett, R.N., and Wallsgrove, R.M. (1994). Secondary metabolites in plant defence mechanisms. New Phytol. **127,** 617-633.

Bennetzen, J.L., Coleman, C., Liu, R., Ma, J., and Ramakrishna, W. (2004). Consistent over-estimation of gene number in complex plant genomes. Curr Opin Plant Biol. **7,** 732-736.

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

Bergey, D.R., Howe, G.A., and Ryan, C.A. (1996). Polypeptide signaling for plant defensive genes exhibits analogies to defense signaling in animals. PNAS **93,** 12053-12058.

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

Biesgen, C., and Weiler, E.W. (1999). Structure and regulation of *OPR1* and *OPR2*, two closely related genes encoding 12-oxophytodienoic acid-10,11-reductases from *Arabidopsis thaliana*. Planta **208,** 155-165.

Biggs, A.R. (1997). Genetic and temporal variation in abscission zone formation in peach leaves in relation to peach canker disease. Can. J. Bot **75,** 717-722.

Bird, P.M., and Ride, J.P. (1981). The resistance of wheat to *Septoria nodorum*: fungal development in relation to host lignification. Physiol Plant Pathol **19,** 289-300.

Birkenmeier, G.F., and Ryan, C.A. (1998). Wound signaling in tomato plants. Evidence that ABA is not a primary signal for defense gene activation. Plant Physiol. **117,** 687-693.

Birkett, M.A., Campbell, C.A., Chamberlain, K., Guerrieri, E., Hick, A.J., Martin, J.L., Matthes, M., Napier, J.A., Pettersson, J., Pickett, J.A., Poppy, G.M., Pow, E.M., Pye, B.J., Smart, L.E., Wadhams, G.H., Wadhams, L.J., and Woodcock, C.M. (2000). New roles for *cis*-jasmone as an insect semiochemical and in plant defense. PNAS **97,** 9329-9334.

Blechert, S., Bockelmann, C., FuBlein, M., Schrader, T.v., Stelmach, B., Niesel, U., and Weiler, E.W. (1999). Structure-activity analyses reveal the existence of two

separate groups of active octadecanoids in elicitation of the tendril-coiling response of *Bryonia dioica* Jacq. Planta **207,** 470-479.

Blee, E. (2002). Impact of phyto-oxylipins in plant defense. Trends Plant Sci. **7,** 315-322.

Boege, K., and Marquis, R.J. (2005). Facing herbivory as you grow up: the ontogeny of resistance in plants. Trends Ecol Evol. **20,** 441-448.

Borek, V., Elberson, L.R., McCaffrey, J.P., and Morra, M.J. (1998). Toxicity of isothiocyanates produced by glucosinolates in *Brassicaceae* species to black vine weevil eggs. J. Agric. Food Chem. **46,** 5318-5323.

Borner, G.H.H., Lilley, K.S., Stevens, T.J., and Dupree, P. (2003). Identification of glycosylphosphatidylinositol-anchored proteins in *Arabidopsis*. A proteomic and genomic analysis. Plant Physiol. **132,** 568-577.

Bors, W., Michel, C., and Saran, M. (1994). Flavonoid antioxidants: rate constants for reactions with oxygen radicals. Methods Enzymol. **234,** 420-429.

Bostock, R.M., and Stermer, B.A. (1989). Perspectives on wound healing in resistance to pathogens. Annu Rev Phytopathol **27,** 343-371.

Bouarab, K., Adas, F., Gaquerel, E., Kloareg, B., Salaun, J.-P., and Potin, P. (2004). The innate immunity of a marine red alga involves oxylipins from both the eicosanoid and octadecanoid pathways. Plant Physiol. **135,** 1838-1848.

Bowles, D.J. (1993). Local and systemic signals in the wound response. Semin Cell Biol **4,** 103-111.

Bowles, D.J. (1994). Signalling events in the wound response of tomato plants. Biochem Soc Symp **60,** 155-164.

Boyington, J.C., Gaffney, B.J., and Amzel, L.M. (1993). The three-dimensional structure of an arachidonic acid 15-lipoxygenase. Science **260,** 1482-1486.

Braam, J. (2005). In touch: plant responses to mechanical stimuli. New Phytol **165,** 373-389.

Braam, J., and Davis, R.W. (1990). Rain-, wind-, and touch-induced expression of calmodulin and calmodulin-related genes in *Arabidopsis*. Cell **60,** 357-364.

Brader, G., Tas, E., and Palva, E.T. (2001). Jasmonate-dependent induction of indole glucosinolates in *Arabidopsis* by culture filtrates of the nonspecific pathogen *Erwinia carotovora*. Plant Physiol. **126,** 849-860.

Braidot, E., Petrussa, E., Micolini, S., Tubaro, F., Vianello, A., and Macri, F. (2004). Biochemical and immunochemical evidences for the presence of lipoxygenase in plant mitochondria. J. Exp. Bot. **55,** 1655-1662.

Braunstein, T.H., Moury, B., Johannessen, M., and Albrechtsen, M. (2002). Specific degradation of 3' regions of GUS mRNA in posttranscriptionally silenced tobacco lines may be related to 5'-3' spreading of silencing. RNA **8,** 1034-1044.

Brent, M.R. (2005). Genome annotation past, present, and future: How to define an ORF at each locus. Genome Res. **15,** 1777-1786.

Brown, I., Trethowan, J., Kerry, M., Mansfield, J., and Bolwell, G.P. (1998). Localization of components of the oxidative cross-linking of glycoproteins and of callose synthesis in papillae formed during the interaction between non-pathogenic strains of *Xanthomonas campestris* and French bean mesophyll cells. Plant J. **15,** 333-343.

Butt, A., Mousley, C., Morris, K., Beynon, J., Can, C., Holub, E., Greenberg, J.T., and Buchanan-Wollaston, V. (1998). Differential expression of a senescence-enhanced metallothionein gene in *Arabidopsis* in response to isolates of *Peronospora parasitica* and *Pseudomonas syringae*. Plant J **16,** 209-221.

Cao, H., Bowling, S.A., Gordon, A.S., and Dong, X. (1994). Characterization of an *Arabidopsis* mutant that is nonresponsive to inducers of systemic acquired resistance. Plant Cell **6,** 1583-1592.

Celenza, J.L., Quiel, J.A., Smolen, G.A., Merrikh, H., Silvestro, A.R., Normanly, J., and Bender, J. (2005). The Arabidopsis *ATR1* Myb transcription factor controls indolic glucosinolate homeostasis. Plant Physiol. **137,** 253-262.

Chang, C.C., Ball, L., Fryer, M.J., Baker, N.R., Karpinski, S., and Mullineaux, P.M. (2004). Induction of *ASCORBATE PEROXIDASE 2* expression in wounded *Arabidopsis* leaves does not involve known wound-signalling pathways but is associated with changes in photosynthesis. Plant J **38,** 499-511.

Cheong, Y.H., Chang, H.S., Gupta, R., Wang, X., Zhu, T., and Luan, S. (2002). Transcriptional profiling reveals novel interactions between wounding, pathogen, abiotic stress, and hormonal responses in *Arabidopsis*. Plant Physiol. **129,** 661-677.

Choi, D., Ward, B.L., and Bostock, R.M. (1992). Differential induction and suppression of potato 3-hydroxy-3-methylglutaryl coenzyme A reductase genes in response to *Phytophthora infestans* and to its elicitor arachidonic acid. Plant Cell **4,** 1333-1344.

Choi, D., Bostock, R.M., Avdiushko, S., and Hildebrand, D.F. (1994). Lipid-derived signals that discriminate wound- and pathogen-responsive isoprenoid pathways in plants: methyl jasmonate and the fungal elicitor arachidonic acid induce different 3-hydroxy-3-methylglutaryl-coenzyme A reductase genes and antimicrobial isoprenoids in *Solanum tuberosum* L. PNAS **91,** 2329-2333.

Chomcyznski, P., and Sacchi, N. (1987). Single-step method of isolation of RNA by guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem **162,** 156-159.

Christmann, A., Moes, D., Himmelbach, A., Yang, Y., Tang, Y., and Grill, E. (2006). Integration of abscisic acid signalling into plant responses. Plant Biol **8,** 314-325.

Church, G.M., and Gilbert, W. (1984). Genomic sequencing. PNAS **81,** 1991-1995.

Clarke, D.D. (1982). The accumulation of cinnamic acid amides in the cell walls of potato tissue an early response to fungal attack. In Active Defense in Plants, R.K.S. Wood, ed (New York: Plenum Press), pp. 321-322.

Clarke, H.R., Davis, J.M., Wilbert, S.M., Bradshaw, H.D.J., and Gordon, M.P. (1994). Wound-induced and developmental activation of a poplar tree chitinase gene promoter in transgenic tobacco. Plant Mol Biol. **25,** 799-815.

Clough, S.J., and Bent, A.F. (1998). Floral dip: a simplified method for*Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J. **16,** 735-743.

Conconi, A., Miquel, M., Browse, J.A., and Ryan, C.A. (1996). Intracellular levels of free linolenic and linoleic acids increase in tomato leaves in response to wounding. Plant Physiol. **111,** 797-803.

Corbineau, F., Rudnicki, R.M., and Come, D. (1988). The effects of methyl jasmonate on sunflower (*Helianthus annus* L) seed germination and seedling development. Plant Growth Regul **7,** 157-169.

Cove, D.J., Knight, C.D., and Lamparter, T. (1997). Mosses as model systems. Trends Plant Sci. **2,** 99-105.

Creelman, R.A., and Mullet, J.E. (1995). Jasmonic acid distribution and action in plants: regulation during development and response to biotic and abiotic stress. PNAS **92,** 4114-4119.

Creelman, R.A., and Mullet, J.E. (1997). Oligosaccharins, brassinolides, and jasmonates: nontraditional regulators of plant growth, development, and gene expression. Plant Cell **9,** 1211-1223.

Creelman, R.A., Tierney, M.L., and Mullet, J.E. (1992). Jasmonic acid/methyl jasmonate accumulate in wounded soybean hypocotyls and modulate wound gene expression. PNAS **89,** 4938-4941.

Croft, K., Juttner, F., and Slusarenko, A.J. (1993). Volatile products of the lipoxygenase pathway evolved from *Phaseolus vulgaris* (L.) leaves inoculated with *Pseudomonas syringae* pv phaseolicola. Plant Physiol. **101,** 13-24.

Cruz Castillo, M., Martinez, C., Buchala, A., Metraux, J.P., and Leon, J. (2004). Gene-specific involvement of beta-oxidation in wound-activated responses in *Arabidopsis*. Plant Physiol. **135,** 85-94.

Cummins, I., Cole, D.J., and Edwards, R. (1999). A role for glutathione transferases functioning as glutathione peroxidases in resistance to multiple herbicides in black-grass. Plant J **18,** 285-292.

Czapski, J., and Saniewski, M. (1992). Stimulation of ethylene production and ethylene-forming enzyme in fruits of the non-ripening *nor* and *rin* tomato mutants by methyl jasmonate. J. Plant Physiol **139,** 265-268.

David, G. (1993). Integral membrane heparan sulfate proteoglycans. FASEB J. **7,** 1023-1030.

Davies, E. (1987). Plant responses to wounding. In A Comprehensive Treatise, E.E.C. P.K. Stumpf, ed (New York: Academic), pp. 234-264.

Davies, E., and Schuster, A. (1981). Intercellular communication in plants: Evidence for a rapidly generated, bidirectionally transmitted wound signal. PNAS **78,** 2422-2426.

Delaney, T.P., Uknes, S., Vernooij, B., Friedrich, L., Weymann, K., Negrotto, D., Gaffney, T., Gut-Rella, M., Kessmann, H., and Ward, E. (1994). A central role of salicylic acid in plant disease resistance. Science **266,** 1247.

Delessert, C., Wilson, I.W., Van Der Straeten, D., Dennis, E.S., and Dolferus, R. (2004). Spatial and temporal analysis of the local response to wounding in *Arabidopsis* leaves. Plant Mol Biol. **55,** 165-181.

