9-LIPOXYGENASE OXYLIPIN PATHWAY IN PLANT RESPONSE TO BIOTIC STRESS

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The activity of plant 9-lipoxygenases (LOXs) influences the outcome of *Arabidopsis thaliana* interaction with pathogen and insects. Evidence provided here indicates that in *Arabidopsis*, 9-LOXs facilitate infestation by *Myzus persicae*, commonly known as the green peach aphid (GPA), a sap-sucking insect, and infection by the fungal pathogen *Fusarium graminearum*. In comparison to the wild-type plant, *lox5* mutants, which are deficient in a 9-lipoxygenase, GPA population was smaller and the insect spent less time feeding from sieve elements and xylem, thus resulting in reduced water content and fecundity of GPA. *LOX5* expression is induced rapidly in roots of GPA-infested plants. This increase in *LOX5* expression is paralleled by an increase in LOX5-synthesized oxylipins in the root and petiole exudates of GPA-infested plants. Micrografting experiments demonstrated that GPA population size was smaller on plants in which the roots were of the *lox5* mutant genotype. Exogenous treatment of *lox5* mutant roots with 9-hydroxyoctadecanoic acid restored water content and population size of GPA on *lox5* mutants. Together, these results suggest that *LOX5* genotype in roots is critical for facilitating insect infestation of *Arabidopsis*. In *Arabidopsis*, 9-LOX function is also required for facilitating infection by *F. graminearum*, which is a leading cause of Fusarium head blight (FHB) disease in wheat and other small grain crops. Loss of *LOX1* and *LOX5* function resulted in enhanced resistance to *F. graminearum* infection. Similarly in wheat, RNA interference-mediated silencing of the 9-LOX homolog TaLpx1, resulted in enhanced resistance to *F. graminearum*. Experiments in *Arabidopsis* indicate that 9-LOXs promote susceptibility to this

fungus by suppressing the activation of salicylic acid-mediated defense responses that are important for basal resistance to this fungus.

The *lox1* and *lox5* mutants were also compromised for systemic acquired resistance (SAR), an inducible defense mechanism that is systemically activated throughout a plant in response to a localized infection. The *lox1* and *lox5* mutants exhibited reduced cell death and delayed hypersensitive response when challenged with an avirulent strain of the bacterial pathogen *Pseudomonas syringae* pv tomato. *LOX1* and *LOX5* functions were further required for the synthesis as well as perception of a SAR-inducing activity present in petiole exudates collected from wild-type avirulent pathogen-challenged leaves. Taken together, results presented here demonstrate that 9-LOX contribute to host susceptibility as well as defense against different biotic stressors.

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COMPREHENSIVE LIST OF ABBREVIATIONS

ABA	Abscisic acid
ACT	Actin
ANOVA	Analysis of variance
AOC	Allene oxide cyclase
Avr	Avirulent
BTH	Benza-thiadiazole-7-carbonic acid
CFU	Colony forming units
DA	Dehydroabietinal
DOX	Dioxygenase
DSI	Disease severity index
EAS	Epoxy alcohol synthase
EDS1	ENHANCED DISEASE SUSCEPTIBILTY1
EDTA	Ethylene diamine tetraacetic acid
EF	Elongation factor
*EIN*2	ETHYLENE INSENSITIVE2
EPG	Electrical penetration graph
EST	Expressed sequence tags
ET	Ethylene
ETI	Effector triggered immunity
FA	Fatty acids
FAD	Fatty acid desaturase
FHB	Fusarium head blight
Fg	*Fusarium graminearum*
FW	Fresh weight
GC-MS	Gas chromatography mass spectrometry
GFP	Green fluorescent protein
GLM	Generalized linear model
GLV	Green leafy volatiles
GPA	Green peach aphid
GUS	β-Glucuronidase
HOD	Hydoxyoctadecadienoic acid

HOT	Hydroxyoctadecatrienoic acid
HP	Hydroperoxides
HPL	Hydroperoxide lyase
HPLC	High performance liquid chromatography
HPOD	Hydroperoxyoctadecadienoic acid
HPOT	Hydroperoxyoctadecatrienoic acid
HR	Hypersensitive response
Hv	*Hardeum vulgare*
ICS	Isochorismate synthase
IPTG	Isopropyl β-D-1-thiogalactopyranoside
ISR	Induced systemic resistance
JA	Jasmonic acid
KOD	Ketooctadecadienoic acid
KOT	Ketooctadecatrienoic acid
LOX	Lipoxygenase
MES	2-(*N*-morpholino)ethanesulfonic acid
MeSA	Methyl salicylate
MJ	Methyl jasmonate
NAE	N-acetyl ethanolamine
NIL	Near isogenic line
NP	Non-probing phase
NPR1	NONEXPRESSOR OF PATHOGENESIS-RELATED GENE1
NS	Not significant
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PAL	Phenylalanine ammonia lyase
PAMP	Pathogen associated molecular pattern
PCR	Polymerase chain reaction
PDF	PLANT DEFENSIN
PP	Pathway phase
PR1	PATHOGENESIS RELATED 1
PRR	Pathogen recognition receptor
Psm	*Pseudomonas syringae* pv. *maculicola*

Pst	*Pseudomonas syringae* pv. *tomato*
PTI	Pathogen triggered immunity
PUFA	Poly unsaturated fatty acids
QTL	Quantitative trait loci
ROS	Reactive oxygen species
SA	Salicylic acid
SAR	Systemic acquired resistance
SDS	Sodium dodecyl sulfate
SEM	Standard error of mean
SEP	Sieve element phase
*SID*2	*SA INDUCTION-DEFICIENT2*
Ta	*Triticum aestivum*
TaLPX	*Tritium aestivum* lipoxygenase
TMS	Trimethylsilyl
TUB	Tubulin
WCI	WHEAT CHEMICALLY INDUCED
WGA	Wheat germ agglutinin
WT	Wild-type
XP	Xylem phase

CHAPTER 1

INTRODUCTION

In the year 2012, the world's population is expected to hit the 7 billion mark and is projected to grow and plateau at approximately 9 billion by the year 2050. Global food production will need to increase by an estimated 50% in order to meet the world's food demands (Godfray et al., 2010; Chakraborty and Newton, 2011). Several constraints will have to be overcome to meet the increased demand. The loss of agricultural land to degradation and conversion to non-food production, the continued impact of pests and disease, lack of availability of good quality water for agriculture coupled with climate change are already posing major challenges in maintaining let alone increase food production (Godfray et al., 2010). The Green Revolution resulted in over 70% increase in yield in the past as a result of the development of F_1 hybrids of maize and semi-dwarf varieties of rice that responded to more irrigation and increased fertilizer application. Yet an estimated 1.02 billion people went hungry in 2009, the highest ever level of world hunger (http://www.ifad.org/). Therefore, radical changes in food production, storage, processing and distribution are required to meet the challenge of feeding the world's population.

Pests and diseases continue to impact food production and quality despite the many decades of research by crop protection scientists on the development of improved methods for their control. An estimated 30-40% of crop yield is lost annually in the fields even in crops where pesticides and cultivars with genetic resistance to pests and diseases are used (Oerke, 2006). The widespread use of agro-chemicals such as fungicides and pesticides to the tune of 3 billion kg every year (Pimentel, 2009), has enabled significant increases in crop yields but has also resulted in the development of more aggressive or chemical-resistant biotypes which can

potentially cause devastating losses. The dependence on pesticides has also resulted in major costs to the environment. Improved crop protection strategies to reduce the environmental impact of pesticides and still prevent losses due to pests and pathogens are needed to increase production and make a substantial contribution to food security.

A major advance in plant biology that will lead to improved and novel approaches to crop protection is an understanding of the genetic and molecular basis of plant immune response to the various biotic stressors. Knowledge about the regulatory genes, signal molecules and defense pathways in plants, will aid in the development of new crop varieties by conventional breeding and/or genetic engineering with increased resistance while at the same time reducing our dependence on pesticides.

1.1 The Plant Immune System

In nature, plants are continually exposed to attacks from various biotic agents like bacteria, fungi, oomycetes, viruses and insects. The outcome of the interaction between the plant and the pest/pathogen is largely determined by preformed constitutive defenses coupled with specific defenses employed against specific invaders (induced defenses). In a majority of the cases, the plant is able to counter and prevent colonization by pests/pathogens (non-host interactions). However, some microbes and insects have acquired genetic adaptations that enable them to overcome or tolerate the plants' constitutive and induced defenses. These pathogens and insect pests are then able to obtain nutrients from plants enabling them to establish and grow resulting in disease and damage of the host plant.

Plants are involved in a continuous co-evolutionary struggle for dominance or 'arms race' with the pests and pathogens that attack them. In the absence of an adaptive immune system,

plants have evolved an innate immune system that recognizes the presence of potential pathogens and initiates effective defenses, whereas successful pathogens have evolved to suppress host responses. Plants integrate mechanical and chemical cues associated with insect and microbial pathogen attack and orchestrate defenses that are specific to each (Walling, 2000; De Vos et al., 2005; Glazebrook, 2005). This first line of defense against a pathogen that is able to overcome the plants constitutive defenses is the primary immune response. This response is activated upon the perception of highly conserved molecules that are common to invading organisms called pathogen associated molecular patterns (PAMPs) (Jones and Dangl, 2006). The recognition of PAMPs by plant pattern-recognition receptors (PRRs), results in the activation of characterized downstream signaling events regulated by salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) resulting in basal resistance or PAMP-triggered immunity (PTI) (Glazebrook, 2005; Chisholm et al., 2006; Jones and Dangl, 2006). However, successful plant pathogens have acquired adaptations in the form of effector molecules that enable them to repress PTI and allow colonization of the plant. Plants in turn have evolved resistance (*R*) genes that enable them to identify pathogen specific effectors and initiate a second line of immune response or effector-triggered immunity (ETI) (Chisholm et al., 2006; Jones and Dangl, 2006). The defense responses activated by the plant depends on the mechanisms used for nutrient retrieval and the lifestyle of the attacker. Despite the differing mechanisms utilized by microbes and insects to procure nutrients, the plants' innate immune responses show conservation (Walling, 2009). The ability of the pathogen to suppress plant defenses and the plants ability to recognize and initiate timely defense against the pathogen determines the final outcome of the interaction.

The activation of ETI, mediated by *R*-gene signaling at the site of infection is often accompanied by a long-lasting and induced disease resistance in the distal healthy parts of the

plant (Durrant and Dong, 2004). This form of immunity protects distal plant parts and even the subsequent generation of progeny in certain cases from a broad spectrum of attackers is referred to as systemic acquired resistance (SAR) (Walters et al., 2007; Jaskiewicz et al., 2010; Luna et al., 2012). SAR is characterized by the generation of a mobile signal generated at the site of infection that establishes systemic immunity. Another example of this form of acquired resistance, induced systemic resistance (ISR), occurs upon the colonization of roots by beneficial soil borne microorganisms such as nonpathogenic rhizobacteria and mycorrhizal fungi leading to induction of pathogen resistance in above ground tissues (van Loon et al., 1998; Pozo and Azcon-Aguilar, 2007). SAR and ISR differ with respect to the nature of the elicitor and also the regulatory pathways involved which are mediated by signaling pathways controlled the phytohormones SA and JA/ET respectively (Walters et al., 2007).