Delessert, C., Kazan, K., Wilson, I.W., Van Der Straeten, D., Manners, J., Dennis, E.S., and Dolferus, R. (2005). The transcription factor ATAF2 represses the expression of pathogenesis-related genes in *Arabidopsis*. Plant J **43,** 745-757.

Delker, C., Stenzel, I., Hause, B., Miersch, O., Feussner, I., and Wasternack, C. (2006). Jasmonate biosynthesis in *Arabidopsis thaliana*--enzymes, products, regulation. Plant Biol **8,** 297-306.

Devoto, A., and Turner, J.G. (2003). Regulation of jasmonate-mediated plant responses in *Arabidopsis*. Ann Bot **92,** 329-337.

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

Devoto, A., Nieto-Rostro, M., Xie, D., Ellis, C., Harmston, R., Patrick, E., Davis, J., Sherratt, L., Coleman, M., and Turner, J.G. (2002). COI1 links jasmonate

signalling and fertility to the SCF ubiquitin-ligase complex in *Arabidopsis*. Plant J **32,** 457-466.

Dixon, D.P., Lapthorn, A., and Edwards, R. (2002). Plant glutathione transferases. Genome Biol. **3,** 1–10.

Dixon, R.A., and Paiva, N.L. (1995). Stress-induced phenylpropanoid metabolism. Plant Cell **7,** 1085-1097.

Doares, S.H., Syrovets, T., Weiler, E.W., and Ryan, C.A. (1995a). Oligogalacturonides and chitosan activate plant defensive genes through the octadecanoid pathway. PNAS **92,** 4095-4098.

Doares, S.H., Narvaez-Vasquez, J., Conconi, A., and Ryan, C.A. (1995b). Salicylic acid inhibits synthesis of proteinase inhibitors in tomato leaves induced by systemin and jasmonic acid. Plant Physiol. **108,** 1741-1746.

Doherty, H.M., Selvendran, R.R., and Bowles, D.J. (1988). The wound response of tomato plants can be inhibited by aspirin and related hydroxy-benzoic acids. Physiol Mol Plant Path. **33,** 377-384.

Donald, R.G.K., and Cashmore, A.R. (1990). Mutation of either G box or I box sequences profoundly affects expression from the *Arabidopsis* rbcS-1A promoter. EMBO J **9,** 1717-1726.

Dong, X. (2004). NPR1, all things considered. Curr Opin Plant Biol **7,** 547-552.

Donofrio, N.M., and Delaney, T.P. (2001). Abnormal callose response phenotype and hypersusceptibility to *Peronospoara parasitica* in defence-compromised *Arabidopsis nim1-1* and salicylate hydroxylase-expressing plants. Mol Plant Microbe Interact **14,** 439-450.

Driscoll, W.J., Komatsu, K., and Strott, C.A. (1995). Proposed active site domain in estrogen sulfotransferase as determined by mutational analysis. PNAS **92,** 12328-12332.

Edwards, R., and Dixon, D.P. (2000). The role of glutathione transferases in herbicide metabolism. In Herbicides and Their Mechanisms of Action, K.R. Cobb AH, ed (Sheffield: Sheffield Academic Press), pp. 38–71.

Eiben, H.G., and Slusarenko, A.J. (1994). Complex spatial and temporal expression of lipoxygenase genes during *Phaseolus vulgaris* (L.) development. Plant J **5,** 123-135.

Eigenbrode, S.D., and Espelie, K.E. (1995). Effects of plant epicuticular lipids on insect herbivores. Annu Rev Entomol **40,** 171-194.

Ellard-Ivey, M., and Douglas, C.J. (1996). Role of jasmonates in the elicitor- and wound-inducible expression of defense genes in parsley and transgenic tobacco. Plant Physiol. **112,** 183-192.

Ellis, C., and Turner, J.G. (2001). The *Arabidopsis* mutant *cev1* has constitutively active jasmonate and ethylene signal pathways and enhanced resistance to pathogens. Plant Cell **13,** 1025-1033.

Ellis, C., Karafyllidis, I., and Turner, J.G. (2002a). Constitutive activation of jasmonate signaling in an *Arabidopsis* mutant correlates with enhanced resistance to *Erysiphe cichoracearum*, *Pseudomonas syringae*, and *Myzus persicae*. Mol Plant Microbe Interact **15,** 1025-1030.

Ellis, C., Karafyllidis, I., Wasternack, C., and Turner, J.G. (2002b). The *Arabidopsis* mutant *cev1* links cell wall signaling to jasmonate and ethylene responses. Plant Cell **14,** 1557-1566.

Emanuelsson, O., Nielsen, H., Brunak, S., and Heijne, G. (2000). Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. J Mol Biol. **300,** 1005-1016.

Eulgem, T. (2005). Regulation of the *Arabidopsis* defense transcriptome. Trends Plant Sci. **10,** 71-78.

Falany, C.N. (1997). Enzymology of human cytosolic sulfotransferases. FASEB J. **11,** 206-216.

Falany, C.N., and Kerl, E.A. (1990). Sulfation of minoxidil by human liver phenol sulfotransferase. Biochem Pharmacol **40,** 1027-1032.

Falany, C.N., Wheeler, J., Oh, T.S., and Falany, J.L. (1994). Steroid sulfation by expressed human cytosolic sulfotransferases. J Steroid Biochem Mol Biol. **48,** 369-375.

Fammartino, A., Cardinale, F., Gobel, C., Mene-Saffrane, L., Fournier, J., Feussner, I., and Esquerre-Tugaye, M.-T. (2007). Characterization of a divinyl ether biosynthetic pathway specifically associated with pathogenesis in tobacco. Plant Physiol. **143,** 378-388.

Farmaki, T., Sanmartin, M., Jimenez, P., Paneque, M., Sanz, C., Vancanneyt, G., Leon, J., and Sanchez-Serrano, J.J. (2007). Differential distribution of the lipoxygenase pathway enzymes within potato chloroplasts. J. Exp. Bot. **58,** 555-568.

Farmer, E.E., and Ryan, C.A. (1990). Interplant communication: airborne methyl jasmonate induces synthesis of proteinase inhibitors in plant leaves. PNAS **87,** 7713-7716.

Farmer, E.E., and Ryan, C.A. (1992). Octadecanoid precursors of jasmonic acid activate the synthesis of wound-inducible proteinase inhibitors. Plant Cell **4,** 129-134.

Farmer, E.E., Johnson, R.R., and Ryan, C.A. (1992). Regulation of expression of proteinase inhibitor genes by methyl jasmonate and jasmonic acid. Plant Physiol. **98,** 995-1002.

Farmer, E.E., Almeras, E., and Krishnamurthy, V. (2003). Jasmonates and related oxylipins in plant responses to pathogenesis and herbivory. Curr Opin Plant Biol. **6,** 372-378.

Farooqui, A.A. (1980). 3'-phosphoadenosine 5'-phosphosulphate metabolism in mammalian tissues. Int J Biochem. **12,** 529-536.

Farooqui, A.A., and Horrocks, L.A. (1985). On the role of sulfolipids in mammalian metabolism. Mol Cell Biochem **66,** 87-95.

Ferreres, F., Gil, M.I., Castaner, M., and Tomas-Barberan, F.A. (1997). Phenolic metabolites in red pigmented lettuce (*Lactuca sativa*). Changes with minimal processing and cold storage. J Agric Food Chem. **45,** 4249-4254.

Feussner, I., and Wasternack, C. (2002). The lipoxygenase pathway. Annu Rev Plant Biol. **53,** 275-297.

Feys, B., Benedetti, C.E., Penfold, C.N., and Turner, J.G. (1994). *Arabidopsis* mutants selected for resistance to the phytotoxin coronatine are male sterile, insensitive to methyl jasmonate, and resistant to a bacterial pathogen. Plant Cell **6,** 751-759.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., and Mello, C.C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature **391,** 806-811.

Fortes, A.M., Coronado, M.J., Testillano, P.S., del Carmen Risueno, M., and Pais, M.S. (2004). Expression of lipoxygenase during organogenic nodule formation from hop internodes. J. Histochem. Cytochem. **52,** 227-241.

Fowler, T.J., Bernhardt, C., and Tierney, M.L. (1999). Characterization and expression of four proline-rich cell wall protein genes in *Arabidopsis* encoding two distinct subsets of multiple domain proteins. Plant Physiol. **121,** 1081-1091.

Frank, W., Decker, E.L., and Reski, R. (2005). Molecular tools to study *Physcomitrella patens*. Plant Biol., 220-227.

Friedman, R., and Hughes, A.L. (2002). Molecular evolution of the NF-kappaB signaling system. Immunogenetics **53,** 964-974.

Fujioka, S., and Yokota, T. (2003). Biosynthesis and metabolism of brassinosteroids. Annu Rev Plant Biol. **54,** 137-164.

Fujioka, S., Choi, Y.-H., Takatsuto, S., Yokota, T., Li, J., Chory, J., and Sakurai, A. (1996). Identification of castasterone, 6-deoxocastasterone, typhasterol and 6-deoxotyphasterol from the shoots of *Arabidopsis thaliana*. Plant Cell Physiol. **37,** 1201-1203.

Gaffney, T., Friedrich, L., Vernooij, B., Negrotto, D., Nye, G., Uknes, S., Ward, E., Kessmann, H., and Ryals, J. (1993). Requirement of salicylic acid for the induction of systemic acquired resistance. Science **261,** 754.

Galli, S.J., and Wershil, B.K. (1996). The two faces of the mast cell. Nature **381,** 21-22.

Gamage, N.U., Tsvetanov, S., Duggleby, R.G., McManus, M.E., and Martin, J.L. (2005). The structure of human SULT1A1 crystallized with estradiol: An insight into active site plasticity and substrate inhibition with multi-ring substrates. J. Biol. Chem. **280,** 41482-41486.

Gamage, N.U., Duggleby, R.G., Barnett, A.C., Tresillian, M., Latham, C.F., Liyou, N.E., McManus, M.E., and Martin, J.L. (2003). Structure of a human carcinogen-converting enzyme, SULT1A1. Structural and kinetic implications of substrate inhibition. J. Biol. Chem. **278,** 7655-7662.

Gan, S., and Amasino, R.M. (1997). Making sense of senescence (Molecular genetic regulation and manipulation of leaf senescence). Plant Physiol. **113,** 313-319.

Gerson, U. (1969). Moss-Arthropod Associations. Bryologist **72,** 495-500.

Gidda, S.K., Miersch, O., Levitin, A., Schmidt, J., Wasternack, C., and Varin, L. (2003). Biochemical and molecular characterization of a hydroxyjasmonate sulfotransferase from *Arabidopsis thaliana*. J. Biol. Chem. **278,** 17895-17900.

Glatt, H., Boeing, H., Engelke, C.E., Ma, L., Kuhlow, A., Pabel, U., Pomplun, D., Teubner, W., and Meinl, W. (2001). Human cytosolic sulphotransferases: genetics, characteristics, toxicological aspects. Mutat Res. **482,** 27-40.

Glombitza, S., Dubuis, P., Thulke, O., Welzl, G., Bovet, L., Götz, M., Affenzeller, M., Geist, B., Hehn, A., and Asnaghi, C. (2004). Crosstalk and differential response to abiotic and biotic stressors reflected at the transcriptional level of effector genes from secondary metabolism. Plant Molecular Biol. **54,** 817-835.

Gould, K.S. (2004). Nature's Swiss army knife: the diverse protective roles of anthocyanins in leaves. J Biomed Biotechnol. **2004,** 314-320.

Gould, K.S., McKelvie, J., and Markham, K.R. (2002). Do anthocyanins function as antioxidants in leaves? Imaging of H_2O_2 in red and green leaves after mechanical injury. Plant Cell Environ. **25,** 1261-1269.

Grechkin, A.N. (2002). Hydroperoxide lyase and divinyl ether synthase. Prostaglandins Other Lipid Mediat **68-69,** 457-470.

Green, T.R., and Ryan, C.A. (1972). Wound-induced proteinase inhibitor in plant leaves: a possible defense mechanism against insects. Science **175,** 776-777.

Grimsley, N.H., Grimsley, J.M., and Hartmann, E. (1981). Fatty acid composition of mutants of the moss *Physcomitrella patens*. Phytochemistry **20,** 1519-1524.

Gu, Y.Q., Pautot, V., Holzer, F.M., and Walling, L.L. (1996). A complex array of proteins related to the multimeric leucine aminopeptidase of tomato. Plant Physiol. **110,** 1257-1266.

Guan, Y., and Nothnagel, E.A. (2004). Binding of arabinogalactan proteins by Yariv phenylglycoside triggers wound-like responses in *Arabidopsis* cell cultures. Plant Physiol. **135,** 1346-1366.

Gupta, V., Willits, M.G., and Glazebrook, J. (2000). *Arabidopsis thaliana EDS 4* contributes to aalicylic acid(SA)-dependent expression of defense responses: Evidence for inhibition of jasmonic acid signaling by SA. Mol Plant Microbe Interact. **13,** 503-511.

Gutierrez, R.A., MacIntosh, G.C., and Green, P.J. (1999). Current perspectives on mRNA stability in plants: multiple levels and mechanisms of control. Trends Plant Sci **4,** 429-438.

Hahlbrock, K., and Scheel, D. (1989). Physiology and molecular biology of phenylpropanoid metabolism. Annu Rev Plant Physiol. **40,** 347-369.

Hahlbrock, K., Bednarek, P., Ciolkowski, I., Hamberger, B., Heise, A., Liedgens, H., Logemann, E., Nurnberger, T., Schmelzer, E., Somssich, I.E., and Tan, J. (2003). Non-self recognition, transcriptional reprogramming, and secondary metabolite accumulation during plant/pathogen interactions. PNAS **100 Suppl 2,** 14569-14576.

Halim, V.A., Vess, A., Scheel, D., and Rosahl, S. (2006). The role of salicylic acid and jasmonic acid in pathogen defence. Plant Biol **8,** 307-313.

Halkier, B.A., and Gershenzon, J. (2006). Biology and biochemistry of glucosinolates. Ann Rev Plant Biol. **57,** 303-333.

Hamberg, M., and Fahlstadius, P. (1990). Allene oxide cyclase: a new enzyme in plant lipid metabolism. Arch Biochem Biophys **276,** 518-526.

Hamilton, A.J., and Baulcombe, D.C. (1999). A species of small antisense RNA in posttranscriptional gene silencing in plants. Science **286,** 950-952.

Hamilton, W.D., and Brown, S.P. (2001). Autumn tree colours as a handicap signal. Proc R Soc Lond B Biol Sci. **268,** 1489-1493.

Hammerschmidt, R., and Kuć, J. (1982). Lignification as a mechanism for induced systemic resistance of cucumber. Physiol Plant Pathol **20,** 61-71.

Hara-Nishimura, I., and Matsushima, R. (2003). A wound-inducible organelle derived from endoplasmic reticulum: a plant strategy against environmental stresses? Curr Opin Plant Biol **6,** 583-588.

Hara, K., Yagi, M., Kusano, T., and Sano, H. (2000). Rapid systemic accumulation of transcripts encoding a tobacco WRKY transcription factor upon wounding. Mol Gen Genet **263,** 30-37.

Hause, B., Weichert, H., Hohne, M., Kindl, H., and Feussner, I. (2000a). Expression of cucumber lipid-body lipoxygenase in transgenic tobacco: lipid-body lipoxygenase is correctly targeted to seed lipid bodies. Planta **210,** 708-714.

Hause, B., Hause, G., Kutter, C., Miersch, O., and Wasternack, C. (2003). Enzymes of jasmonate biosynthesis occur in tomato sieve elements. Plant Cell Physiol **44,** 643-648.

Hause, B., Stenzel, I., Miersch, O., Maucher, H., Kramell, R., Ziegler, J., and Wasternack, C. (2000b). Tissue-specific oxylipin signature of tomato flowers: allene oxide cyclase is highly expressed in distinct flower organs and vascular bundles. Plant J **24,** 113-126.

He, J.X., Gendron, J.M., Yang, Y., Li, J., and Wang, Z.Y. (2002a). The GSK3-like kinase BIN2 phosphorylates and destabilizes BZR1, a positive regulator of the brassinosteroid signaling pathway in *Arabidopsis*. PNAS **99,** 10185-10190.

He, Y., Fukushige, H., Hildebrand, D.F., and Gan, S. (2002b). Evidence supporting a role of jasmonic acid in *Arabidopsis* leaf senescence. Plant Physiol. **128,** 876-884.

Heitz, T., Bergey, D.R., and Ryan, C.A. (1997). A gene encoding a chloroplast-targeted lipoxygenase in tomato leaves is transiently induced by wounding, systemin, and methyl jasmonate. Plant Physiol. **114,** 1085-1093.

Helliwell, C., and Waterhouse, P. (2003). Constructs and methods for high-throughput gene silencing in plants. Methods **30,** 289-295.

Herde, O., Pena-Cortes, H., Fuss, H., Willmitzer, L., and Fisahn, J. (1999). Effects of mechanical wounding, current application and heat treatment on chlorophyll fluorescence and pigment composition in tomato plants. Physiol. Plant. **105,** 179-184.

Herman, G., Lehmann, J., Peterson, A., Sembdner, G., Weidhase, R.A., and Parthier, B. (1989). Species and tissue specificity of jasmonate-induced abundant proteins. J. Plant Physiol **134,** 703-709.

Hilpert, B., Bohlmann, H., op den Camp, R.O., Przybyla, D., Miersch, O., Buchala, A., and Apel, K. (2001). Isolation and characterization of signal transduction mutants of *Arabidopsis thaliana* that constitutively activate the octadecanoid pathway and form necrotic microlesions. Plant J **26,** 435-446.

Himber, C., Dunoyer, P., Moissiard, G., Ritzenthaler, C., and Voinnet, O. (2003). Transitivity-dependent and -independent cell-to-cell movement of RNA silencing. EMBO **22,** 4523-4533.

Hobkirk, R. (1985). Steroid sulfotransferase and steroid sulfate sulfatases: characteristics and biological roles. Can J Biochem Cell Biol **63,** 1127-1144.

Hohlfeld, H., Strack, D., Keller, H., Wray, V., Hahlbrock, K., and Scheel, D. (1996). Changes in the accumulation of soluble and cell wall-bound phenolics in elicitor-treated cell suspension cultures and fungus-infected leaves of *Solanum tuberosum*. Phytochemistry **42,** 389-396.

Holt, B.F., 3rd, Hubert, D.A., and Dangl, J.L. (2003). Resistance gene signaling in plants--complex similarities to animal innate immunity. Curr Opin Immunol. **15,** 20-25.

Holton, T.A., and Cornish, E.C. (1995). Genetics and biochemistry of anthocyanin biosynthesis. Plant Cell **7,** 1071-1083.

Hong, Z., Delauney, A.J., and Verma, D.P.S. (2001). A cell plate-specific callose synthase and its interaction with phragmoplastin. Plant Cell **13,** 755-768.

Howe, G.A. (2004). Jasmonates as signals in the wound response. J Plant Growth Regulation **23,** 223-237.

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

Howe, G.A., and Schilmiller, A.L. (2002). Oxylipin metabolism in response to stress. Curr Opin Plant Biol. **5,** 230-236.

Hubank, M., and Schatz, D.G. (1994). Identifying differences in mRNA expression by representational difference analysis of cDNA. Nucleic Acids Res **22,** 5640-5648.

Hückelhoven, R. (2004). BAX Inhibitor-1, an ancient cell death suppressor in animals and plants with prokaryotic relatives. Apoptosis **9,** 299-307.

Huxtable, R.J., and Lafranconi, W.M. (1986). Biochemistry of Sulfur (New York: Plenum), pp. 293-358.

Ichimura, K., Mizoguchi, T., Yoshida, R., Yuasa, T., and Shinozaki, K. (2000). Various abiotic stresses rapidly activate Arabidopsis MAP kinases ATMPK4 and ATMPK6. Plant J **24,** 655-665.

Ishiguro, S., Kawai-Oda, A., Ueda, J., Nishida, I., and Okada, K. (2001). The *DEFECTIVE IN ANTHER DEHISCIENCE* gene encodes a novel phospholipase A1 catalyzing the initial step of jasmonic acid biosynthesis, which synchronizes pollen maturation, anther dehiscence, and flower opening in *Arabidopsis*. Plant Cell **13,** 2191-2209.

Ishihara, A., Kawata, N., Matsukawa, T., and Iwamura, H. (2000). Induction of *N*-hydroxycinnamoyltyramine synthesis and tyramine *N*-hydroxycinnamoyltransferase (THT) activity by wounding in maize leaves. Biosci Biotechnol Biochem **64,** 1025-1031.

Itoh, A., and Howe, G.A. (2001). Molecular cloning of a divinyl ether synthase. . J. Biol. Chem. **276,** 3620-3627.

Jacobs, A.K., Lipka, V., Burton, R.A., Panstruga, R., Strizhov, N., Schulze-Lefert, P., and Fincher, G.B. (2003). An *Arabidopsis* callose synthase, *GSL5*, is required for wound and papillary callose formation. Plant Cell **15,** 2503-2513.

Jensen, A.B., Raventos, D., and Mundy, J. (2002). Fusion genetic analysis of jasmonate-signalling mutants in *Arabidopsis*. Plant J **29,** 595-606.

Johnson, P.R., and Ecker, J.R. (1998). The ethylene gas signal transduction pathway: a molecular perspective. Annu Rev Genet. **32,** 227-254.

Johnson, R., Narvaez, J., An, G., and Ryan, C. (1989). Expression of proteinase inhibitors I and II in transgenic tobacco plants: effects on natural defense against *Manduca sexta* larvae. PNAS **86,** 9871-9875.

Jose, M., and Puigdomenech, P. (1993). Structure and expression of genes coding for structural proteins of the plant cell wall. New Phytol. **125,** 259-282.

Kachroo, A., and Kachroo, P. (2007). Salicylic acid-, jasmonic acid- and ethylene-mediated regulation of plant defense signaling. Genet Eng (N Y) **28,** 55-83.

Kakuta, Y., Pedersen, L.G., Pedersen, L.C., and Negishi, M. (1998a). Conserved structural motifs in the sulfotransferase family. Trends Biochem Sci **23,** 129-130.

Kakuta, Y., Petrotchenko, E.V., Pedersen, L.C., and Negishi, M. (1998b). The sulfuryl transfer mechanism. Crystal structure of a vanadate complex of estrogen sulfotransferase and mutational analysis. J Biol Chem. **273,** 27325-27330.

Kakuta, Y., Pedersen, L.G., Carter, C.W., Negishi, M., and Pedersen, L.C. (1997). Crystal structure of estrogen sulphotransferase. Nat Struct Biol. **4,** 904-908.

Katoh, K., Misawa, K., Kuma, K.-i., and Miyata, T. (2002). MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucl. Acids Res. **30,** 3059-3066.

Katoh, K., Kuma, K.-i., Toh, H., and Miyata, T. (2005). MAFFT version 5: improvement in accuracy of multiple sequence alignment. Nucl. Acids Res. **33,** 511-518.

Kawano, T. (2003). Roles of the reactive oxygen species-generating peroxidase reactions in plant defense and growth induction. Plant Cell Rep. **21,** 829-837.

Kenrick, P., and Crane, P.R. (1997). The origin and early evolution of plants on land. Nature **389,** 33-39.

Kernan, A., and Thornburg, R.W. (1989). Auxin levels regulate the expression of a wound-inducible proteinase inhibitor II-chloramphenicol acetyl transferase gene fusion *in vitro* and *in vivo*. Plant Physiol. **91,** 73-78.