1.1.1 Systemic Acquired Resistance

SAR is induced by pathogens that cause necrosis, either as disease symptom or as a part of the hypersensitive response (HR) triggered during ETI. HR is associated with the rapid production of reactive oxygen species (ROS) and programmed cell death at the site of infection providing a physical and chemical barrier that limits further spread of the pathogen. Although, an HR is not essential for SAR and the generation of the long-distance signal (Cameron et al., 1994; Mishina and Zeier, 2007), its appearance advents the onset of SAR in most cases. At the molecular level, SAR is characterized by the activation of a specific set of pathogenesis-related (*PR*) genes encoding proteins with antimicrobial properties in both local infected and distal uninfected tissues (Van Loon et al., 2006). This is associated with increased accumulation of SA in local and systemically in distant tissues. The importance of SA in SAR is further highlighted

by genetic studies with mutants and transgenic plants that are impaired in SA signaling. The activation of *PR* gene expression and development of SAR is impaired in these lines highlighting the importance of SA in SAR signaling (Durrant and Dong, 2004). A key component of SA-mediated signaling during SAR is regulated by the protein NPR1 (NONEXPRESSOR OF PR GENES1) (Dong, 2004; Durrant and Dong, 2004). The gene was identified in several genetic screens conducted to identify genes involved in SA signaling (Cao et al., 1994; Delaney et al., 1995; Glazebrook et al., 1996; Shah et al., 1997). *Arabidopsis npr1* mutants are able to accumulate SA upon pathogen infection but fail to exhibit SAR (Delaney et al., 1995; Shah et al., 1997). Upon activation by SA, *NPR1* along with TGA transcription factors activates the expression of *PR* and other genes that are necessary for SAR (Dong, 2004).

The systemic enhancement of defenses during SAR implies the presence of a mobile signal(s) that is generated at the site of infection and aids in the establishment of SAR in distal uninfected plant parts. In recent years, major advances have been made in identifying the nature of the mobile signal. Several metabolites have been proposed as candidate SAR signals. Lipids or lipid-derived molecules have been implicated in this process (Maldonado et al., 2002; Nandi et al., 2004; Chaturvedi et al., 2008). In tobacco plants, an SA derivative, methyl salicylate and an unidentified lipid-derived molecule act as the mobile signal (Park et al., 2007; Liu et al., 2011). The metabolite, azelaic acid was identified in petiole exudates (pet-ex) of plants in which SAR was induced suggesting that it may be a mobile signal although millimolar quantities of the compound are required (Jung et al., 2009). Recently, a diterpenoid, dehydroabietinal, was identified in petiole exudates of plants treated with an avirulent pathogen which is able to initiate SAR in a SA-dependant manner in picomolar quantities (Chaturvedi et al., 2012). The

identification of several potential signal molecules suggests that plants have evolved several mechanisms by which they can efficiently induce SAR in response to various pathogens.

The continuous activation of defenses in the plant has a high metabolic cost resulting in reduced plant fitness. In economically important crops, this is undesirable since reduced fitness results in low yields. SAR is however, a widely observed phenomenon in plants resulting in a state of heightened alertness by which plants are able to combat pathogens more quickly and effectively with seemingly low impact on metabolic costs and fitness (Heidel et al., 2004; Traw et al., 2007). Recent evidence suggests that the large scale chromatin remodeling that occurs during SAR allows for epigenetic inheritance of the state of heightened alertness to the next generation of offspring (Jaskiewicz et al., 2010; Luna et al., 2012; Slaughter et al., 2012). This finding has major implications in crop systems, where 'alert' or disease-resistant offspring can be produced by deliberately exposing parent plants to diseases or a priming treatment. Furthermore, genetic engineering has allowed for targeted manipulations of genes of the SAR pathway to enhance resistance to pests and pathogens. Transgenic crop plants either over-expressing or constitutively expressing *NPR1*, exhibit enhanced resistance to a variety of pathogens in tomato and cotton and also in monocot crops like rice and wheat (Lin et al., 2004; Chern et al., 2005; Makandar et al., 2006; Parkhi et al., 2010). Additionally, the conclusive identification of the SAR signal molecule(s) has widespread implications in agriculture.

1.2 Plant Oxylipins

A large body of research implies an important role for oxidized lipids, more commonly known as oxylipins, not only in plant development but also in defense against various pests and pathogens (Blée, 2002; Howe and Schilmiller, 2002; Andreou et al., 2009; Mosblech et al., 2009,

2010). In plants, oxylipins play diverse roles. They are not only thought to stimulate signals resulting in the mounting of plant defenses, but also have antimicrobial properties, provide building units to generate physical barriers by inducing lignification (Kishimoto et al., 2006) against pathogen invasion, regulate plant cell death and are also involved in senescence by inducing rapid chlorophyll breakdown and plastid protein turnover (Reinbothe et al., 2009). In addition, Jasmonic acid (JA), one of the best studied oxylipins is a phytohormone (La Camera et al., 2004; Shah, 2005).

Plant oxylipins are a diverse class of lipid metabolites that are derived from the initial oxidation of polyunsaturated fatty acids. The first step in the synthesis of oxylipins involves the formation of fatty acid hydroperoxides either by autooxidation, or by the action of enzymes like lipoxygenases (LOXs) and α-dioxygenases (α-DOX) (Feussner and Wasternack, 2002; Mosblech et al., 2009) (Figure 1.1). Further modifications of the fatty acid hydroperoxides is catalyzed by other enzymatic activities, including those initiated by allene oxides synthase (AOS), divinyl ether synthase (DES), epoxy alcohol synthase (EAS), reductase, LOXs and hydroperoxide lyase (HPL), resulting in a range of biologically active compounds. These include fatty acid hydroperoxides, hydroxy-, oxo-, or keto-fatty acids, divinyl ethers, volatile aldehydes, oxo-acids and the plant hormone, jasmonic acid (Figure 1.1) (Blée, 2002; Feussner and Wasternack, 2002; Mosblech et al., 2009). The enzymes involved in the synthesis of oxylipins are diverse and the pathway results in a vast array of compounds with varied physiological properties. In mammals, the arachidonic acid cascade results in oxylipins which play a major role in inflammatory processes and in stress response to infections and allergies (Blée, 2002). The occurrence and formation of oxylipins not only in plants and mammals but also in fungi, algae and bacteria is

leading to a substantial increase in our understanding of the role of oxylipins in cellular development and stress responses (Andreou et al., 2009).

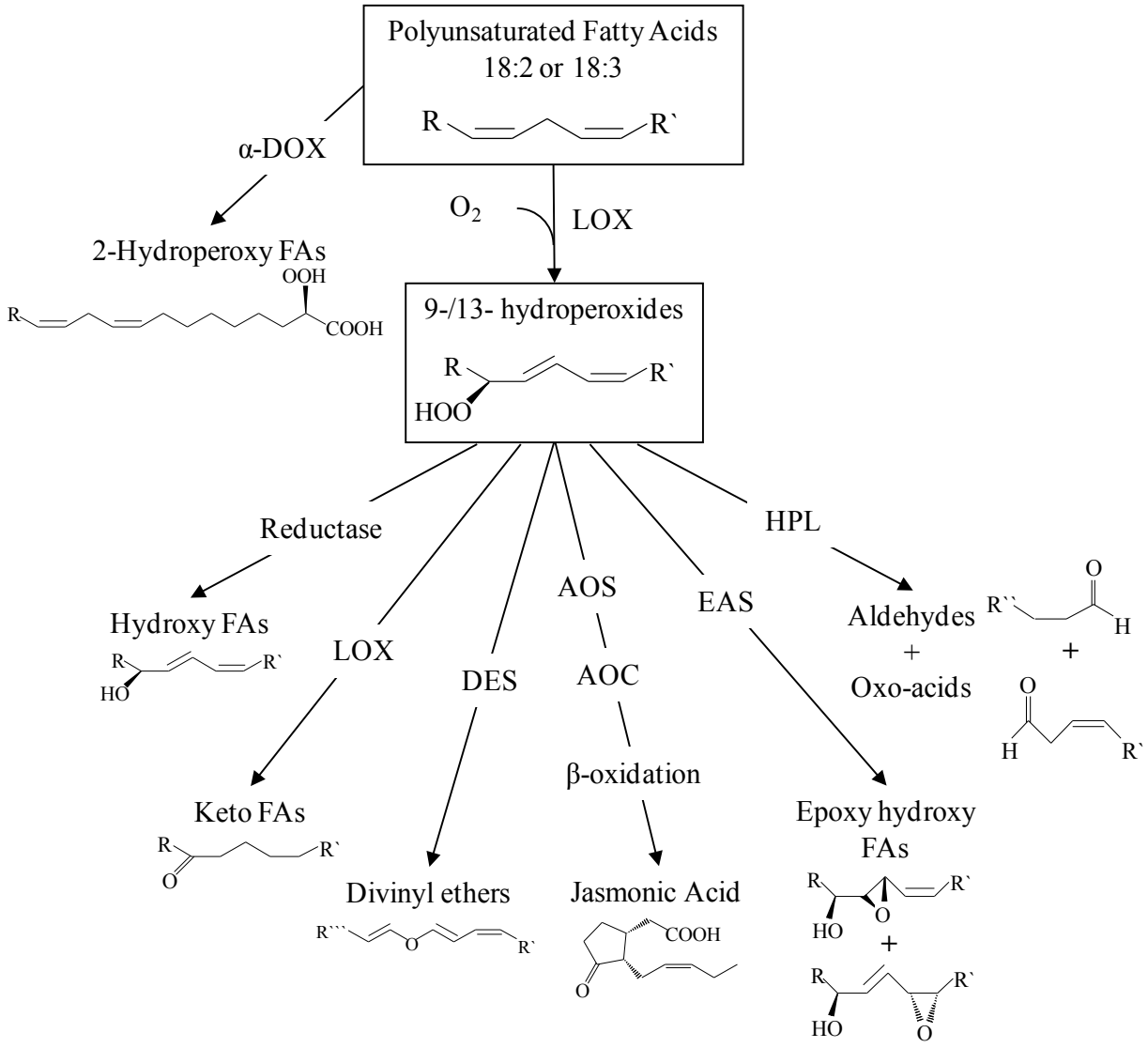

Figure 1. 1 Major pathways of oxylipin biosynthesis in plants from linoleic (18:2) or linolenic acid (18:3). LOX, Lipoxygenase; α-DOX, α-dioxygenase; DES, divinyl ether synthase; AOS, Allene oxide synthase; AOC, Allene oxide cyclase EPS, Epoxy alcohol synthase; HPL, Hydroperoxide lyase; FAs, Fatty acids.

1.2.1 Lipoxygenases

A large body of evidence implies a crucial physiological role for jasmonic acid and its

derivatives in plant development and response to various stresses (Wasternack, 2007; Balbi and Devoto, 2008; Chehab and Braam, 2012). The relevance of 9-LOX derived oxylipins in plant physiological processes is only recently becoming apparent, aspects of which are reviewed below.

Lipoxygenases catalyze the introduction of molecular oxygen into polyunsaturated fatty acids (PUFAs) to yield corresponding hydroperoxides. They are a family of non-heme, iron containing dioxygenases that occur not only in plants and mammals but also in fungi, algae and bacteria (Andreou et al., 2009). LOXs are multifunctional enzymes catalyzing at least three distinct reactions in plants. The first being the oxygenation of substrates, mainly linoleic (18:2) and linolenic acid (18:3), the second being the conversion of hydroperoxy fatty acids to hydroxy fatty acids (Figure 1.2) and finally the formation of epoxy leukotrienes (Feussner and Wasternack, 2002; Bannenberg et al., 2009). However, under physiological conditions, the first reaction is more prevalent in plants. In plants, distinct LOX isozymes are classified on the basis of the positional specificity of oxygenation of linoleic and linoleic acids. Isozymes that add a hydroperoxy group at the carbon atom 9 are referred to as 9-LOXs and those that add to the carbon atom 13 are referred to as 13-LOXs (Figure 1.2). The oxygenation of linoleic or linolenic by LOXes with differing specificities results in the formation of two groups of compounds, the (9*S*)-hydroperoxy and the (13*S*)-hydroperoxy derivates of polyunsaturated fatty acids (Figure 1.2). The specificity of the LOX is determined by the space within the active site and the orientation of the substrate (Kühn et al., 1990; Liavonchanka and Feussner, 2006).