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

Kinkema, M., Fan, W., and Dong, X. (2000). Nuclear localization of NPR1 Is required for activation of *PR* gene expression. Plant Cell **12,** 2339-2350.

Kiyosue, T., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1993). Characterization of two cDNAs (*ERD11* and *ERD13*) for dehydration-inducible genes that encode putative glutathione S-transferases in *Arabidopsis thaliana* L. FEBS Lett. **335,** 189-192.

Kjellen, L., and Lindahl, U. (1991). Proteoglycans: structures and interactions. Annu Rev Biochem. **60,** 443-475.

Knight, H., and Knight, M.R. (2001). Abiotic stress signalling pathways: specificity and cross-talk. Trends Plant Sci **6,** 262-267.

Koch, T., Bandemer, K., and Boland, W. (1997). Biosynthesis of *cis*-jasmone. A pathway for the inactivation and the disposal of the plant stress hormone jasmonic acid in the gas phase. Helv Chim Acta **80,** 838.

Koda, Y., Omer, E.S.A., Yoshihara, T., Shibata, H., Sakamura, S., and Okazawa, Y. (1988). Isolation of a specific potato tuber-inducing substance from potato leaves. Plant Cell Physiol. **29,** 1047.

Koiwa, H., Bressan, R.A., and Hasegawa, P.M. (1997). Regulation of proteinase inhibitors and plant defense. Trends Plant Sci **2,** 379-384.

Koizumi, M., Yamaguchi-Shinozaki, K., Tsuji, H., and Shinozaki, K. (1993). Structure and expression of two genes that encode distinct drought-inducible cysteine proteinases in *Arabidopsis thaliana*. Gene **129,** 175-182.

Kubigsteltig, II, and Weiler, E.W. (2003). *Arabidopsis* mutants affected in the transcriptional control of allene oxide synthase, the enzyme catalyzing the entrance step in octadecanoid biosynthesis. Planta **217,** 748-757.

Kubigsteltig, I., Laudert, D., and Weiler, E.W. (1999). Structure and regulation of the Arabidopsis thaliana allene oxide synthase gene. Planta **208,** 463-471.

Kunkel, B.N., and Brooks, D.M. (2002). Cross talk between signaling pathways in pathogen defense. Curr. Opin. Plant Biol **5,** 325-331.

Lacomme, C., and Roby, D. (1996). Molecular cloning of a sulfotransferase in *Arabidopsis thaliana* and regulation during development and in response to infection with pathogenic bacteria. Plant Mol Biol. **30,** 995-1008.

Lam, W.S., Yang, X., and Makaroff, C.A. (2005). Characterization of *Arabidopsis thaliana* SMC1 and SMC3: evidence that AtSMC3 may function beyond chromosome cohesion. J Cell Sci. **118,** 3037-3048.

Lambert, J.J., Belelli, D., Hill-Venning, C., and Peters, J.A. (1995). Neurosteroids and GABAA receptor function. Trends Pharmacolo Sci. **16,** 295-303.

Laudert, D., and Weiler, E.W. (1998). Allene oxide synthase: a major control point in *Arabidopsis thaliana* octadecanoid signalling. Plant J. **15,** 675-684.

Laudert, D., Schaller, F., and Weiler, E.W. (2000). Transgenic *Nicotiana tabacum* and *Arabidopsis thaliana* plants overexpressing allene oxide synthase. Planta **211,** 163-165.

Laudert, D., Hennig, P., Stelmach, B.A., Muller, A., Andert, L., and Weiler, E.W. (1997). Analysis of 12-oxo-phytodienoic acid enantiomers in biological samples by capillary gas chromatography-mass spectrometry using cyclodextrin stationary phases. Analytical biochemistry **246,** 211-217.

Lawton, K., Weymann, K., Friedrich, L., Vernooij, B., Uknes, S., and Ryals, J. (1995). Systemic acquired resistance in *Arabidopsis* requires salicylic acid but not ethylene. Mol Plant Microbe Interact. **8,** 863-870.

LeBrasseur, N.D., MacIntosh, G.C., Perez-Amador, M.A., Saitoh, M., and Green, P.J. (2002). Local and systemic wound-induction of RNase and nuclease activities in *Arabidopsis*: RNS1 as a marker for a JA-independent systemic signaling pathway. Plant J. **29,** 393-403.

Lechner, E., Achard, P., Vansiri, A., Potuschak, T., and Genschik, P. (2006). F-box proteins everywhere. Curr Opin Plant Biol. **9,** 631-638.

Lee, G.I., and Howe, G.A. (2003). The tomato mutant *spr1* is defective in systemin perception and the production of a systemic wound signal for defense gene expression. Plant J. **33,** 567-576.

Leon, J., Rojo, E., and Sanchez-Serrano, J.J. (2001). Wound signalling in plants. J. Exp. Bot. **52,** 1-9.

Leon, J., Rojo, E., Titarenko, E., and Sanchez-Serrano, J.J. (1998). Jasmonic acid-dependent and -independent wound signal transduction pathways are differentially regulated by Ca2+/calmodulin in *Arabidopsis thaliana*. Mol Gen Genet. **258,** 412-419.

Lequeu, J., Fauconnier, M.-L., Chammai, A., Bronner, R., and Blee, E. (2003). Formation of plant cuticle: evidence for the occurrence of the peroxygenase pathway. Plant J. **36,** 155-164.

Li, C., Williams, M.M., Loh, Y.T., Lee, G.I., and Howe, G.A. (2002a). Resistance of cultivated tomato to cell content-feeding herbivores is regulated by the octadecanoid-signaling pathway. Plant Physiol. **130,** 494-503.

Li, J. (2005). Brassinosteroid signaling: from receptor kinases to transcription factors. Curr Opin Plant Biol. **8,** 526-531.

Li, J., and Chory, J. (1997). A putative leucine-rich repeat receptor kinase involved in brassinosteroid signal transduction. Cell **90,** 929-938.

Li, J., and Nam, K.H. (2002). Regulation of brassinosteroid signaling by a GSK3/SHAGGY-like kinase. Science **295,** 1299-1301.

Li, J., Brader, G., and Palva, E.T. (2004a). The WRKY70 transcription factor: a node of convergence for jasmonate-mediated and salicylate-mediated signals in plant defense. Plant Cell **16,** 319-331.

Li, J., Wen, J., Lease, K.A., Doke, J.T., Tax, F.E., and Walker, J.C. (2002b). BAK1, an *Arabidopsis* LRR receptor-like protein kinase, interacts with BRI1 and modulates brassinosteroid signaling. Cell **110,** 213-222.

Li, L., Li, C., Lee, G.I., and Howe, G.A. (2002c). Distinct roles for jasmonate synthesis and action in the systemic wound response of tomato. PNAS **99,** 6416-6421.

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

Lightner, J., Pearce, G., Ryan, C.A., and Browse, J. (1993). Isolation of signaling mutants of tomato (*Lycopersicon esculentum*). Mol Gen Genet. **241,** 595-601.

Lin, C., and Thomashow, M.F. (1992a). A cold-regulated *Arabidopsis* gene encodes a polypeptide having potent cryoprotective activity. Biochem Biophys Res Commun. **183,** 1103-1108.

Lin, C., and Thomashow, M.F. (1992b). DNA sequence analysis of a complementary DNA for cold-regulated *Arabidopsis* gene *cor15* and characterization of the COR 15 Polypeptide. Plant Physiol. **99,** 519-525.

Lintala, M., Allahverdiyeva, Y., Kidron, H., Piippo, M., Battchikova, N., Suorsa, M., Rintamaki, E., Salminen, T.A., Aro, E.-M., and Mulo, P. (2007). Structural and functional characterization of ferredoxin-NADP+-oxidoreductase using knock-out mutants of *Arabidopsis*. Plant J, **49,** 1041-1052.

Linthorst, H.J., van der Does, C., Brederode, F.T., and Bol, J.F. (1993). Circadian expression and induction by wounding of tobacco genes for cysteine proteinase. Plant Mol Biol. **21,** 685-694.

Lisitsyn, N.A. (1995). Representational difference analysis: finding the differences between genomes. Trends Genet. **11,** 303-307.

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

Liyama, K., Lam, T.B.T., and Stone, B.A. (1994). Covalent cross-links in the cell wall. Plant Physiol. **104,** 315-320.

Logemann, J., Mayer, J.E., Schell, J., and Willmitzer, L. (1988). Differential expression of genes in potato tubers after wounding. PNAS **85,** 1136-1140.

Lopez, R., Dathe, W., Bruckner, C., and Miersch, O. (1987). Jasmonic acid in different parts of the developing soybean fruit. Biochem Physiol Pflanzenphysiol. **182,** 195-201.

Lorenzo, O., Piqueras, R., Sanchez-Serrano, J.J., and Solano, R. (2003). ETHYLENE RESPONSE FACTOR1 integrates signals from ethylene and jasmonate pathways in plant defense. Plant Cell **15,** 165-178.

Lorenzo, O., Chico, J.M., Sanchez-Serrano, J.J., and Solano, R. (2004). JASMONATE-INSENSITIVE1 encodes a MYC transcription factor essential to discriminate between different jasmonate-regulated defense responses in *Arabidopsis*. Plant Cell **16,** 1938-1950.

Lucinski, Schmid, Jansson, and Klimmek. (2006). Lhca5 interaction with plant photosystem I. FEBS Lett. **580,** 6485-6488.

Luo, C., and Zheng, L. (2000). Independent evolution of Toll and related genes in insects and mammals. Immunogenetics **51,** 92-98.

Luu-The, V., Bernier, F., Dufort, I., and Labrie, F. (1996). Molecular biology of steroid sulfotransferases. Ann N Y Acad Sci. **784,** 137-148.

Maleck, K., Levine, A., Eulgem, T., Morgan, A., Schmid, J., Lawton, K.A., Dangl, J.L., and Dietrich, R.A. (2000). The transcriptome of *Arabidopsis thaliana* during systemic acquired resistance. Nat Genet. **26,** 403-410.

Malone, M. (1996). Rapid, long-distance signal transmission in higher plants. Adv Bot Res. **22,** 163-228.

Marchler-Bauer, A., Anderson, J.B., Derbyshire, M.K., DeWeese-Scott, C., Gonzales, N.R., Gwadz, M., Hao, L., He, S., Hurwitz, D.I., Jackson, J.D., Ke, Z., Krylov, D., Lanczycki, C.J., Liebert, C.A., Liu, C., Lu, F., Lu, S., Marchler, G.H., Mullokandov, M., Song, J.S., Thanki, N., Yamashita, R.A., Yin, J.J., Zhang, D., and Bryant, S.H. (2007). CDD: a conserved domain database for interactive domain family analysis. Nucl Acids Res **35,** D237-240.

Marrs, K.A. (1996). The functions and regulation of glutathione S-transferases in plants. Annu Rev Plant Physiol Plant Mol Biol **47,** 127-158.

Martin-Tanguy, J., Cabanne, F., Perdrizet, E., and Martin, C. (1978). The distribution of hydroxycinnamic acid amides in flowering plants. Phytochemistry **17,** 1927-1928.

Mason, H.S., and Mullet, J.E. (1990). Expression of two soybean vegetative storage protein genes during development and in response to water deficit, wounding, and jasmonic acid. Plant Cell **1990,** 569-579.

Matsuda, F., Morino, K., Miyashita, M., and Miyagawa, H. (2003). Metabolic flux analysis of the phenylpropanoid pathway in wound-healing potato tuber tissue using stable isotope-labeled tracer and LC-MS spectroscopy. Plant Cell Physiol **44,** 510-517.

Matsui, K. (2006). Green leaf volatiles: hydroperoxide lyase pathway of oxylipin metabolism. Curr. Opin. Plant Biol. **9,** 274-280.

Matsui, K., Hijiya, K., Tabuchi, Y., and Kajiwara, T. (1999). Cucumber cotyledon lipoxygenase during postgerminative growth. Its expression and action on lipid bodies. Plant Physiol. **119,** 1279-1288.