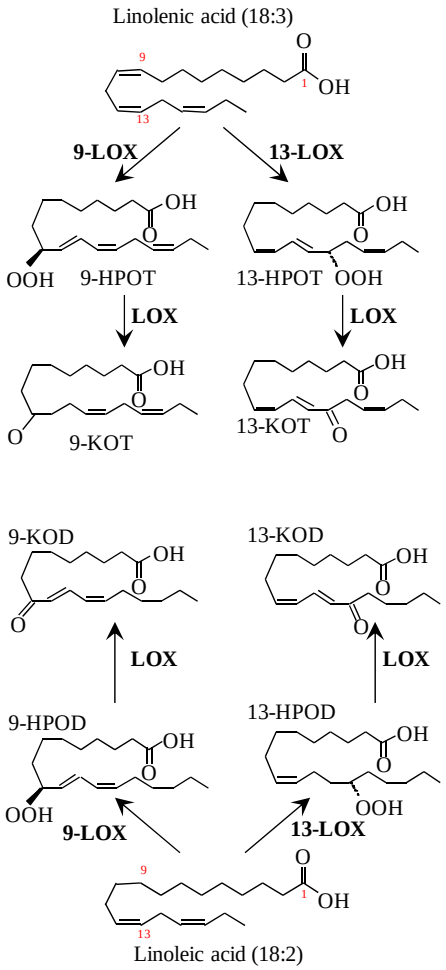

Figure 1.2 The lipoxygenase reactions.

Although a majority of LOXs strongly prefer free fatty acids as substrates, a few instances of LOXs active on polyunsaturated fatty acids in phospholipids have been reported (Liavonchanka and Feussner, 2006). An important role for the lipoxygenase pathway is the turnover of *N*-Acetylethanolamines (NAEs). In plants, NAEs are most abundant in dessciated seeds, however several important functional roles for these compounds is emerging (Kilaru et al., 2007; Coulon et al., 2011). The depletion of polyunsaturated NAEs during early stages of seedling development is necessary for normal seedling growth and the lipoxygenase mediated oxygenation has been suggested to be essential for this process (Kilaru et al., 2011). Furthermore, the competitive inhibition of LOX-mediated oxidation by lauroylethanolamine

(NAE 12:0) is thought to interfere with LOX-mediated defense responses as NAE 12:0 prevents the accumulation of wound induced JA (Keereetaweep et al., 2010). Additional research to determine the physiological role of the NAE-oxylipins in planta is needed.

Lipoxygenase catalyzed production of hydroperoxy and keto FAs from linolenic and linoleic acids. Numbers in red indicate the carbon atom. LOX, Lipoxygenase; 9-HPOD, 9(*S*)-Hydroperoxy-10(*E*),12(*Z*)-octadecadienoic acid; 9-HPOT, 9(*S*)-Hydroperoxy-10(*E*),12(*Z*)-octadecatrienoic acid; 13-HPOD, 13(*S*)-Hydroperoxy-9(*Z*),11(*E*) ,15(*Z*)-octadecadienoic acid; 13-HPOT, 13(*S*)-Hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid; 9-KOD, 9-keto-10(*E*),12(*Z*)-octadecadienoic acid; 9-KOT, 9-keto-10(*E*),12(*Z*),15(Z)-octadecatrienoic acid; 13-KOD, 13-keto-9(*Z*),11(*E*)-octadecadienoic acid; 13-KOT, 13-keto-9(*Z*),11(*E*),15(Z)-octadecatrienoic acid.

The increase in availability of sequence information shows that LOXs are present in all plants analyzed and are normally present as a multigene family. The number of members in the family can vary widely, for instance there are 6 members in *Arabidopsis* and at least 12 and 14 in maize (*Zea mays*) and potato (*Solanum tuberosum*) respectively (Feussner and Wasternack, 2002; Gao et al., 2007). The families consists mainly of both 9-/13-LOXs, however in a few cases, LOXs with dual specificities have also been identified (Hughes et al., 2001).

The physiological functions of the various LOX isoforms can be determined in part by their intra-cellular localization. LOX isoforms have been identified in virtually every compartment of a cell including the cytosol, stroma, vacuole, lipid bodies or in association with different membranes (Mosblech et al., 2009). Additionally, studies in *Arabidopsis* on LOX expression have revealed that LOXs are expressed in different plant tissues at different times during plant development (Vellosillo et al., 2007). It is possible that the occurrence of different

LOX isoforms in specific locations coupled with their temporal differentiation leads to the formation of various pools of hydroperoxy fatty acids. These pools in turn act as substrates for alternative pathways of further conversion resulting in a range of products which have different effects on physiology. A better understanding of the localization and temporal control of LOXes is therefore necessary to help decipher their function and role in plant development and response to biotic stressors.

1.2.2 Physiological Roles of 9-LOX-derived Oxylipins

The oxylipins generated via the LOX pathway are functionally diverse and influence several physiological processes. LOX-derived oxylipins have been implicated in plant growth and development, senescence, resistance to pathogens and insect pests and in response to environmental stress (Blée, 2002; Howe and Schilmiller, 2002; Mosblech et al., 2010). Much of our current understanding of the physiological roles of LOXs derives from studies on oxylipins derived via 13-LOXs. Following oxygenation of C18:3 by 13-LOXs, the 13- hydroperoxy derivatives produced are further metabolized by two major branches of the oxylipin pathway. One branch results in jasmonates and other octadecanoids produced via the AOS branch also known as the octadecanoid pathway (Figure 1.1). Jasmonates and their octadecanoid precursors are involved in the regulation of several physiological processes in plants ranging from stress response to development. During normal plant growth and development, they are involved in carbon partitioning, mechanotransduction, senescence and reproductive development (Balbi and Devoto, 2008; Reinbothe et al., 2009; Mosblech et al., 2010). Stress responses include defenses against insect and microbial pathogens as wells as responses to drought, UV radiation, ozone, and other abiotic stresses (Wasternack, 2007; Chehab and Braam, 2012).

The second branch involves HPL which cleaves hydroperoxy fatty acids to produce volatile C-6 aldehydes, alcohols and their esters which are collectively known as green leaf volatiles (GLV) (Feussner and Wasternack, 2002). GLVs are the compounds that are responsible for the 'aroma' of many plants, flowers and fruits. The physiological roles of GLVs are yet to be determined but research suggests that GLVs play important roles in communication within and between plants. In addition GLVs allow plants to communicate with other plants and organisms, specifically insects that surround them (Matsui, 2006).

The significance of 13-LOX-derived oxylipins in plant development and response to abiotic and biotic stresses is better understood than oxylipins derived from the 9-LOX pathway. Emerging evidence suggest 9-LOXs play important roles during plant development and in various physiological processes. In potato, a specific 9-LOX is expressed transiently during tuber growth and is required to maintain tuber size (Kolomiets et al., 2001). Genetic studies in *Arabidopsis* and maize demonstrate that 9-LOXs are involved in the regulation of lateral root growth and development (Vellosillo et al., 2007; Gao et al., 2008). In maize, *ZmLOX3,* a 9-LOX also controls plant germination and senescence (Gao et al., 2008). The expression of 9-LOXs and increased LOX activity is observed during germination (Feussner and Wasternack, 2002) and during seed development in almonds (Santino et al., 2005) and in maize (Jensen et al., 1997). In barley, activity of both 9-/13-LOXs in seeds influences the quality of malt produced resulting in the production of beer-deteriorating substances (Hirota et al., 2006). In wheat, LOX activity leads to oxidative degradation of carotenoid pigments in seeds that are essential for the bright color of pasta and semolina (Borrelli et al., 1999). Although, wheat lipoxygenases have not been characterized, it is possible that both 9-/13-LOXs are active in seeds given that a number of LOX isozymes have been identified during seed development (Shiiba et al., 1991).

1.2.3 9-LOX-derived Oxylipins in Plant Defense

The defense-related functions of the 9-LOX pathway are not fully understood. The role of 9-LOXs in defense is likely related to the synthesis of fatty acid hydroperoxides (Rusterucci et al., 1999; Gobel et al., 2001), regulation of plant defenses (Knight et al., 2001; La Camera et al., 2004), antimicrobial activity (Prost et al., 2005), volatiles with signaling functions (Matsui, 2006) and as signal molecules involved in cross-kingdom communication (Christensen and Kolomiets, 2011). The induction of enzymes of the 9-LOX pathway along with an increase in the levels of corresponding fatty hydroperoxides in response to infection by certain pests and pathogens further confirms the hypothesis that the 9-LOX pathway plays an important role in plant defense.

1.2.3.1 In Hypersensitive Reaction

During infection by avirulent or incompatible pathogens, plants display rapid localized cell death at the site of attempted attack. This process termed hypersensitive reaction (HR) limits the spread of the pathogen from the site of infection and is a result of several metabolic changes in the cell, one of them being lipid peroxidation. The involvement of 9-LOX derived oxylipins in lipid peroxidation during HR has been demonstrated for specific cases. For instance, the levels of 9-LOX transcripts and related products increase in tobacco and potato in response to treatment with fungal elicitors or infection with pathogens (Rusterucci et al., 1999; Gobel et al., 2001; Göbel et al., 2002; Montillet et al., 2005). In transgenic tobacco with reduced expression of pathogen-inducible 9-LOX, susceptibility to *Phytopthora parasitica* pv. *nicotianae* increased and conversely constitutive expression resulted in decreased susceptibility to the same pathogen (Rancé et al., 1998; Mène-Saffrané et al., 2003). In tobacco, 9-LOXs along with oxidative

processes contribute to lipid peroxidation during HR induced by the avirulent pathogen *Pseudomonas syringae* pv. *syringae* (Montillet et al., 2005). In cotton plants, the induction of HR is associated with *GhLOX1*, a 9-LOX, during infection with an incompatible pathogen *Xanthomonas campestris* pv. *malvacearum* (Jalloul et al., 2002; Marmey et al., 2007). However, in transgenic potato with reduced expression of 9-LOX, the timing and extent of tissue necrosis and HR due to treatment with avirulent *P. syringae* pv. *maculicola* was unaltered but autooxidative lipid peroxidation and 13-LOX-mediated oxylipin synthesis was more prominent (Gobel et al., 2003). This suggests that although 9-LOX dependent peroxidation is a feature of HR, the origin of lipid hydroperoxides does not influence HR in potato.

1.2.3.2 Regulation of Plant Defense

In response to pathogens or pests, oxylipins produced via the 9-LOX pathway are thought to contribute to resistance by acting as signal molecules that modulate plant responses through interactions with hormones involved in defense. Rustérucci et al. (1999) suggest that 9-LOX generated 9-HPOD or 9-HPOT act as signal molecules with neighboring cells during HR. In cotton, *GhLOX1* is associated with salicylic acid (SA) and jasmonic acid (JA) accumulation during HR (Marmey et al., 2007). The 9-LOX product 9-HOT, induces the deposition of callose and production of reactive oxygen species (ROS) which are characteristic stress responses that occur during pathogen infection (Vellosillo et al., 2007). Microarray studies revealed that a subset of genes induced by 9-HOT in roots, are also induced in leaves in response to 9-HOT treatment or infection by *Pseudomonas*. Additionally, *noxy2* (*non-responding to oxylipins 2*), a mutant that is insensitive to 9-HOT displayed enhanced susceptibility to *Pseudomonas* infection (Vellosillo et al., 2007). Identification of two *noxy* mutants, *noxy6* and *noxy22* as constitutive

ethylene (ET) mutants suggest a negative interaction of ET with 9-HOT signaling (López et al., 2011). Further analysis indicated that ET impairs 9-HOT response whereas 9-HOT interfered with ET signaling pathway implying an antagonistic interaction between the two. Lopez et al. (2011) suggest that 9-LOX and ET pathways exert tight control over oxidative stress during pathogen infection enabling the plant to survive and achieve full resistance.

A different 9-LOX product, 9-KOT, produced by the dehydration of 9-HPOT has also been implicated in plant defense against bacteria (Vicente et al., 2011). Pre-treatment of *Arabidopsis* with 9-KOT reduced the severity of symptoms observed in response to infection by a virulent pathogen, *P. syringae* pv *tomato* DC3000 (*Pst* DC3000). Levels of 9-KOT accumulate in response to the bacterial infection and *lox1dox1* mutants that are unable to synthesize 9-KOT display local susceptibility and are partially impaired in systemic acquired resistance (SAR). 9-KOT treatment also induces the expression of a subset of genes that are upregulated in response to infection by both virulent and avirulent pathogens, in oxidative and osmotic stress and also by the plant hormones, SA and abscisic acid (ABA) (Vicente et al., 2011). The up-regulation of a wide variety of genes in response to 9-KOT treatment suggests a role for the 9-LOX pathway in contributing plant adaptation to various biotic and abiotic stresses it encounters in the environment. Further genetic studies involving crosses of 9-LOX pathway mutants with mutants of other defense pathways will provide an insight into the extent of cross-talk between 9-LOX-derived oxylipins and defense hormones in response to biotic and abiotic stresses.

A role for α-DOX derived oxylipins in plant defense response is also emerging. In plants, α-DOXs catalyze the formation of 2-hydroperoxy fatty acids from PUFA that are subsequently transformed to corresponding alcohols or aldehydes (Hamberg et al., 1999). In addition to the direct participation of these compounds in plant defense, α-DOXs can also influence the 9-LOX

pathway as a result of substrate depletion (Vicente et al., 2011). Studies in tobacco and *Arabidopsis* have revealed that α-DOX activity regulates defenses by regulating oxidative stress and cell death in addition to possessing antibacterial properties (De León et al., 2002; Hamberg et al., 2003; Vicente et al., 2011).

The products of the 9-LOX pathway have also been proposed to function as suppressors of plant defense responses. In maize a 9-LOX, *ZmLOX3* displays root-specific expression and in the mutant, *lox3-4*, reduced root length and plant height was observed (Gao et al., 2007). The mutation of *ZmLOX3* also resulted in overproduction of the major defense hormones, SA, JA and ET and the overexpression of defense-related genes that they regulate (Gao et al., 2007). Further research is however needed to determine the identity of 9-LOX products involved in suppression of defense hormones and if a similar phenomenon also occurs in leaves and in other plant species.

1.2.3.3 Antimicrobial Properties

Of the several oxylipins that are produced in response to pathogen attack some of them have been shown to have antimicrobial properties. Oxylipins derived via the 13-LOX pathway, 13-HPOT and 13-HOT have an inhibitory effect on the growth of several fungi infecting the oilseed rape, *Brassica napus* (Granér et al., 2003). Volatiles produced via 13-LOX activity, *cis*-3-hexenol and trans-2-hexenal show antimicrobial properties and inhibit the growth of *Ps* pv *phaseolicola* in vitro (Croft et al., 1993). Two divinyl ether fatty acids, colneleic and colnelenic acids inhibit the growth of *Phytophthora infestans* which causes late blight on potato (Weber et al., 1999). Several epoxy- or polyhydroxylated fatty acids have also been suggested to possess antifungal or antioomycete properties (Blée, 1998). In a comprehensive study by Prost et

al.(2005), the antimicrobial properties of 43 naturally occurring oxylipins against 13 plant pathogens including bacteria, oomycetes and fungi was surveyed in vitro. Among the most active oxylipins displaying antimicrobial properties were oxylipins derived via the 9-/13-LOX pathway including 9-HPOD, 9-HPOT, 9-HOD, 9-HOT and 9-KOT (Prost et al., 2005; Vicente et al., 2011). Interestingly, several of the most active oxylipins are also regulators of plant defenses suggesting that these oxylipins play a dual role in plant defense.

1.2.3.4 Signal Molecules in Cross-Kingdom Communication

Although 9-LOX products by themselves are not volatile, aldehydes and oxo-acids produced by the action of hydroperoxide lyase (*HPL*) enzyme on 9-LOX products are volatile. These compounds posses antimicrobial properties *in vitro* and their production is enhanced in response to mechanical stress or herbivore/pathogen attacks, resulting in the production of a distinctive scent or aroma (Hatanaka, 1993; Kishimoto et al., 2005). For example, green peach aphid (*Myzus* persicae Sulzer) feeding on potato plants exclusively induces the production of volatiles derived via the 9-LOX pathway (Gosset et al., 2009). In transgenic potato plants with anti-sense mediated *HPL* depletion, aphids show a two-fold increase in fecundity (Vancanneyt et al., 2001). Therefore, the GLVs produced via *HPL* activity are thought to negatively affect herbivore performance. An additional role for the volatile compounds are in attracting natural enemies of insect pests and engineering plants to emit modified GLV blends has been suggested as an approach to enhance plant resistance (Matsui, 2006; Shiojiri et al., 2006). The observation that plant 9-LOX derived oxylipins are present in the aphid gut (Harmel et al., 2007) further suggests that these compounds directly impact aphid performance. In addition, GLVs also function as potent signal molecules both within and between plants (Matsui, 2006). For instance,

treatment of *Arabidopsis* with GLV enhances resistance against a necrotrophic pathogen, *Botrytis cinerea* (Kishimoto et al., 2005). In uninfested-maize plants that were treated with a continuous flow of (*Z*)-3-hexenol produced by herbivore-infested plants, the expression of several defense genes was upregulated (Farag et al., 2005) implying a role for GLVs in plant to plant communication.

Oxylipins are also produced by several genera of fungi including those that are plant pathogenic. Fungal oxylipins include oxylipins derived from oleic, linoleic and linolenic acids, collectively termed *psi*-factors, are important regulators of sexual and asexual spore development, promote or repress conidiation and act as signals for mycotoxin production (Brodhagen and Keller, 2006; Tsitsigiannis and Keller, 2007; Christensen and Kolomiets, 2011). The structures of fungal oxylipins or *psi*-factors are similar to plant oxylipins, particularly those produced via the 9-/13-LOX pathways such as 9-HPOD and 13-HPOD, giving rise to the hypothesis that these compounds are involved in cross-kingdom communication. Indeed, 9-HPOD treatment of *Aspergillus* cultures results in increased conidiation and also induces mycotoxin production (Aflatoxin, AF, in the case of *Aspergillus*) whereas 13-HOD treatment suppressed the production of AF (Burow et al., 1997; Calvo et al., 1999). Evidence supporting reciprocal cross-talk between plants and fungi comes from genetic studies where a maize 9-LOX, *ZmLOX3* was engineered into an *A. nidulans* mutant that is unable to produce conidia (Brodhagen et al., 2008). The restoration of conidia production in *A. nidulans* mutant which expressed *ZmLOX3* implies that plant 9-LOX derived oxylipins are involved in cross-talk. Further support for the role of 9-LOX-derived oxylipins in regulating fungal sporulation and mycotoxin production is provided by studies using a maize mutant in which the function of *ZmLOX3* is abolished. The *ZmLOX3* knock-out mutant displays reduced conidiation and

mycotoxin accumulation upon infection of kernels by *Fusarium verticillioides*. Reduced disease severity and conidiation was also observed in response to leaf and stalk infection by *F. verticillioides*, *Colletotrichum gramincola* and *Cochlibolus heterstrophus* (Gao et al., 2007). However, the same knock-out mutant is susceptible to infection by the seed pathogens, *A. falvus* and *A. nidulans* (Gao et al., 2009) suggesting that the oxylipin-mediated cross-talk between the host and the pathogen is pathogen specific.

1.3. Concluding Remarks

The role of oxylipins in plant development and response to biotic and abiotic stresses appear to be as diverse as the oxylipins themselves. Oxylipins not only directly affect pathogens and pests but are also involved in defense signaling both within the plant and between the affected organisms and the surrounding plants. Although, the biosynthesis and signaling functions of 13-LOX derived oxylipins such as the plant hormone JA are well studied, the importance of other oxylipins such as those derived from 9-LOXs is not well understood. Toward this goal, I have utilized genetic, molecular and biochemical approaches to better understand the role of the 9-LOX pathway in plant response to biotic stresses that include a phloem-feeder, a fungal pathogen and in systemic acquired resistance during infection by an avirulent pathogen. Chapter 2 presents the role of 9-LOX derived oxylipins as susceptibility factors in plant interaction with green peach aphid. I provide genetic and biochemical evidence that indicates that roots are the source of 9-LOX-derived oxylipins that facilitate shoot colonization of *Arabidopsis* by GPA by promoting insect feeding from sieve elements and water consumption from xylem. Chapter 3 attempts to elucidate the role of 9-LOX pathway in systemic acquired resistance. The evidence presented suggests that 9-LOXs are required for the generation

and perception of the SAR signal. Chapter 4 highlights an additional role for 9-LOXs during *Arabidopsis* interaction with a fungal pathogen that normally infects wheat spikes, *Fusarium graminearum* Schwabe (*Fg*). The data presented show that *Fg* is able to colonize and grow in *Arabidopsis* leaves and further that 9-LOXs contribute to host susceptibility to *Fg* infection by suppressing SA-mediated signaling. As an applied aspect, putative wheat LOXs have been cloned and RNAi-expressing transgenic wheat to silence/reduce the expression of wheat LOXs in order to enhance resistance to FHB.

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CHAPTER 2

Arabidopsis LOX5 ACTIVITY IN ROOTS PROMOTES APHID PERFORMANCE ON FOLIAGE*

2.1 Abstract

The influence of root-derived metabolites and signal molecules on interaction of the above ground plant organs with pathogen and insects is an emerging theme. The results presented here show that roots are a source of oxylipins that assist infestation of *Arabidopsis thaliana* shoots by green peach aphid (GPA, *Myzus persicae* Sülzer), an agronomically important phloem sap-consuming insect pest. GPA population on mutants lacking the *LOX5*-encoded 9-lipoxygenase was smaller compared to wild type *Arabidopsis*. Infestation by GPA resulted in the failure to accumulate a GPA fecundity promoting activity in the *lox5* mutants. GPA also had difficulty feeding from sieve elements and tapping into the xylem of *lox5* mutant, thus resulting in reduced water content in the insect. The expression of *LOX5* was rapidly induced in the roots of GPA-infested plants and associated with an increase in levels of LOX5-synthesized oxylipins in roots and petiole exudates of GPA-colonized plants. Application of the LOX5-synthesized 9-hydroxyoctadecadienoic acid (9-HOD) to roots restored water content and population size of GPA on the *lox5* mutant, thus confirming that a LOX5-synthesized oxylipin or product thereof, promotes insect infestation in foliage. The influence of *LOX5* genotype in roots of *Arabidopsis* on GPA performance on the shoots was confirmed in experiments involving micrografting. These results highlight the importance of root-derived factors in plant interaction with GPA colonizing the foliage.

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2.2 Introduction

2.2.1 Aphids

The members of the superfamily Aphidoidea comprising of more than 4400 species are small soft bodied insects, all of whom are phloem-feeders (Blackman and Eastop, 2000). Of these, around 250 species are among the most destructive pests on cultivated plants in temperate regions (Dixon, 1998; Blackman and Eastop, 2000; Dedryver et al., 2010) especially when their attack is associated with the transmission of phytopathogenic viruses. 'Aphid' is the common name given to insects that belong to the superfamily Aphidoidea, within the order Hemiptera and suborder Sternorrhyncha. Their success as phytophagous insects can be attributed in part to their ability to attack and feed from virtually every plant organ, either above or below the ground, including the bark. The wingless morphs of aphids attain high population densities on host plants due to a shortened pre-reproductive time (Braendle et al., 2006). The ability of the wingless morphs to reproduce asexually via parthenogenesis contributes to the doubling in the rate of population growth. With the declining nutritional quality of the host plant, aphids are able to invest in the production of winged morphs that are less prolific but are able to disperse and colonize new host plants (Powell et al., 2006).