Matsushima, R., Kondo, M., Nishimura, M., and Hara-Nishimura, I. (2003a). A novel ER-derived compartment, the ER body, selectively accumulates a beta-glucosidase with an ER-retention signal in *Arabidopsis*. Plant J **33,** 493-502.

Matsushima, R., Hayashi, Y., Kondo, M., Shimada, T., Nishimura, M., and Hara-Nishimura, I. (2002). An endoplasmic reticulum-derived structure that is induced under stress conditions in *Arabidopsis*. Plant Physiol. **130,** 1807-1814.

Matsushima, R., Hayashi, Y., Yamada, K., Shimada, T., Nishimura, M., and Hara-Nishimura, I. (2003b). The ER body, a novel endoplasmic reticulum-derived structure in *Arabidopsis*. Plant Cell Physiol **44,** 661-666.

Mauricio, R. (1998). Cost of resistance to natural enemies in field populations of the annual plant *Arabidopsis thaliana*. Am. Nat. **151,** 20-28.

McConn, M., Hugly, S., Browse, J., and Somerville, C. (1994). A mutation at the *fad8* locus of *Arabidopsis* identifies a second chloroplast σ-3 desaturase. Plant Physiol. **106,** 1609-1614.

McConn, M., Creelman, R.A., Bell, E., Mullet, J.E., and Browse, J. (1997). Jasmonate is essential for insect defense in *Arabidopsis*. PNAS **94,** 5473-5477.

McCully, M.E., Canny, M.J., and Huang, C.X. (2004). The management of extracellular ice by petioles of frost-resistant herbaceous plants. Ann Bot **94,** 665-674.

McGurl, B., Orozco-Cardenas, M., Pearce, G., and Ryan, C.A. (1994). Overexpression of the prosystemin gene in transgenic tomato plants generates a systemic signal that constitutively induces proteinase inhibitor synthesis. PNAS **91,** 9799-9802.

Meyer, A., Miersch, O., Buttner, C., Dathe, W., and Sembdner, G. (1984). Occurrence of the plant growth regulator jasmonic acid in plants. J Plant Growth Regul **3,** 1-8.

Meyer, S., Lauterbach, C., Niedermeier, M., Barth, I., Sjolund, R.D., and Sauer, N. (2004). Wounding enhances expression of *AtSUC3*, a sucrose transporter from *Arabidopsis* sieve elements and sink tissues. Plant Physiol. **134,** 684-693.

Mhiri, C., Morel, J.B., Vernhettes, S., Casacuberta, J.M., Lucas, H., and Grandbastien, M.A. (1997). The promoter of the tobacco Tnt1 retrotransposon is induced by wounding and by abiotic stress. Plant Mol Biol. **33,** 257-266.

Miersch, O., and Wasternack, C. (2000). Octadecanoid and jasmonate signaling in tomato (*Lycopersicon esculentum* Mill.) leaves: endogenous jasmonates do not induce jasmonate biosynthesis. Biol Chem. **381,** 715-722.

Miki, D., Itoh, R., and Shimamoto, K. (2005). RNA silencing of single and multiple members in a gene family of rice. Plant Physiol. **138,** 1903-1913.

Minor, W., Steczko, J., Stec, B., Otwinowski, Z., Bolin, J.T., Walter, R., and Axelrod, B. (1996). Crystal structure of soybean lipoxygenase L-1 at 1.4 Å resolution. Biochemistry **35,** 10687-10701.

Mishra, N.S., Tuteja, R., and Tuteja, N. (2006). Signaling through MAP kinase networks in plants. Arch Biochem Biophys **452,** 55-68.

Mizoguchi, T., Ichimura, K., Yoshida, R., and Shinozaki, K. (2000). MAP kinase cascades in Arabidopsis: their roles in stress and hormone responses. Results Probl Cell Differ **27,** 29-38.

Mol, J., Grotewold, E., and Koes, R. (1998). How genes paint flowers and seeds. Trends Plant Sci. **3,** 212-217.

Moore, K.L. (2003). The biology and enzymology of protein tyrosine O-sulfation. J. Biol. Chem. **278,** 24243-24246.

Moran, P.J., Cheng, Y., Cassell, J.L., and Thompson, G.A. (2002). Gene expression profiling of *Arabidopsis thaliana* in compatible plant-aphid interactions. Arch Insect Biochem Physiol **51,** 182-203.

Mueller, L.A., Goodman, C.D., Silady, R.A., and Walbot, V. (2000). AN9, a petunia glutathione S-transferase required for anthocyanin sequestration, is a flavonoid-binding protein. Plant Physiol. **123,** 1561-1570.

Mulder, G.J. (1990). Sulfation. In Conjugation Reactions in Drug Metabolism, G.J. Mulder, ed (New York: Taylor & Francis), pp. 107-161.

Muller-Dieckmann, H.J., and Schulz, G.E. (1994). The structure of uridylate kinase with its substrates, showing the transition state geometry. J Mol Biol. **236,** 361-367.

Murfett, J., Wang, X.J., Hagen, G., and Guilfoyle, T.J. (2001). Identification of *Arabidopsis* histone deacetylase *HDA6* mutants that affect transgene expression. Plant Cell **13,** 1047-1061.

Nakamura, S., and Hatanaka, A. (2002). Green-leaf-derived C6-aroma compounds with potent antibacterial action that act on both Gram-negative and Gram-positive bacteria. J Agric Food Chem. **50,** 7639-7644.

Nam, K.H., and Li, J. (2002). BRI1/BAK1, a receptor kinase pair mediating brassinosteroid signaling. Cell **110,** 203-212.

Narlikar, G.J., Fan, H.-Y., and Kingston, R.E. (2002). Cooperation between complexes that regulate chromatin structure and transcription. Cell **108,** 475-487.

Narvaez-Vasquez, J., and Ryan, C.A. (2004). The cellular localization of prosystemin: a functional role for phloem parenchyma in systemic wound signaling. Planta **218,** 360-369.

Narvaez-Vasquez, J., Florin-Christensen, J., and Ryan, C.A. (1999). Positional specificity of a phospholipase A activity induced by wounding, systemin, and oligosaccharide elicitors in tomato leaves. Plant Cell **11,** 2249-2260.

Nawrath, C., and Metraux, J.P. (1999). Salicylic acid induction-deficient mutants of *Arabidopsis* express *PR-2* and *PR-5* and accumulate high levels of camalexin after pathogen inoculation. Plant Cell **11,** 1393-1404.

Neff, M.M., Nguyen, S.M., Malancharuvil, E.J., Fujioka, S., Noguchi, T., Seto, H., Tsubuki, M., Honda, T., Takatsuto, S., Yoshida, S., and Chory, J. (1999). *BAS1*: A gene regulating brassinosteroid levels and light responsiveness in *Arabidopsis*. PNAS **96,** 15316-15323.

Negrel, J., and Martin, C. (1984). The biosynthesis of feruloyltyramine in *Nicotiana tabacum*. Phytochemistry **23,** 2797-2801.

Negrel, J., Javelle, F., and Paynot, M. (1993). Wound-induced tyramine hydroxycinnamoyl transferase in potato (*Solanum tuberosum*) tuber discs. J. Plant Physiol. **142,** 518-524.

Negrel, J., Pollet, B., and Lapierre, C. (1996). Ether-linked ferulic acid amides in natural and wound periderms of potato tuber. Phytochemistry **43,** 1195-1199.

Newman, M.A., von Roepenack-Lahaye, E., Parr, A., Daniels, M.J., and Dow, J.M. (2001). Induction of hydroxycinnamoyl-tyramine conjugates in pepper by *Xanthomonas campestris*, a plant defense response activated by *hrp* gene-dependent and *hrp* gene-independent mechanisms. Mol Plant Microbe Interact **14,** 785-792.

Nimchuk, Z., Eulgem, T., Holt, B.F., 3rd, and Dangl, J.L. (2003). Recognition and response in the plant immune system. Annu Rev Genet. **37,** 579-609.

Nishiyama, T., Fujita, T., Shin-I, T., Seki, M., Nishide, H., Uchiyama, I., Kamiya, A., Carninci, P., Hayashizaki, Y., Shinozaki, K., Kohara, Y., and Hasebe, M. (2003). Comparative genomics of *Physcomitrella patens* gametophytic transcriptome and *Arabidopsis thaliana*: Implication for land plant evolution. PNAS **100,** 8007-8012.

Niyogi, K.K., and Fink, G.R. (1992). Two anthranilate synthase genes in *Arabidopsis*: defense-related regulation of the tryptophan pathway. Plant Cell **4,** 721-733.

Noguchi, T., Fujioka, S., Choe, S., Takatsuto, S., Yoshida, S., Yuan, H., Feldmann, K.A., and Tax, F.E. (1999). Brassinosteroid-insensitive dwarf mutants of *Arabidopsis* accumulate brassinosteroids. Plant Physiol. **121,** 743-752.

O'Donnell, P.J., Calvert, C., Atzorn, R., Wasternack, C., Leyser, H.M.O., and Bowles, D.J. (1996). Ethylene as a signal mediating the wound response of tomato plants. Science **274,** 1914-1917.

Orozco-Cardenas, M., and Ryan, C.A. (1999). Hydrogen peroxide is generated systemically in plant leaves by wounding and systemin via the octadecanoid pathway. PNAS **96,** 6553-6557.

Orozco-Cardenas, M.L., and Ryan, C.A. (2002). Nitric oxide negatively modulates wound signaling in tomato plants. Plant Physiol. **130,** 487-493.

Orozco-Cardenas, M.L., and Ryan, C.A. (2003). Polygalacturonase beta-subunit antisense gene expression in tomato plants leads to a progressive enhanced wound response and necrosis in leaves and abscission of developing flowers. Plant Physiol. **133,** 693-701.

Orozco-Cardenas, M.L., Narvaez-Vasquez, J., and Ryan, C.A. (2001). Hydrogen peroxide acts as a second messenger for the induction of defense genes in tomato plants in response to wounding, systemin, and methyl jasmonate. Plant Cell **13,** 179-191.

Østergaard, L., Peterson, M., Mattsson, O., and Mundy, J. (2002). An *Arabidopsis* callose synthase. Plant Mol Biol. **49,** 559-566.

Park, J.-H., Halitschke, R., Kim, H.B., Baldwin, I.T., Feldmann, K.A., and Feyereisen, R. (2002). A knock-out mutation in allene oxide synthase results in male sterility and defective wound signal transduction in *Arabidopsis* due to a block in jasmonic acid biosynthesis. Plant J. **31,** 1-12.

Parry, G., and Estelle, M. (2006). Auxin receptors: a new role for F-box proteins. Curr Opin Cell Biol. **18,** 152-156.

Parsons, T.J., Bradshaw, H.D., and Gordon, M.P. (1989). Systemic accumulation of specific mRNAs in response to wounding in poplar trees. PNAS **86,** 7895-7899.

Pasqualini, J.R., Schatz, B., Varin, C., and Nguyen, B.L. (1992). Recent data on estrogen sulfatases and sulfotransferases activities in human breast cancer. J Steroid Biochem Mol Biol. **41,** 323-329.

Pautot, V., Holzer, F.M., and Walling, L.L. (1991). Differential expression of tomato proteinase inhibitor I and II genes during bacterial pathogen invasion and wounding. Mol Plant Microbe Interact **4,** 284-292.

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

Pearce, R.B. (1996). Antimicrobial defences in the wood of living trees. New Phytol **132,** 203-233.

Peck, S.C., and Kende, H. (1998). Differential regulation of genes encoding 1-aminocyclopropane-1-carboxylate (ACC) synthase in etiolated pea seedlings: effects of indole-3-acetic acid, wounding, and ethylene. Plant Mol Biol. **38,** 977-982.

Pena-Cortes, H., Fisahn, J., and Willmitzer, L. (1995). Signals involved in wound-induced proteinase inhibitor II gene expression in tomato and potato plants. PNAS **92,** 4106-4113.

Pena-Cortes, H., Sanchez-Serrano, J.J., Mertens, R., Willmitzer, L., and Prat, S. (1989). Abscisic acid is involved in the wound-induced expression of the *proteinase inhibitor II* gene in potato and tomato. PNAS **86,** 9851-9855.

Pena-Cortés, H., Albrecht, T., Prat, S., Weiler, E.W., and Willmitzer, L. (1993). Aspirin prevents wound-induced gene expression in tomato leaves by blocking jasmonic acid biosynthesis. Planta **191,** 123-128.

Penninckx, I.A., Thomma, B.P., Buchala, A., Metraux, J.P., and Broekaert, W.F. (1998). Concomitant activation of jasmonate and ethylene response pathways is required for induction of a plant defensin gene in *Arabidopsis*. Plant Cell **10,** 2103-2113.

Petersen, M., Brodersen, P., Naested, H., Andreasson, E., Lindhart, U., Johansen, B., Nielsen, H.B., Lacy, M., Austin, M.J., and Parker, J.E. (2000). *Arabidopsis MAP Kinase 4* negatively regulates systemic acquired resistance. Cell **103,** 1111-1120.

Pichersky, E., and Gang, D.R. (2000). Genetics and biochemistry of secondary metabolites in plants: an evolutionary perspective. Trends Plant Sci **5,** 439-445.

Pieterse, C.M.J., Ton, J., and Van Loon, L.C. (2001). Cross-talk between plant defence signalling pathways: boost or burden. AgBiotechNet **3,** 1-8.

Piotrowski, M., Schemenewitz, A., Lopukhina, A., Muller, A., Janowitz, T., Weiler, E.W., and Oecking, C. (2004). Desulfoglucosinolate sulfotransferases from *Arabidopsis thaliana* catalyze the final step in the biosynthesis of the glucosinolate core structure. J Biol Chem. **279,** 50717-50725.

Porta, H., and Rocha-Sosa, M. (2002). Plant lipoxygenases. Physiological and molecular features. Plant Physiol. **130,** 15-21.

Potuschak, T., Lechner, E., Parmentier, Y., Yanagisawa, S., Grava, S., Koncz, C., and Genschik, P. (2003). EIN3-dependent regulation of plant ethylene hormone signaling by two *Arabidopsis* F Box proteins EBF1 and EBF2. Cell **115,** 679-689.

Priestley, J.H., and Woffenden, L.M. (1922). Physiological studies in plant anatomy. V. Causal factors in cork formation. New Phytol**,** 252-268.

Qi, B., Fraser, T., Mugford, S., Dobson, G., Sayanova, O., Butler, J., Napier, J.A., Stobart, A.K., and Lazarus, C.M. (2004). Production of very long chain polyunsaturated omega-3 and omega-6 fatty acids in plants. Nat Biotechnol. **22,** 739-745.

Quilliam, R.S., Swarbrick, P.J., Scholes, J.D., and Rolfe, S.A. (2006). Imaging photosynthesis in wounded leaves of *Arabidopsis thaliana*. J Exp Bot **57,** 55-69.

Rask, L., Andreasson, E., Ekbom, B., Eriksson, S., Pontoppidan, B., and Meijer, J. (2000). Myrosinase: gene family evolution and herbivore defense in *Brassicaceae*. Plant Mol Biol. **42,** 93-113.

Reinbothe, S., Reinbothe, C., and Parthier, B. (1993). Methyl jasmonate-regulated translation of nuclear-encoded chloroplast proteins in barley (*Hordeum vulgare* L. cv. salome). J. Biol. Chem. **268,** 10606-10611.

Reski, R. (1998). Development, genetics and molecular biology of mosses. Bot Acta **111,** 1-15.

Reyes, L.F., and Cisneros-Zevallos, L. (2003). Wounding stress increases the phenolic content and antioxidant capacity of purple-flesh potatoes (*Solanum tuberosum* L.). J. Agric. Food Chem. **51,** 5296-5300.

Reymond, P., and Farmer, E.E. (1998). Jasmonate and salicylate as global signals for defense gene expression. Curr Opin Plant Biol **1,** 404-411.

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

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

Ride, J.P. (1978). The role of cell wall alterations in resistance to fungi. Physiol Mol Plant Pathol **31,** 349-360.

Ride, J.P., and Barber, M.S. (1987). The effects of various treatments on induced lignification and the resistance of wheat to fungi. Physiol Mol Plant Pathol **31,** 349-360.

Rojo, E., Leon, J., and Sanchez-Serrano, J.J. (1999). Cross-talk between wound signalling pathways determines local versus systemic gene expression in *Arabidopsis thaliana*. Plant J **20,** 135-142.

Rose, J.K.C., Braam, J., Fry, S.C., and Nishitani, K. (2002). The XTH family of enzymes involved in xyloglucan endotransglucosylation and endohydrolysis: Current perspectives and a new unifying nomenclature. Plant Cell Physiol. **43,** 1421-1435.

Rouleau, M., Marsolais, F., Richard, M., Nicolle, L., Voigt, B., Adam, G., and Varin, L. (1999). Inactivation of brassinosteroid biological activity by a salicylate-inducible steroid sulfotransferase from *Brassica napus*. J Biol Chem. **274,** 20925-20930.

Roxas, V.P., Smith, R.K., Jr., Allen, E.R., and Allen, R.D. (1997). Overexpression of glutathione S-transferase/glutathione peroxidase enhances the growth of transgenic tobacco seedlings during stress. Nat Biotechnol. **15,** 988-991.

Roy, A.B. (1981). Sulfotransferases. In Sulfation of Drugs and Related Compounds, G.J. Mulder, ed (Boca Raton, FL: CRC Press), pp. 83–130.

Royo, J., Vancanneyt, G., Perez, A.G., Sanz, C., Stormann, K., Rosahl, S., and Sanchez-Serrano, J.J. (1996). Characterization of three potato lipoxygenases with distinct enzymatic activities and different organ-specific and wound-regulated expression patterns. J. Biol. Chem. **271,** 21012-21019.

Rupprecht, R. (1997). The neuropsychopharmacological potential of neuroactive steroids. J Psychiatr Res **31,** 297-314.

Ryals, J.A., Neuenschwander, U.H., Willits, M.G., Molina, A., Steiner, H.Y., and Hunt, M.D. (1996). Systemic acquired resistance. Plant Cell **8,** 1809-1819.

Ryan, C.A. (1974). Assay and biochemical properties of the proteinase inhibitor-inducing factor, a wound hormone 1. Plant Physiol. **54,** 328-332.

Sambrook, J., and Russell, D.W. (2001). Molecular Cloning. (Cold Spring Harbor, NY: CSHL Press).

Sanchez-Serrano, J., Schmidt, R., Schell, J., and Willmitzer, L. (1986). Nucleotide sequence of proteinase inhibitor II encoding cDNA of potato (*Solanum tuberosum*) and its mode of expression. Mol Gen Genet. **203,** 15-20.

Sanchez, P., de Torres Zabala, M., and Grant, M. (2000). *AtBI-1*, a plant homologue of *Bax Inhibitor-1*, suppresses Bax-induced cell death in yeast and is rapidly upregulated during wounding and pathogen challenge. Plant J. **21,** 393-399.

Sanders, P.M., Lee, P.Y., Biesgen, C., Boone, J.D., Beals, T.P., Weiler, E.W., and Goldberg, R.B. (2000). The *Arabidopsis DELAYED DEHISCENCE1* gene encodes an enzyme in the jasmonic acid synthesis pathway. Plant Cell **12,** 1041-1061.

Saniewski, M., and Czapski, J. (1983). The effect of methyl jasmonate on lycopene and ß-carotene accumulation in ripening red tomatoes. Experienta **39,** 1373-1374.

Sato, K., Ueda, T., and Nakano, A. (1999). The *Arabidopsis thaliana RER1* gene family: its potential role in the endoplasmic reticulum localization of membrane proteins. Plant Mol Biol. **41,** 815-824.

Schaefer, D., Zryd, J.P., Knight, C.D., and Cove, D.J. (1991). Stable transformation of the moss *Physcomitrella patens*. Mol Gen Genet **226,** 418-424.

Schaefer, D.G. (2001). Gene targeting in *Physcomitrella patens*. Curr Opin Plant Biol **4,** 143-150.

Schaefer, D.G., and Zryd, J.P. (1997). Efficient gene targeting in the moss *Physcomitrella patens*. Plant J **11,** 1195-1206.

Schaller, F., Schaller, A., and Stintzi, A. (2005). Biosynthesis and metabolism of jasmonates. J Plant Growth Regul **23,** 179-199.

Scheer, J.M., and Ryan, C.A., Jr. (2002). The systemin receptor SR160 from *Lycopersicon peruvianum* is a member of the LRR receptor kinase family. PNAS **99,** 9585-9590.

Scherp, P., Grotha, R., and Kutschera, U. (2001). Occurrence and phylogenetic significance of cytokinesis-related callose in green algae, bryophytes, ferns and seed plants. Plant Cell Rep. **20**.

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

Schwachtje, J., Minchin, P.E., Jahnke, S., van Dongen, J.T., Schittko, U., and Baldwin, I.T. (2006). SNF1-related kinases allow plants to tolerate herbivory by allocating carbon to roots. PNAS **103,** 12935-12940.

Seki, M., Narusaka, M., Ishida, J., Nanjo, T., Fujita, M., Oono, Y., Kamiya, A., Nakajima, M., Enju, A., Sakurai, T., Satou, M., Akiyama, K., Taji, T., Yamaguchi-Shinozaki, K., Carninci, P., Kawai, J., Hayashizaki, Y., and Shinozaki, K. (2002). Monitoring the expression profiles of 7000 *Arabidopsis* genes under drought, cold and high-salinity stresses using a full-length cDNA microarray. Plant J. **31,** 279-292.

Sembdner, G., and Parthier, B. (1993). The biochemistry and the physiological and molecular actions of jasmonates. Ann Rev Plant Physiol **44,** 569-589.

Senger, T., Wichard, T., Kunze, S., Gobel, C., Lerchl, J., Pohnert, G., and Feussner, I. (2005). A multifunctional lipoxygenase with fatty acid hydroperoxide cleaving activity from the moss *Physcomitrella patens*. J Biol Chem. **280,** 7588-7596.

Seo, H.S., Song, J.T., Cheong, J.J., Lee, Y.H., Lee, Y.W., Hwang, I., Lee, J.S., and Choi, Y.D. (2001). Jasmonic acid carboxyl methyltransferase: a key enzyme for jasmonate-regulated plant responses. PNAS **98,** 4788-4793.

Shewry, P.R. (2003). Tuber storage proteins. Ann Bot **91,** 755-769.

Siedow, J.N. (1991). Plant lipoxygenase: structure and function. Annu Rev Plant Biol. **42,** 145-188.

Sijen, T., Fleenor, J., Simmer, F., Thijssen, K.L., Parrish, S., Timmons, L., Plasterk, R.H.A., and Fire, A. (2001). On the role of RNA amplification in dsRNA-triggered gene silencing. Cell **107,** 465-476.

Skowyra, D., Craig, K.L., Tyers, M., Elledge, S.J., and Harper, J.W. (1997). F-Box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex. Cell **91,** 209-219.

Smalle, J., and Vierstra, R.D. (2004). The ubiquitin 26S proteosome proteolytic pathway. Ann Rev Plant Biol **55,** 555-590.

Smith, D.W., Johnson, K.A., Bingman, C.A., Aceti, D.J., Blommel, P.G., Wrobel, R.L., Frederick, R.O., Zhao, Q., Sreenath, H., Fox, B.G., Volkman, B.F., Jeon, W.B., Newman, C.S., Ulrich, E.L., Hegeman, A.D., Kimball, T., Thao, S., Sussman, M.R., Markley, J.L., and Phillips, G.N., Jr. (2004). Crystal structure of At2g03760, a putative steroid sulfotransferase from *Arabidopsis thaliana*. Proteins **57,** 854-857.