Aphid feeding impacts plant productivity in numerous ways. As phloem feeders, they consume profuse amounts of photoassimilates necessary for plant growth and also alter source-sink patterns (Blackman and Eastop, 2000; Goggin, 2007; Giordanengo et al., 2010).During feeding, they inject salivary proteins that may function as effectors that manipulate host cellular processes to improve palatibility of the host by the insect (Bos et al., 2010). Nearly 275 out of the 600 insect transmitted phytopathogenic viruses are vectored and transmitted by aphids with a devasting impact on plant productivity (Brault et al., 2010). The sugar-rich 'honey dew', which

is expelled in copious amounts by aphids, enable growth of filamentous saprophytic fungi (sooty moulds) that hinder photsynthetic acitivity (Dedryver et al., 2010), thus further impacting host productivity.

Depending on the plants they infest, aphids are classified as generalists, that feed on many species, and as specialists, that feed on a set of closely related species. For example, the host range of *Brevicoryne brassicae* (cabbage aphid) and *Lipaphis erysimi* (mustard aphid) is limited to related cruciferous species (Blackman and Eastop, 2000). By contrast, a generalist aphid like *Myzus persicae* Sülzer, more commonly known as the green peach aphid (GPA), has a wide host range that exceeds 50 plant families and is thus considered polyphagous (Blackman and Eastop, 2000). It has been suggested that generalist aphids use nutritional cues to make their host selections (Powell et al., 2006), while specialist aphids utilize plant secondary metabolites as cues for host recognition, feeding and oviposition (Raybould and Moyes, 2001; Macel and Vrieling, 2003).

2.2.2 Aphid Saliva

Aphids obtain nutrients from the phloem of the host plant. Their mouthparts are modified into narrow, slender and flexible piercing-sucking structures called stylets. After landing on plants, the stylets are inserted into the leaf tissue. The first insertions that occur, last for less than one minute and seem to provide enough information regarding the suitability of the plant as a host (Powell et al., 2006). Once a suitable host is found, the aphid stylet is inserted between epidermal cells and the mouthparts follow an intercellular path between the primary and secondary cell walls (Tjallingii and Esch, 1993). During the penetration process, aphids continuously secrete gelling saliva that contains proteins, phospholipids and conjugated

carbohydrates (Miles, 1999; Cherqui and Tjallingii, 2000). This gelling saliva lubricates and hardens around the stylet, forming a sheath that is left behind after stylet withdrawal (Tjallingii and Esch, 1993). Occasionally the stylets transiently puncture epidermal, mesophyll and parenchyma cells to sample cell contents (Pollard, 1973; Tjallingii and Esch, 1993). During this process, a small amount of 'watery' saliva is secreted and a mixture of saliva/cytosol is ingested (Tjallingii and Esch, 1993; Martin et al., 1997; Tjallingii, 2006). These intracellular probes are suggested to provide additional information regarding the quality of the host plant and also the location of the stylet within the plant tissue. Once the sieve element is reached, the 'watery' saliva is injected periodically during the whole phloem-feeding period (Prado and Tjallingii, 2007), and likely aids in the reversal of phloem occlusion and suppression of plant defenses (Will et al., 2007; Will et al., 2009).

The composition of the watery saliva varies between species and contains a more complex mixture of hydrolytic enzymes (Cherqui and Tjallingii, 2000; Will et al., 2009). The watery saliva also contains proteins/peptides that can either elicit (De Vos and Jander, 2009) and/or suppress host plant defense responses (Cherqui and Tjallingii, 2000; Will et al., 2007). The wide variation in the composition of the watery saliva between different aphid species is thought to be a factor that determines the host-plant range of an aphid (Carolan et al., 2009).

Once the sieve element is located, aphids feed for prolonged periods on the phloem sap (Tjallingii, 1988; Dixon, 1998). This is necessitated due to the fact that the plant phloem sap although consisting of high concentrations of sugars contains low concentrations of the amino acids histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine that are essential for aphid growth; (Wilkinson and Douglas, 2003; Douglas, 2006). The combined concentration of essential amino acids in the phloem sap of *Vicia faba* (broad bean)

plants represents only 8.2% of the total amino acid content (Douglas, 2006).The ability of aphids to synthesize essential amino acids and lipids from the sugar rich phloem sap they consume is dependent entirely on the bacterial endosymbiont, *Buchnera* sp., present in the cytoplasm of 60 to 70 huge specialized cells in the aphid called the bacteriocytes (Febvay et al., 1999). Sequencing of the genome of *Buchnera* sp. APS, revealed that the endosymbiont contains genes encoding proteins involved in the synthesis of amino acids that are essential for the aphid (Shigenobu et al., 2000).

2.2.3 Aphid Adaptations to Feeding on a High Osmolarity Diet

Aphids have developed adaptations to overcome the problems posed by the consumption of phloem sap, which is a sugar-rich and thus a diet with high osmolarity. The osmolarity of the phloem sap is considerably higher than that of the aphid haemolymph (Douglas, 2003). If not controlled, the resultant osmotic potential gradient between the aphid gut and the hemolymph could result in the transfer of water from body fluids to the gut and thus result in dehydration of the aphid (Ashford et al., 2000). The osmotic potential of the gut contents is thought to be regulated by two sucrose-related physiological processes. First, sucrose is hydrolyzed by α-glucosidase to yield fructose and glucose. The fructose is then assimilated by the aphid, whereas a second enzyme, transglycosidase polymerizes the glucose into oligosaccharides that is expelled in the honeydew (Ashford et al., 2000; Cristofoletti et al., 2003; Karley et al., 2005).

The observation that non-dehydrated aphids periodically also ingest xylem sap has led to the proposition of water consumption being another mechanism utilized by aphids to regulate the osmotic potential of the gut (Spiller et al., 1990; Tjallingii and Esch, 1993; Powell and Hardie, 2002; Douglas, 2006). The xylem sap has lower osmotic potential than the phloem sap and is

nutritionally poorer than phloem sap containing only a few salts and amino acids (Malone et al., 1999). The poor nutritional quality of the xylem sap is thought to be unlikely to make up for the energy involved in actively sucking xylem sap (Malone et al., 1999).Therefore, xylem sap ingestion is thought to be considered as 'drinking' rather than as feeding. On wheat plants treated with a neonicotinoid insecticide, thiamethoxam, xylem sap ingestion was disrupted in the bird cherry-oat aphid (*Rhopalosiphum padi*). This resulted in a significant reduction in the water content in the aphid and size of the insect population (Daniels et al., 2009). Furthermore, the duration of xylem consumption by the potato aphid (*Macrosiphum euphorbiae*, Thomas) is higher when the aphids are either artificially starved or pre-fed on artificial diets with a high sucrose concentrations (Pompon et al., 2009; Pompon et al., 2011). These observations further support the hypothesis that xylem sap consumption contributes to osmoregulation in phloem-sap feeding insects.

2.2.4 Plant Defense Against Aphids

Plants have evolved various means of protecting themselves against attacks by aphids. All plants possess certain characteristics/defenses that make them non-hosts for a particular aphid. Relatively little is known about plant characteristics that make them unsuitable as hosts for some aphids but not others (Goggin, 2007). In general, plant defense against aphids can be classified as antibiosis, antixenosis and tolerance. Antixenosis impacts insect behavior that deters aphid infestation, and antibiosis impacts insect physiology resulting in adverse impacts on insect growth, development and/or reproduction (Smith, 2005). The ability of the infested plant to be able to produce biomass that is comparable to an un-infested plant is termed tolerance (Reese et al., 1994).

Plant defense against aphids include constitutive defenses that include physical and chemical barriers that exist prior to insect attack, and inducible defenses that are activated upon perception of aphid attack (Chen, 2008). Among the first plant features that aphids encounter is the presence of trichomes. A high density of trichomes, either simple or glandular, on the plant surface can prevent infestation (Musetti and Neal, 1997). Trichomes not only hamper aphid movement and stylet insertion but glandular trichomes produce toxins that can repel or even kill aphids (Avé et al., 1987; Goffreda et al., 1989; Tissier, 2012). Constitutive defenses can also be chemical in nature. Many plants naturally produce secondary metabolites that are involved in direct or indirect defenses (Goggin, 2007; Chen, 2008). For example, sulfur-containing glucosinolates produced by *Brassicaceae* family plants are toxic to aphids (Mewis et al., 2005; Mewis et al., 2006; Kim and Jander, 2007). However, a few species of aphids have adapted to thrive on *Brassicaceae*. For example, the cabbage aphid, *Brevicoryne brassicae*, utilizes these compounds as chemical cues in host identification and has evolved detoxification mechanisms to break down glucosinolates that enable then to colonize cabbage plants that are normally toxic to other generalist herbivores (Gols et al., 2008). Tomato and potato, members of *Solanaceae*, contain glycosidic alkaloids that make them unpalatable to many aphids. Another example is nicotine produced by *Nicotiana* spp, which renders the plants unpalatable to generalist aphids. Nicotine accumulates in the roots from where it is translocated via the xylem to the shoots of aphid-infested plants (Guerrieri and Digilio, 2008). In addition to secondary metabolites, other major defensive chemicals that are produced by plants that limit aphid survival include several plant proteins, lectins, inhibitors of insect digestive enzymes, amino acid deaminases and polyphenol oxidases (Goggin, 2007; Chen, 2008)

Aphid feeding induces changes in the plant transcriptome and numerous microarray studies with several plant species have helped identify mechanisms that putatively contribute to defenses against aphids (Moran et al., 2002; Voelckel et al., 2004; Zhu-Salzman et al., 2004; Park et al., 2006; Thompson and Goggin, 2006; Smith and Boyko, 2007; Smith et al., 2010). Genes involved in oxidative burst including ROS production, carbohydrate metabolism, amino acid metabolism, cell wall metabolism and remodeling, and photosynthesis are upregulated in plants that are resistant to aphid infestation (Smith and Boyko, 2007). In addition, aphid feeding induces transcripts associated with signaling pathways controlled by plant hormones involved in defense such as jasmonic acid (JA), salicylic acid (SA), ethylene (ET), abscisic acid (ABA) and gibberellic acid (GA) (Moran and Thompson, 2001; Moran et al., 2002; Thompson and Goggin, 2006; Goggin, 2007; Smith and Boyko, 2007; Anstead et al., 2010). The role of JA, SA and ET in plant defense against aphids varies in different plant species and appears to depend on the aphid species.

In various plant species, aphid feeding induces the expression of genes associated with SA signaling and suppression of transcripts associated with JA signaling (Moran and Thompson, 2001; Moran et al., 2002; Zhu-Salzman et al., 2004; Pegadaraju, 2005). Exogenous application of the SA analog, benza-thiadiazole-7-carbonic acid (BTH) and exposure to methyl jasmonic acid (MJ) vapors reduced aphid populations on susceptible genotypes in tomato and in *Arabidopsis* (Moran and Thompson, 2001; Cooper et al., 2004; Zhu-Salzman et al., 2004). However, aphid bioassays on *Arabidopsis* SA and JA biosynthetic and insensitive mutants suggested that SA-mediated defenses have either a positive or neutral role, whereas JA restricts the size of aphid populations (Ellis et al., 2002; Pegadaraju, 2005; Mewis et al., 2006). Given the antagonistic interaction between the SA and JA signaling pathways in plant defense against

pathogens (Kunkel and Brooks, 2002), several studies imply the possibility that aphids suppress resistance conferred by the JA signaling pathway by inducing SA signaling (Moran and Thompson, 2001; Zhu-Salzman et al., 2004; Gao et al., 2007a). The role of ET in defense response to aphid is relatively less well understood. In a few cases, it has been shown that the ET signaling pathway and downstream genes are highly induced in resistant plants after aphid infestation (Moran and Thompson, 2001; Anstead et al., 2010). Although, the plant defense hormones, SA, JA and ET are involved in plant-aphid interactions, further work will be needed to determine their contribution to infestation by different aphids.

2.2.5 Oxylipins in Plant-Stress Response

Oxylipins, which encompass a large family of oxidized fatty acids, play pivotal roles as signaling molecules and protective compounds in plant response to biotic stress, including defense against insects (Blée, 2002; Prost et al., 2005). Oxylipins are also implicated in cross-kingdom communication between plants and pathogenic fungi (Christensen and Kolomiets, 2011). The role of oxylipins in plant defense against aphids has largely focused on jasmonates that are derived by the action of 13-LOXs. However, aphid attack also activates the production of 9-LOX derived oxylipins (Gosset et al., 2009). These oxylipins are present in the phloem sap of infested plants and are also ingested by aphids (Harmel et al., 2007). The role of these oxylipins is plant-aphid interaction is not clearly understood. The first step in the synthesis of oxylipins involves the formation of fatty acid hydroperoxides either by autooxidation, or by the action of enzymes like lipoxygenases (LOXs) and α-dioxygenases (Feussner and Wasternack, 2002; Mosblech et al., 2009). Spontaneous or enzymatic modification of fatty acid hydroperoxides yields an additional array of oxylipins (Feussner and Wasternack, 2002;

Mosblech et al., 2009). The *Arabidopsis thaliana* genome contains six *LOX* genes, which encode proteins that are classified as 9- and 13-LOXs based on their ability to incorporate oxygen either at the C-9 or C-13 position of the fatty acid, yielding the 9- or 13-fatty acid hydroperoxides, respectively (Liavonchanka and Feussner, 2006). *Arabidopsis LOX1* and *LOX5* encode 9-LOXs, whereas *LOX2*, *LOX3*, *LOX4* and *LOX6* encode 13-LOXs (Bannenberg et al., 2009). 9-LOX-derived oxylipins are also involved in plant growth and development, and in the stress response. For example, 9-LOX-derived oxylipins are involved in lateral root development in *Arabidopsis* and maize (*Zea mays*) (Vellosillo et al., 2007; Gao et al., 2008), and in defense against bacterial pathogens in pepper (*Capsicum annuum*) and *Arabidopsis* (Vellosillo et al., 2007; Hwang and Hwang, 2010; López et al., 2011), and against fungal pathogens and nematodes in maize (Gao et al., 2007b; Gao et al., 2008; Gao et al., 2009).

The co-evolution of plants and their insect pests has resulted in the development of complex interactions between them. Whereas plants have evolved a variety of defense and tolerance mechanisms against insect herbivory (Howe and Jander, 2008), insects have evolved countering strategies to bypass and/or overcome the barriers employed by plants (Hogenhout and Bos, 2011). Much of our current understanding of molecular mechanisms that influence plant interaction with insects arises from studies dealing with response of the above ground shoot foliage to insect attack (Howe and Jander, 2008). However, although less well studied, physiological changes in the roots also impact the severity of infestation in the shoots (Erb et al., 2009). For example, a variety of secondary metabolites (e.g. nicotine) that are involved in protecting above ground tissues are synthesized in roots from where they are transported to shoots in response to insect infestation (Baldwin et al., 1994; Erb et al., 2009; Morita et al., 2009). Here, the evidence provided shows that in *Arabidopsis* the *LOX5*-encoded 9-LOX-

derived oxylipin(s) functions as a susceptibility factor in plant interaction with GPA. *LOX5* facilitates insect feeding from sieve elements and water consumption from xylem. Genetic and biochemical evidence provided here demonstrates that roots are the source of a *LOX5*-derived oxylipin(s) that facilitates colonization of *Arabidopsis* shoots by GPAs, thus highlighting the dependence of GPAs on root-derived oxylipins for colonizing *Arabidopsis*.

2.3 Results

2.3.1 *LOX5* Predisposes *Arabidopsis* to Infestation by GPA

Although 9-LOX derived oxylipins accumulate in phloem sap and can also be recovered from aphids (Harmel et al., 2007), their direct role in plant-aphid interaction has not been addressed. *Arabidopsis* contains two 9-LOX-encoding genes, *LOX1* and *LOX5* (Bannenberg et al., 2009). While LOX1 is localized to the plastids, LOX5 exhibits extraplastidic localization (Figure 2.1). To determine if either of these genes have a role in plant aphid interaction, a no-choice bioassay was conducted in which 20 adult apterous GPA were released on each wild-type (WT) *Arabidopsis* accession Columbia-0 plant and on mutant plants that lack *LOX1* or *LOX5* functions. Two days post infestation, the size of the insect population on each plant was determined. As shown in Figure 2.2A, GPA population size on the *Arabidopsis lox5-1* and *lox5-3* mutants, which contain T-DNA insertions within the *LOX5* coding sequence (Figure 2.3 A-C), was significantly smaller than on wild-type (WT) plants. Ectopic expression of *LOX5* or a *LOX5-GFP* fusion from the *Cauliflower mosaic virus 35S* promoter attenuated the *lox5-1*-conferred enhanced resistance to GPA (Figure 2.2B), thus confirming that a *LOX5*-dependent activity is a susceptibility factor that predisposes *Arabidopsis* to infestation by GPA. By comparison to the *lox5* mutants, insect numbers on *lox1-1* and *lox1-3* were higher and comparable to that on the

WT (Figure 2.2A). Furthermore, in comparison to the WT and *lox1-1* single mutant, insect numbers were comparably low on the *lox1-1 lox5-1* double mutant and the *lox5-1* single mutant plant (Figure 2.2B), thus indicating that unlike *LOX5*, *LOX1* does not have a discernible role in *Arabidopsis*-GPA interaction. This difference in the participation of *LOX1* and *LOX5* in plant-GPA interaction may in part be attributable to the differences in their sub-cellular localization.

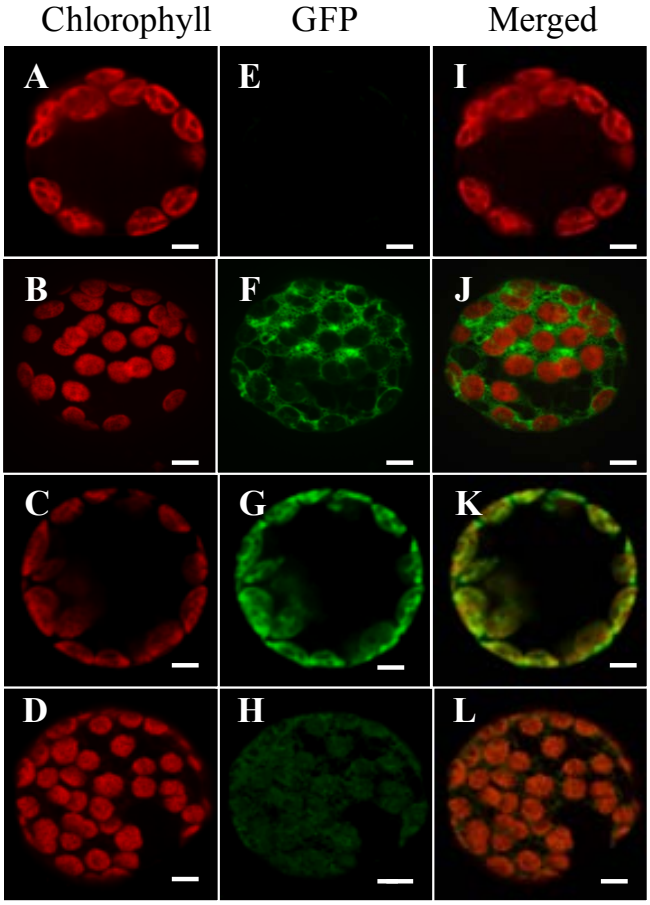

Figure 2.1. LOX1 and LOX5 are localized to different sub-cellular compartments. Confocal laser scanning images of representative protoplasts showing chlorophyll autofluorescence (left panel, A to D), GFP fluorescence (middle panel, E to H), and merged images of chlorophyll autofluorescence and GFP fluorescence (right panel, I to L). GFP fluorescence is compared in protoplasts isolated from WT (A, E, I), 35S:GFP (B, F, J), 35S:LOX1-GFP (C, G, K) and 35S:LOX5-GFP (D, H, L). Bar=5 μm

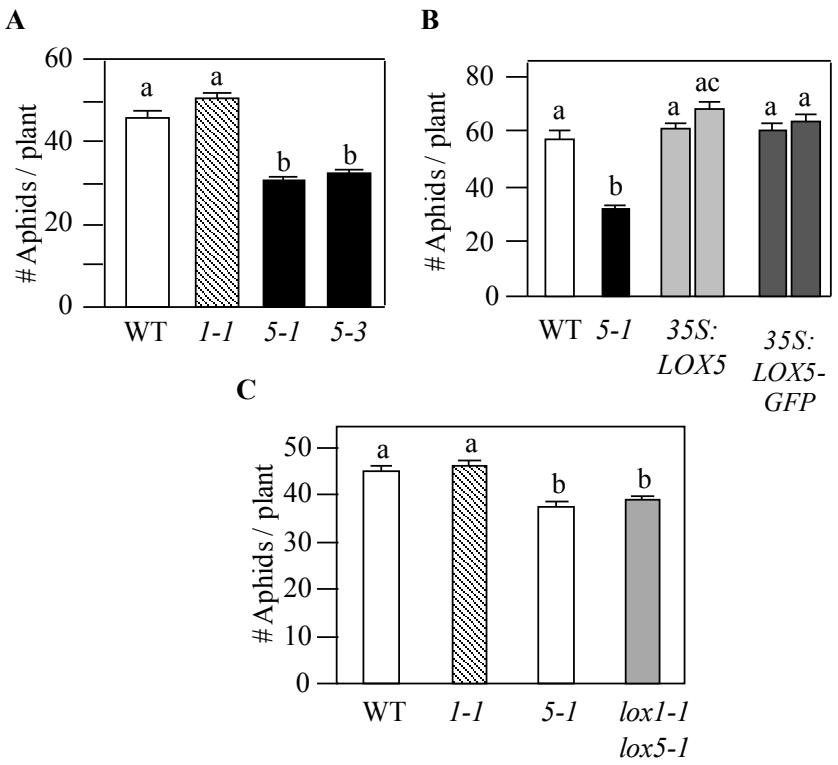

Figure 2.2. *Arabidopsis lox5* mutants are predisposed to GPA infestation. **A.** No-Choice test. Comparison of total numbers of GPAs (adults + nymphs) on WT and 9-LOX mutants, *lox1-1*, *lox1-3*, *lox5-1* and *lox5-3*. **B.** No-Choice test. Comparison of total number of GPAs on WT, *lox5-1*, *lox5-3* and two independent *35S:LOX5* and *35S:LOX5-GFP* transgenic lines each in the *lox5-1* and *lox5-3* backgrounds. **C.** No-Choice test: Comparison of GPA numbers on the *lox1-1* and *lox5-1* single mutants and the *lox1-1 lox5-1* double mutant. In **A** to **C**, all values are means from 12 plants $\pm$ standard error (SEM). Different letters above the bars indicate values that are significantly different ($P < 0.05$) from each other by GLM/ANOVA.