Smith, N.A., Singh, S.P., Wang, M.B., Stoutjesdijk, P.A., Green, A.G., and Waterhouse, P.M. (2000). Total silencing by intron-spliced hairpin RNAs. Nature **407,** 319-320.

Solomon, M., Belenghi, B., Delledonne, M., Menachem, E., and Levine, A. (1999). The involvement of cysteine proteases and protease inhibitor genes in the regulation of programmed cell death in plants. Plant Cell **11,** 431-444.

Spoel, S.H., Koornneef, A., Claessens, S.M., Korzelius, J.P., Van Pelt, J.A., Mueller, M.J., Buchala, A.J., Metraux, J.P., Brown, R., Kazan, K., Van Loon, L.C., Dong, X., and Pieterse, C.M. (2003). NPR1 modulates cross-talk between salicylate- and jasmonate-dependent defense pathways through a novel function in the cytosol. Plant Cell **15,** 760-770.

Staskawicz, B.J., Mudgett, M.B., Dangl, J.L., and Galan, J.E. (2001). Common and contrasting themes of plant and animal diseases. Science **292,** 2285-2289.

Staswick, P.E. (1988). Soybean vegetative storage protein structure and gene expression. Plant Physiol. **87,** 250-254.

Staswick, P.E. (1989a). Preferential loss of an abundant storage protein from soybean pods during seed development. Plant Physiol. **90,** 1252-1255.

Staswick, P.E. (1989b). Developmental regulation and the influence of plant sinks on vegetative storage protein gene expression in soybean leaves. Plant Physiol. **89,** 309-315.

Staswick, P.E. (1990). Novel regulation of vegetative storage protein genes. Plant Cell **2,** 1-6.

Staswick, P.E. (1992). Jasmonate, genes, and fragrant signals. Plant Physiol. **99,** 804-807.

Staswick, P.E., and Tiryaki, I. (2004). The oxylipin signal jasmonic acid is activated by an enzyme that conjugates it to isoleucine in *Arabidopsis*. Plant Cell **16,** 2117-2127.

Staswick, P.E., Su, W., and Howell, S.H. (1992). Methyl jasmonate inhibition of root growth and induction of a leaf protein are decreased in an *Arabidopsis thaliana* mutant. PNAS **89,** 6837-6840.

Staswick, P.E., Yuen, G.Y., and Lehman, C.C. (1998). Jasmonate signaling mutants of *Arabidopsis* are susceptible to the soil fungus *Pythium irregulare*. Plant J. **15,** 747-754.

Staswick, P.E., Tiryaki, I., and Rowe, M.L. (2002). Jasmonate response locus *JAR1* and several related *Arabidopsis* genes encode enzymes of the firefly luciferase superfamily that show activity on jasmonic, salicylic, and indole-3-acetic acids in an assay for adenylation. Plant Cell **14,** 1405-1415.

Steczko, J., Donoho, G.P., Clemens, J.C., Dixon, J.E., and Axelrod, B. (1992). Conserved histidine residues in soybean lipoxygenase: functional consequences of their replacement. Biochemistry **31,** 4053-4057.

Stenzel, I., Hause, B., Maucher, H., Pitzschke, A., Miersch, O., Ziegler, J., Ryan, C.A., and Wasternack, C. (2003a). Allene oxide cyclase dependence of the wound response and vascular bundle-specific generation of jasmonates in tomato - amplification in wound signalling. Plant J. **33,** 577-589.

Stenzel, I., Hause, B., Miersch, O., Kurz, T., Maucher, H., Weichert, H., Ziegler, J., Feussner, I., and Wasternack, C. (2003b). Jasmonate biosynthesis and the allene oxide cyclase family of *Arabidopsis thaliana*. Plant Mol Biol. **51,** 895-911.

Steponkus, P.L., Uemura, M., Joseph, R.A., Gilmour, S.J., and Thomashow, M.F. (1998). Mode of action of the *COR15a* gene on the freezing tolerance of *Arabidopsis thaliana*. PNAS **95,** 14570-14575.

Stintzi, A., and Browse, J. (2000). The *Arabidopsis* male-sterile mutant, *opr3*, lacks the 12-oxophytodienoic acid reductase required for jasmonate synthesis. PNAS **97,** 10625-10630.

Stintzi, A., Weber, H., Reymond, P., Browse, J., and Farmer, E.E. (2001). Plant defense in the absence of jasmonic acid: the role of cyclopentenones. PNAS **98,** 12837-12842.

Stintzi, A., Heitz, T., Prasad, V., Wiedemann-Merdinoglu, S., Kauffmann, S., Geoffroy, P., Legrand, M., and Fritig, B. (1993). Plant 'pathogenesis-related' proteins and their role in defense against pathogens. Biochimie **75,** 687-706.

Stone, B.A., and Clarke, A.E. (1992). Chemistry and Biology of (1→3)-β-D-Glucans. . (Victoria, Australia: La Trobe University Press).

Stone, B.A., Evans, N.A., Bonig, I., and Clarke, A.E. (1985). The application of Sirofluor, a chemically defined fluorochrome from aniline blue for the histochemical detection of callose. Protoplasma **122,** 191-195.

Stotz, H.U., Kroymann, J., and Mitchell-Olds, T. (1999). Plant-insect interactions. Curr Opin Plant Biol **2,** 268-272.

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

Stratmann, J.W. (2003). Long distance run in the wound response--jasmonic acid is pulling ahead. Trends Plant Sci **8,** 247-250.

Strott, C.A. (1996). Steroid sulfotransferases. Endocr Rev. **17,** 670-697.

Strott, C.A., and Higashi, Y. (2003). Cholesterol sulfate in human physiology: what's it all about? J. Lipid Res. **44,** 1268-1278.

Sturm, A., and Chrispeels, M.J. (1990). cDNA cloning of carrot extracellular beta-fructosidase and its expression in response to wounding and bacterial infection. Plant Cell **2,** 1107-1119.

Sun, C.W., and Callis, J. (1997). Independent modulation of *Arabidopsis thaliana* polyubiquitin mRNAs in different organs and in response to environmental changes. Plant J. **11,** 1017-1027.

Susi, P., Hohkuri, M., Wahlroos, T., and Kilby, N.J. (2004). Characteristics of RNA silencing in plants: similarities and differences across kingdoms. Plant Mol Biol. **54,** 157-174.

Swiatek, A., Dongen, W.V., Esmans, E.L., and Onckelen, H.V. (2004). Metabolic fate of jasmonates in tobacco bright yellow-2 cells. Plant Physiol. **135,** 161-172.

Swofford, D.L. (2002). PAUP*. Phylogenetic Analysis Using parsimony (* and Other Methods). Version 4. (Sunderland, MA: Sinauer Associates).

Szczegielniak, J., Klimecka, M., Liwosz, A., Ciesielski, A., Kaczanowski, S., Dobrowolska, G., Harmon, A.C., and Muszynska, G. (2005). A wound-responsive and phospholipid-regulated maize calcium-dependent protein kinase. Plant Physiol. **139,** 1970-1983.

Taki, N., Sasaki-Sekimoto, Y., Obayashi, T., Kikuta, A., Kobayashi, K., Ainai, T., Yagi, K., Sakurai, N., Suzuki, H., Masuda, T., Takamiya, K.-i., Shibata, D., Kobayashi, Y., and Ohta, H. (2005). 12-oxo-phytodienoic acid triggers expression of a distinct set of genes and plays a role in wound-induced gene expression in *Arabidopsis*. Plant Physiol. **139,** 1268-1283.

Tamari, G., Borochov, A., Atzorn, R., and Weiss, D. (1995). Methyl jasmonate induces pigmentation and flavonoid gene expression in petunia corollas: A possible role in wound response. Physiol. Plant. **94,** 45-50.

Tang, G., Reinhart, B.J., Bartel, D.P., and Zamore, P.D. (2003). A biochemical framework for RNA silencing in plants. Genes Dev. **17,** 49-63.

Tatsuki, M., and Mori, H. (1999). Rapid and transient expression of 1-aminocyclopropane-1-carboxylate synthase isogenes by touch and wound stimuli in tomato. Plant Cell Physiol **40,** 709-715.

Thaler, J.S., Owen, B., and Higgins, V.J. (2004). The role of the jasmonate response in plant susceptibility to diverse pathogens with a range of lifestyles. Plant Physiol. **135,** 530-538.

Theodoulou, F.L., Job, K., Slocombe, S.P., Footitt, S., Holdsworth, M., Baker, A., Larson, T.R., and Graham, I.A. (2005). Jasmonic acid levels are reduced in COMATOSE ATP-binding cassette transporter mutants. Implications for transport of jasmonate precursors into peroxisomes. Plant Physiol. **137,** 835-840.

Thomma, B., Eggermont, K., Tierens, K., and Broekaert, W.F. (1999). Requirement of functional *ethylene-insensitive 2* gene for efficient resistance of *Arabidopsis* to infection by *Botrytis cinerea*. Plant Physiol. **121,** 1093-1101.

Thomma, B., Eggermont, K., Penninckx, I., Mauch-Mani, B., Vogelsang, R., Cammue, B.P.A., and Broekaert, W.F. (1998). Separate jasmonate-dependent and salicylate-dependent defense-response pathways in arabidopsis are essential for resistance to distinct microbial pathogens. PNAS **95,** 15107-15111.

Thompson, J.D., Higgins, D.G., and Gibson, T.J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence

weighting, position-specific gap penalties and weight matrix choice. Nucl. Acids Res. **22,** 4673-4680.

Tierens, K.F.M.J., Thomma, B.P.H.J., Brouwer, M., Schmidt, J., Kistner, K., Porzel, A., Mauch-Mani, B., Cammue, B.P.A., and Broekaert, W.F. (2001). Study of the role of antimicrobial glucosinolate-derived isothiocyanates in resistance of *Arabidopsis* to microbial pathogens. Plant Physiol. **125,** 1688-1699.

Ting, J.P., and Davis, B.K. (2005). CATERPILLER: a novel gene family important in immunity, cell death, and diseases. Annu Rev Immunol. **23,** 387-414.

Titarenko, E., Rojo, E., Leon, J., and Sanchez-Serrano, J.J. (1997). Jasmonic acid-dependent and -independent signaling pathways control wound-induced gene activation in *Arabidopsis thaliana*. Plant Physiol. **115,** 817-826.

Turner, J.G., Ellis, C., and Devoto, A. (2002). The jasmonate signal pathway. Plant Cell **14 Suppl,** S153-164.

Ueda, J., and Kato, J. (1981). Promotive effect of methyl jasmonate on oat leaf senescence in the light. Z. Pflanzenphysiol **103,** 357-359.

Ueda, J., and Kato, J. (1982). Inhibition of cytokinin-induced plant growth by jasmonic acid and its methyl ester. Physiol Plant **54,** 249-254.

Urwin, P.E., Atkinson, H.J., Waller, D.A., and McPherson, M.J. (1995). Engineered oryzacystatin-I expressed in transgenic hairy roots confers resistance to *Globodera pallida*. Plant J **8,** 121-131.

Vaistij, F.E., Jones, L., and Baulcombe, D.C. (2002). Spreading of RNA targeting and DNA methylation in RNA silencing requires transcription of the target gene and a putative RNA-dependent RNA polymerase. Plant Cell **14,** 857-867.

van de Ven, W.T., LeVesque, C.S., Perring, T.M., and Walling, L.L. (2000). Local and systemic changes in squash gene expression in response to silverleaf whitefly feeding. Plant Cell **12,** 1409-1423.

Van der Biezen, E.A., and Jones, J.D. (1998). Plant disease-resistance proteins and the gene-for-gene concept. Trends Biochem Sci **23,** 454-456.

Van Houdt, H., Bleys, A., and Depicker, A. (2003). RNA target sequences promote spreading of RNA silencing. Plant Physiol. **131,** 245-253.

Van Loon, L.C., and Van Strien, E.A. (1999). The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. Physiol Mol Plant Pathol. **55,** 85-97.