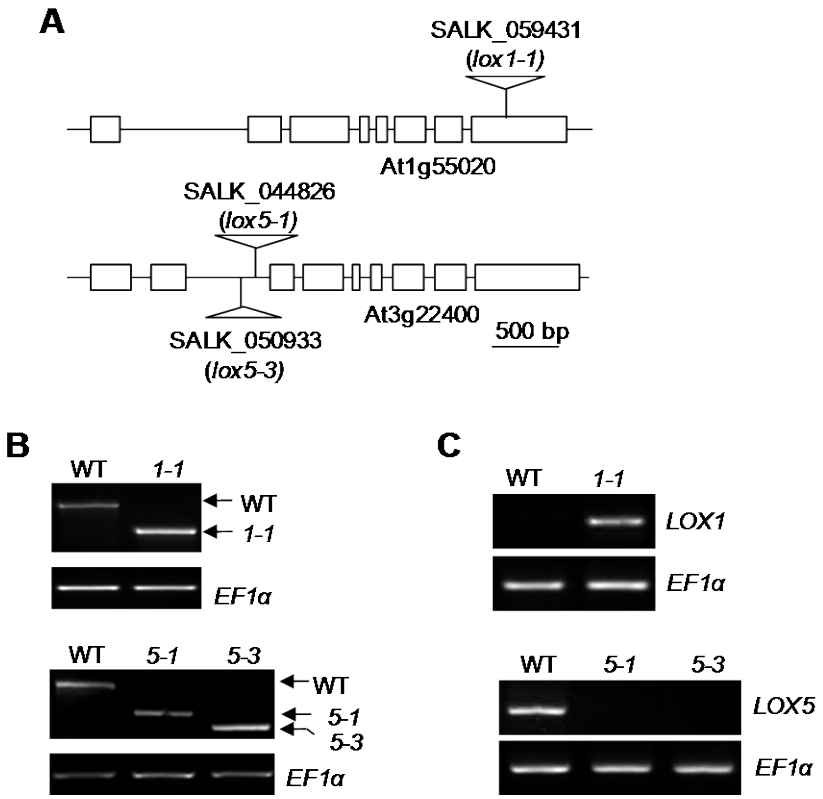

Figure 2.3. Characterization of 9-LOX mutants of *Arabidopsis*. **A.** Diagrammatic representation of the genomic structures of *LOX1* and *LOX5* indicating the location of T-DNA insertions in SALK_059431 (*lox1-1*), SALK_044826 (*lox5-1*) and SALK_050933 (*lox5-3*) alleles. Exons are represented as open boxes. **B.** Genomic PCR analysis from DNA collected from leaves of WT, *lox1-1* (*1-1*), *lox5-1* (*5-1*) and *lox5-3* (*5-3*) to verify the presence of the T-DNA insertions in the respective lines. Gene-specific primers that were used along with the left border primer (LBb1.3) are shown in Table 2.2. The *EF1α* gene provided a control for PCR. **C.** RT-PCR analysis of RNA collected from roots of WT, *lox1-1* (*1-1*), *lox5-1* (*5-1*) and *lox5-3* (*5-3*) to verify loss of expression of T-DNA insertion lines. Gene-specific primers for *LOX1* and *LOX5* used are as listed in Table 2.2. Expression of the *EF1α* gene served as a control for RT-PCR.

2.3.2 GPA-infested *lox5* Lacks a Fecundity Promoting Activity in Vascular Sap

In *Arabidopsis,* antixenosis (deter insect behavior) and antibiosis (adversely impact insect growth, development and/or reproduction) mechanisms limit GPA infestation (Pegadaraju et al., 2005; Louis et al., 2010a; Louis et al., 2010b). Adult GPA were provided with the choice of settling on either WT or *lox5-1* mutant to determine if *LOX5* contributes to antixenosis. The

number of insects settling on WT and *lox5-1* were comparable over a 48 h period (Figure 2.4A) indicating that lack of *LOX5* did not deter insects from settling on *lox5-1* mutants.

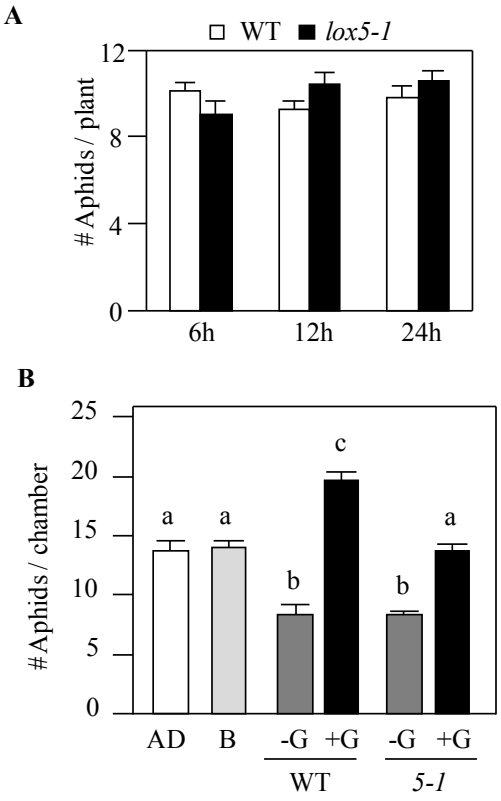

Figure 2.4. *LOX5* is required for fecundity promoting activity in vascular sap. Choice Assay. GPA was given the choice between WT and the *lox5-1* mutant plants. Twenty aphids were released equidistant from a WT plant and a *lox5-1* mutant plant, growing in the same pot. The number of adult GPA that had settled on each plant was determined at 6, 12 and 24 h later. Values are means from 8 plants ± standard error (SEM). **B.** Artificial diet assay. Comparison of GPA numbers on petiole exudates (Pet-ex) collected from un-infested (-G) and GPA-infested (+G) WT and *lox5-1* plants. Artificial diet alone (AD) and diet supplemented with buffer (B) used to collect Pet-ex provided the controls. Values are means (± SEM) of six replicates for each treatment. In A and B, different alphabets above the bars indicate values that are significantly different from each other ($P < 0.05$; GLM/ANOVA).

Although, an antibiotic activity that limits GPA fecundity on artificial diet is present in vascular sap enriched petiole exudates of un-infested *Arabidopsis* (Pegadaraju et al., 2005; Louis et al., 2010a; Louis et al., 2010b), petiole exudates from leaves of GPA-infested WT plants promoted insect fecundity on an artificial diet (Figure 2.4B), thus suggesting that GPA infestation

manipulates host physiology to make vascular sap more suitable for consumption. Although *LOX5* did not impact the accumulation of the antibiotic activity in petiole-exudates from un-infested leaves (Figure 2.4B), petiole exudates collected from GPA-infested leaves of *lox5-1* was less effective in promoting GPA fecundity than comparable petiole exudates from WT leaves (Figure 2.4B). Taken together, the data suggests that *LOX5* is required for the GPA-infestation associated alterations in host physiology that make the vascular sap more conducive to the insect.

2.3.3 GPA Feeding Behavior is Altered on the *Arabidopsis lox5* Mutant

Electrical penetration graph (EPG) analysis provides a sensitive technique to study insect feeding behavior on plants (Walker, 2000). EPG has been used to compare the impact of WT and mutant *Arabidopsis* genotypes on GPA feeding behavior (Pegadaraju et al., 2007; Louis et al., 2010a; Louis et al., 2010b; Singh et al., 2011). In EPG a wired insect that is part of a low voltage circuit is released on a plant and its activity electrically monitored. The pattern of different waveforms generated provide insights into the time spent by insect in different activities, including the non-probing phase (NP) when the insect stylet is not inserted into the plant, the pathway phase (PP) when the insect stylet although inserted in the plant tissue is outside the phloem and xylem, the xylem phase (XP) when the insect is drinking from the xylem, and the sieve-element phase (SEP) when the stylet is in the sieve element. Comparisons of GPA feeding behavior on the WT and *lox5-1* mutant plants uncovered differences in the time spent by GPA in SEP on these genotypes. As shown in Figure 2.5A and Table 2.1, GPAs spent less time in SEP on *lox5-1* than the WT plant.

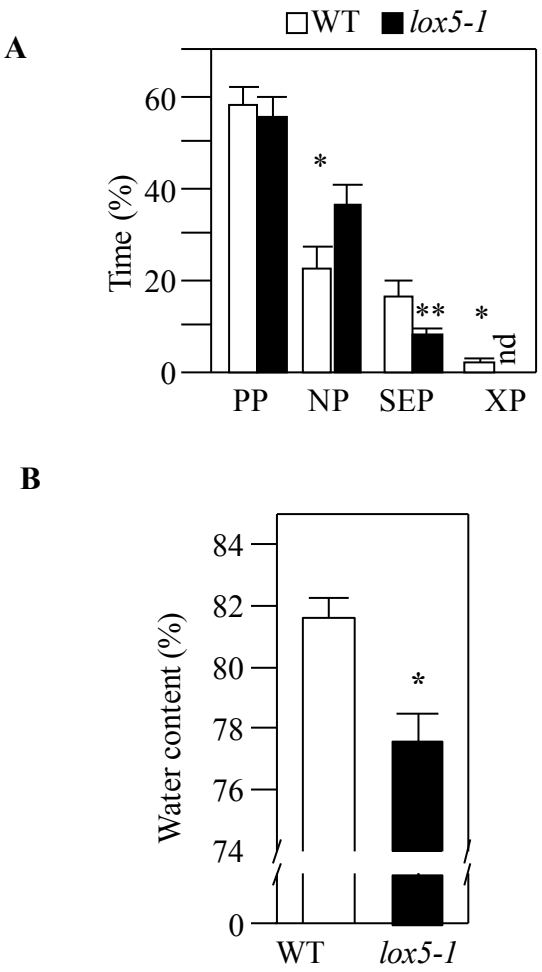

Figure 2.5. GPA is unable to tap into xylem and spends less time in sieve elements of the *Arabidopsis lox5* mutant. **A.** Electrical penetration graph analysis of GPA feeding behavior. Percentage times spent by GPA on various activities on WT and *lox5-1* mutant are shown. Each value is the mean (± SEM) of 16 replications. Nonparametric Kruskal-Wallis test was used to determine significant differences. Significant differences in time spent by GPA in individual activities on WT versus *lox5-1* mutant are indicated by asterisks (*, $P < 0.05$; **, $P < 0.1$). Abbreviations: PP, Pathway phase; NP, Non-probing phase; SEP, Sieve element phase; XP, Xylem phase. **B.** Percentage body water content in GPA reared on WT and *lox5-1* mutant plants. Insects were starved for 12 h prior to release of twenty adult GPA on each WT and *lox5-1*. 12 h later, 30-40 insects from two plants were collected and pooled. The fresh weight and dry weight of the pooled insects were measured to calculate the % water content. Each bar represents the mean of % water content ± SEM ($n = 6$). Asterisk indicates significant difference from WT ($P<0.05$; *t*-test).