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

Vance, C.P., Kirk, T.K., and Sherwood, R.T. (1980). Lignification as a mechanism of disease resistance. Annu Rev Phytopathol **18,** 259-288.

Vaucheret, H., Vazquez, F., Crete, P., and Bartel, D.P. (2004). The action of ARGONAUTE1 in the miRNA pathway and its regulation by the miRNA pathway are crucial for plant development. Genes Dev. **18,** 1187-1197.

Verma, D.P., and Hong, Z. (2001). Plant callose synthase complexes. Plant Mol Biol. **47,** 693-701.

Veronese, P., Ruiz, M.T., Coca, M.A., Hernandez-Lopez, A., Lee, H., Ibeas, J.I., Damsz, B., Pardo, J.M., Hasegawa, P.M., Bressan, R.A., and Narasimhan, M.L. (2003). In defense against pathogens. Both plant sentinels and foot soldiers need to know the enemy. Plant Physiol. **131,** 1580-1590.

Verpoorte, R., and Memelink, J. (2002). Engineering secondary metabolite production in plants. Curr Opin Biotech. **13,** 181-187.

Vert, G., Nemhauser, J.L., Geldner, N., Hong, F., and Chory, J. (2005). Molecular mechanisms of steroid hormone signaling in plants. Annu Rev Cell Dev Biol **21,** 177-201.

Vick, B.A. (1993). Oxygenated fatty acids of the lipoxygenase pathway. In Lipid Metabolism in Plants, T.S. Moore, ed (CRC Press), pp. 167-191.

Vick, B.A., and Zimmerman, D.C. (1983). The biosynthesis of jasmonic acid: a physiological role for plant lipoxygenase. Biochem Biophys Res Commun **111,** 470-477.

Vick, B.A., and Zimmerman, D.C. (1984). Biosynthesis of jasmonic acid by several plant species. Plant Physiol. **75,** 458-461.

Vijayan, P., Shockey, J., Levesque, C.A., Cook, R.J., and Browse, J. (1998). A role for jasmonate in pathogen defense of *Arabidopsis*. PNAS **95,** 7209-7214.

Villegas, M., and Brodelius, P.E. (1990). Elicitor-induced hydroxycinnamoyl-CoA: tyramine hydroxycinnamoyltransferase in plant cell suspension cultures. Physiol. Plant. **78,** 414-420.

Vogt, T., Pollak, P., Tarlyn, N., and Taylor, L.P. (1994). Pollination- or wound-induced kaempferol accumulation in petunia stigmas enhances seed production. Plant Cell **6,** 11-23.

Von Dahl, C.C., and Baldwin, I.T. (2004). Methyl jasmonate and *cis*-jasmone do not dispose of the herbivore-induced jasmonate burst in *Nicotiana attenuata*. Physiol Plant **120,** 474-481.

von Malek, B., van der Graaff, E., Schneitz, K., and Keller, B. (2002). The Arabidopsis male-sterile mutant *dde2-2* is defective in the A*LLENE OXIDE SYNTHASE* gene encoding one of the key enzymes of the jasmonic acid biosynthesis pathway. Planta **216,** 187-192.

Wagner, G.J. (1991). Secreting glandular trichomes: More than just hairs. Plant Physiol. **96,** 675-679.

Walker-Simmons, M., and Ryan, C.A. (1977). Wound-induced accumulation of trypsin inhibitor activities in plant leaves: survey of several plant genera. Plant Physiol. **59,** 437-439.

Walker-Simmons, M., Jin, D., West, C.A., Hadwiger, L., and Ryan, C.A. (1984). Comparison of proteinase inhibitor-inducing activities and phytoalexin elicitor activities of a pure fungal endopolygalacturonase, pectic fragments, and chitosans. Plant Physiol. **76,** 833.

Walle, T., and Walle, U.K. (1992). Stereoselective sulfate conjugation of 4-hydroxypropranolol and terbutaline by the human liver phenolsulfotransferases. Drug Metab Dispos. **20,** 333-336.

Ward, E.R., Uknes, S.J., Williams, S.C., Dincher, S.S., Wiederhold, D.L., Alexander, D.C., Ahl-Goy, P., Metraux, J.P., and Ryals, J.A. (1991). Coordinate gene activity in response to agents that induce systemic acquired resistance. Plant Cell **3,** 1085-1094.

Wasternack, C., Stenzel, I., Hause, B., Hause, G., Kutter, C., Maucher, H., Neumerkel, J., Feussner, I., and Miersch, O. (2006). The wound response in tomato--role of jasmonic acid. J Plant Physiol **163,** 297-306.

Waterhouse, P.M., Graham, M.W., and Wang, M.B. (1998). Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. PNAS **95,** 13959-13964.

Weber, H. (2002). Fatty acid-derived signals in plants. Trends Plant Sci. **7,** 217-224.

Weiler, E.W., Albrecht, T., Groth, B., Xia, Z., Luxem, M., Liss, H., Andert, L., and Spengler, P. (1993). Evidence for the involvement of jasmonates and their octadecanoid precursors in the tendril coiling response of *Bryonia dioica*. Phytochemistry **32,** 591-600.

Welin, B.V., Olson, A., Nylander, M., and Palva, E.T. (1994). Characterization and differential expression of dhn/lea/rab-like genes during cold acclimation and drought stress in *Arabidopsis thaliana*. Plant Mol Biol. **26,** 131-144.

Wesley, S.V., Helliwell, C.A., Smith, N.A., Wang, M., Rouse, D.T., Liu, Q., Gooding, P.S., Singh, S.P., Abbott, D., Stoutjesdijk, P.A., Robinson, S.P., Gleave, A.P.,

Green, A.G., and Waterhouse, P.M. (2001). Construct design for efficient, effective and high-throughput gene silencing in plants. Plant J. **27,** 581-590.

Wheeler, H. (1974). Cell wall and plasmalemma modifications in diseased and injured plant tissues. Can. J. Bot **52,** 1005-1009.

Wichard, T., Gobel, C., Feussner, I., and Pohnert, G. (2004). Unprecedented lipoxygenase/hydroperoxide lyase pathways in the moss *Physcomitrella patens*. Angew Chem **44,** 158-161.

Wilce, M.C., and Parker, M.W. (1994). Structure and function of glutathione *S*-transferases. Biochim. Biophys. Acta **1205,** 1-18.

Wildermuth, M.C., Dewdney, J., Wu, G., and Ausubel, F.M. (2001). Isochorismate synthase is required to synthesize salicylic acid for plant defence. Nature **414,** 562-565.

Wildon, D.C., Thain, J.F., Minchin, P.E.H., Gubb, I.R., Reilly, A.J., Skipper, Y.D., Doherty, H.M., O'Donnell, P.J., and Bowles, D.J. (1992). Electrical signalling and systemic proteinase inhibitor induction in the wounded plant. Nature **360,** 62-65.

Wilen, R.W., van Rooijen, G.J., Pearce, D.W., Pharis, R.P., Holbrook, L.A., and Moloney, M.M. (1991). Effects of jasmonic acid on embryo-specific processes in *Brassica* and *Linum* oilseeds. Plant Physiol. **95,** 399-405.

Williams, J., Bulman, M., Huttly, A., Phillips, A., and Neill, S. (1994). Characterization of a cDNA from *Arabidopsis thaliana* encoding a potential thiol protease whose expression is induced independently by wilting and abscisic acid. Plant Mol Biol. **25,** 259-270.

Wittstock, U., and Gershenzon, J. (2002). Constitutive plant toxins and their role in defense against herbivores and pathogens. Curr Opin Plant Biol. **5,** 300-307.

Wu, C.H., Caspar, T., Browse, J., Lindquist, S., and Somerville, C. (1988). Characterization of an *HSP70* cognate gene family in *Arabidopsis*. Plant Physiol. **88,** 731-740.

Xie, D.X., Feys, B.F., James, S., Nieto-Rostro, M., and Turner, J.G. (1998). *COI1*: an *Arabidopsis* gene required for jasmonate-regulated defense and fertility. Science **280,** 1091-1094.

Xu, L., Liu, F., Lechner, E., Genschik, P., Crosby, W.L., Ma, H., Peng, W., Huang, D., and Xie, D. (2002). The SCF(COI1) ubiquitin-ligase complexes are required for jasmonate response in *Arabidopsis*. Plant Cell **14,** 1919-1935.

Xu, Y., Chang, P., Liu, D., Narasimhan, M.L., Raghothama, K.G., Hasegawa, P.M., and Bressan, R.A. (1994). Plant defense genes are synergistically induced by ethylene and methyl jasmonate. Plant Cell **6,** 1077-1085.

Yamada, K., Nishimura, M., and Hara-Nishimura, I. (2004). The slow wound-response of *γVPE* is regulated by endogenous salicylic acid in *Arabidopsis*. Planta **218,** 599-605.

Yamaguchi-Shinozaki, K., Koizumi, M., Urao, S., and Shinozaki, K. (1992). Molecular cloning and characterization of 9 cDNAs for genes that are responsive to desiccation in *Arabidopsis thaliana*: sequence analysis of one cDNA clone that encodes a putative transmembrane channel protein. Plant Cell Physiol. **33,** 217.

Yamasaki, H., Uefuji, H., and Sakihama, Y. (1996). Bleaching of the red anthocyanin induced by superoxide radical. Arch Biochem Biophys **332,** 183-186.

Yoon, Y., Pope, J., and Wolfe, J. (2003). Freezing stresses and hydration of isolated cell walls. Cryobiology **46,** 271-276.

Yoshihara, T., Omer, E.S.A., Koshino, H., Sakamura, S., Kikuta, Y., and Koda, Y. (1989). Structure of a tuber-inducing stimulus from potato leaves (*Solanum tuberosum* L.). Agric Biol Chem. **53,** 2835-2837.

Young Jr, W.F., Gorman, C.A., and Weinshilboum, R.M. (1988). Triiodothyronine: a substrate for the thermostable and thermolabile forms of human phenol sulfotransferase. Endocrinology **122,** 1816-1824.

Yu, D., Chen, C., and Chen, Z. (2001). Evidence for an important role of WRKY DNA binding proteins in the regulation of *NPR1* gene expression. Plant Cell **13,** 1527-1540.

Yu, S.J., Kim, S., Lee, J.S., and Lee, D.H. (1998). Differential accumulation of transcripts for ACC synthase and ACC oxidase homologs in etiolated mung bean hypocotyls in response to various stimuli. Mol Cells **8,** 350-358.

Zegzouti, H., Jones, B., Marty, C., Lelievre, J.M., Latche, A., Pech, J.C., and Bouzayen, M. (1997). *ER5*, a tomato cDNA encoding an ethylene-responsive LEA-like protein: characterization and expression in response to drought, ABA and wounding. Plant Mol Biol. **35,** 847-854.

Zhang, Y., Fan, W., Kinkema, M., Li, X., and Dong, X. (1999). Interaction of NPR1 with basic leucine zipper protein transcription factors that bind sequences required for salicylic acid induction of the *PR-1* gene. PNAS **96,** 6523-6528.

Ziegler, J., Stenzel, I., Hause, B., Maucher, H., Hamberg, M., Grimm, R., Ganal, M., and Wasternack, C. (2000). Molecular cloning of allene oxide cyclase. The enzyme establishing the stereochemistry of octadecanoids and jasmonates. J Biol Chem. **275,** 19132-19138.

Zimmerman, D.C., and Coudron, C.A. (1979). Identification of traumatin, a wound hormone, as 12-oxo-*trans*-10-dodecenoic acid. Plant Physiol. **63,** 536-541.

CURRICULUM VITA

Chi-Tai Conan Tang

ACADEMIC BACKGROUND

Ph.D., Plant Molecular & Cell Biology
Rutgers University, New Brunswick, NJ
Plant Biology Graduate Program
October 2007

B.A., Biology
University of Pennsylvania, Philadelphia, PA,
August 1994

RESEARCH EXPERIENCE

Isolation of Homeobox Genes from Axolotls	1993-1994
The Wound Response in Land Plants	1994-2006