Table 2 1 Mean time (h) ± standard error (SEM) spent by GPAs for various activities on *Arabidopsis* wild-type (WT) and 9-LOX mutants, *lox5-1* plants in 8h of recording

Parameters	WT	*lox5-1*	*P*-values
Time spent in non-probing phase (NP)[a]	1.89 ± 0.39	3.08 ± 0.44	0.055*
Time spent in pathway phase (PP)[a]	4.63 ± 0.44	4.26 ± 0.48	0.678
Time spent in sieve element phase (s-SEP)[a]	1.29 ± 0.29	0.66 ± 0.12	0.056*
Time spent in xylem phase (XP)[a]	0.19 ± 0.06	nd	
Time to first probe [a]	0.19 + 0.05	0.16 ± 0.04	0.910
Time to first sieve element phase (f-SEP) [a]	2.78 ± 0.52	1.93 ± 0.38	0.122
Time to first xylem phase [a]	4.58 ± 0.84	nd	

EPG Recording was conducted on 16 aphids on plants of each genotype. Recording with each insect was conducted for 8 hours. [a]Each value in hours is the average ± SEM of 16 replications. Parameters that are significantly different (Kruskal-Wallis test) between GPAs reared on WT versus *lox5-1* mutant are indicated by asterisks. nd, none detected

GPAs also had difficulty tapping into the xylem of *lox5-1* compared to the WT plant. No XP was observed in more than 100 h of EPG recording involving 16 insects on the *lox5-1* mutant plant (Table 2.1). The reduced time spent by GPA in SEP and XP on the *lox5-1* mutant was compensated by an increase in NP on *lox5-1* than the WT plant. These results suggest that a product of LOX5-activity facilitates GPA feeding from sieve elements and water consumption from xylem.

To determine if the absence of XP in GPAs reared on *lox5-1* impacts their hydration status, water content was evaluated in GPAs collected from WT and *lox5-1* plants.
As shown in Figure 2.5B, mean body water content of GPA reared on WT plants was significantly higher than on GPAs collected from *lox5-1*, thus confirming that inability of GPA to tap into the xylem is associated with lower water content. This data suggests that a LOX5-synthesized oxylipin(s) or derived product(s) facilitates GPAs' ability to tap into xylem and phloem, and simultaneously promote insect fecundity.

2.3.4 LOX5-Activity in Roots Promotes GPA Performance on Leaves

The expression of *LOX5* is low in *Arabidopsis* leaves as indicated by a survey of publicly available microarray data sets (http://www.genevestigator.com/) and qRT-PCR analysis (Figure 2.6). *LOX5* expression was not significantly altered in GPA-infested leaves (Figure 2.6). Considering that GPA is unable to tap into the xylem of the *lox5-1* and that the general flow of the xylem contents is from roots to shoots, I hypothesized that *LOX5* function might be required in roots of GPA-infested plants. Indeed, *LOX5* expression was rapidly induced in the roots within 3 h of release of GPA on the leaves and sustained at elevated levels through the 48 h duration of the experiment (Figure 2.7A).

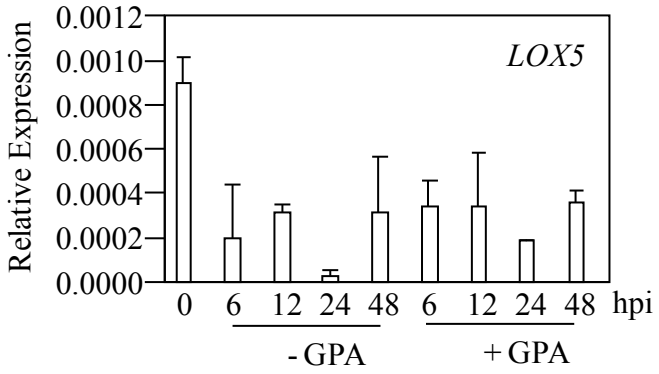

Figure 2.6. *LOX5* expression if leaves of GPA infested *Arabidopsis.* qRT-PCR analysis of *LOX5* expression in leaves of un-infested (-GPA) and GPA-infested (+GPA) wild-type plants. *LOX5* transcript levels have been normalized to the expression of *EF1α*. Each bar represents the mean (±SD) (*n* =3). *LOX5* expression in leaves of the *lox5-1* mutant was undetectable.

To test whether *LOX5* activity in roots facilitates infestation of *Arabidopsis* shoots by GPA, reciprocal micrografting of seedlings was used to generate chimeric *LOX5*/*lox5* plants that contained WT *LOX5* scions (shoots) and *lox5* mutant rootstock, and *lox5*/*LOX5* chimera that contained *lox5* scion and *LOX5* rootstock. *LOX5*/*LOX5* and *lox5*/*lox5* self grafted plants were used as WT and *lox5* controls.

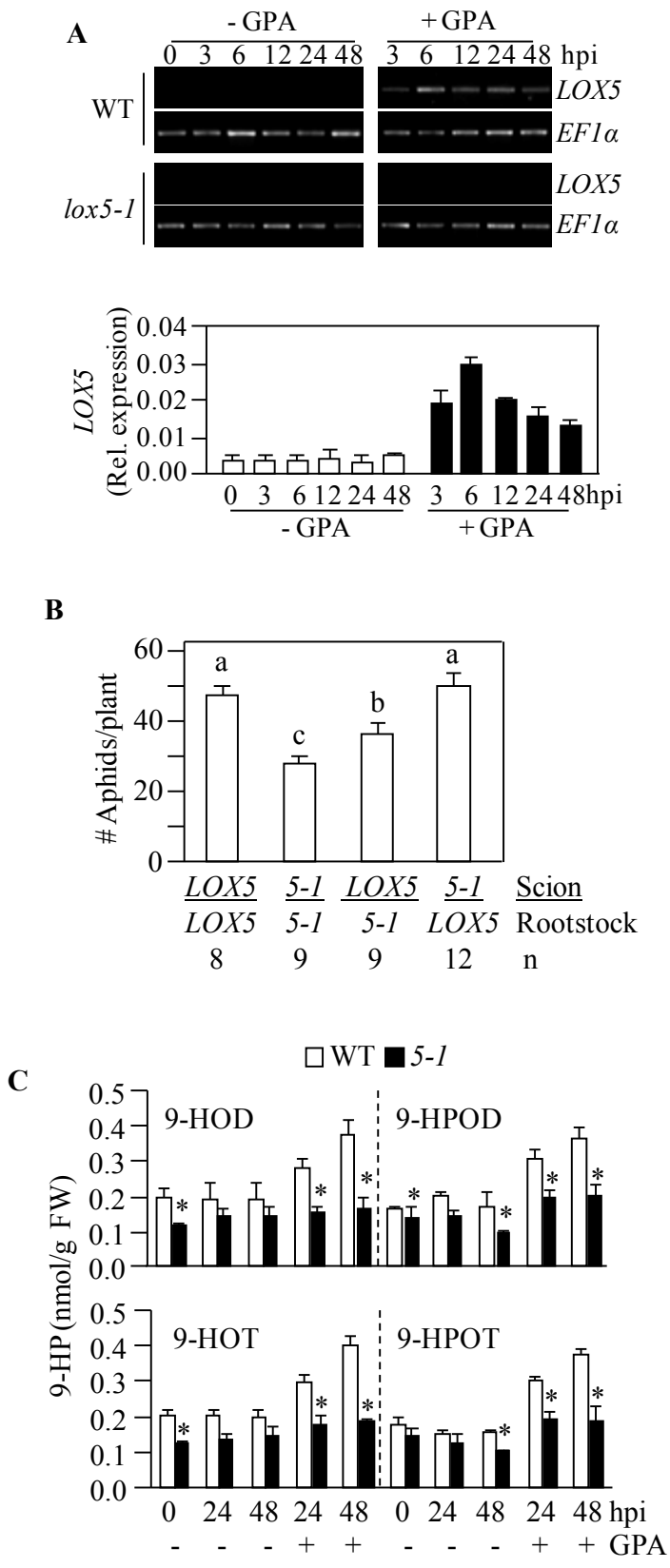

Figure 2.7. *LOX5* activity in roots promotes GPA infestation on shoots. **A.** *LOX5* expression in roots of un-infested (-GPA) and GPA-infested (+GPA) WT and *lox5-1* (*5-1*) plants. RT-PCR (upper panel) and qRT-PCR (lower panel) were performed on RNA from roots of un-infested and GPA-infested plants harvested at 0, 3, 6, 12, 24 and 48 hours post infestation (hpi). The *EF1α* gene provided the control for RT-PCR. For qRT-PCR, expression level of *LOX5* relative to expression level of *EF1α* at each time point is presented (n=3). **B.** *LOX5* function in roots facilitates GPA infestation on shoots. No-choice assay comparison of GPA numbers on self- and reciprocal-grafts between WT and *lox5-1* (*5-1*) plants. The genotype of the scion/rootstock (shoot/root) for each graft combination is indicated below the bars (Mean ± SEM). Different alphabets above the bars indicate values that are significantly different from each other ($P<0.05$; GLM/ANOVA). The numbers (n) of plants used for each graft combination are indicated below each bar. **C.** 9-HP concentrations in roots of un-infested and GPA-infested WT and *lox5-1* (*5-1*) plants. Root tissues were collected at 0, 24 and 48 h after GPA-infestation. Each bar represents the mean (± SEM) concentration of 9-HOD, 9-HPOD, 9-HOT and 9-HPOT (n=5) presented as nmol/g fresh weight (FW) of roots. Asterisk (*) indicates significant difference ($P<0.05$; *t*-test) from WT for that time.

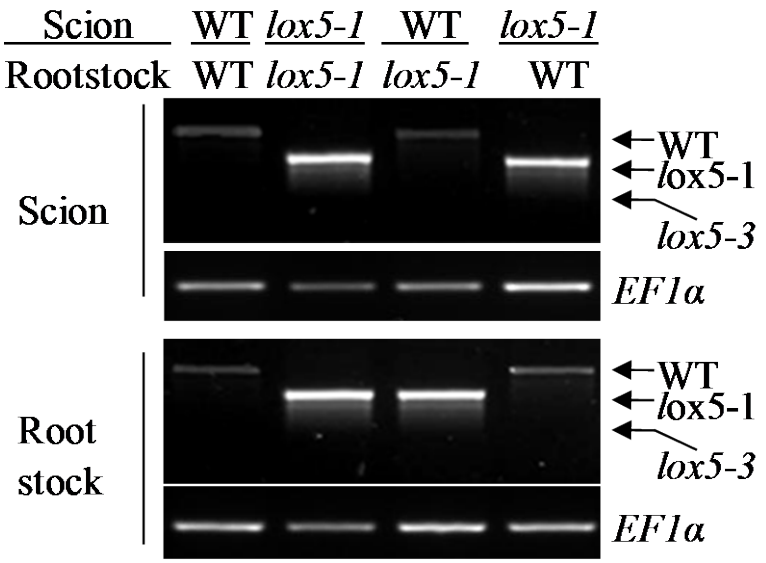

Figure 2.8. Verification of graft integrity. DNA was extracted from shoots (scions) and roots of different graft combinations. DNA from the individuals in each graft combination was pooled and used for genomic PCR analysis. The presence of T-DNA insertions was verified by multiplex PCR using gene-specific primers along with the T-DNA left border primer (LBb1.3). The *EF1α* gene provides a control for PCR.

Allele-specific PCR was used to confirm the genotype of the scion and rootstock (Figure 2.8). These plants were used in no-choice assays to determine the impact of root genotype at the *LOX5* locus on aphid performance in the shoots. It was anticipated that if *LOX5* function in the roots facilitates GPA infestation in the shoots than GPA population should be smaller on chimeric plants that have a *lox5* mutant rootstock compared to plants with a WT *LOX5* rootstock. GPA population on the control *lox5*/*lox5* self-graft was as expected smaller than on the *LOX5*/*LOX5* plant (Figure 2.7B). GPA population was also significantly smaller on the *LOX5*/*lox5* chimera, which contains a WT *LOX5* scion and a *lox5* mutant rootstock, than on the *LOX5*/*LOX5* plant. By contrast, GPA population on the *lox5*/*LOX5* chimera, which contains a *lox5* mutant scion and a WT *LOX5* rootstock, was comparable to that on the *LOX5*/*LOX5* plant and significantly higher than on the *LOX5*/*lox5* and *lox5*/*lox5* plants. These results taken along with the up-regulation of

LOX5 in roots of GPA-infested plants confirm that *LOX5* activity in roots facilitates colonization of the shoots by GPA.

2.3.5 9-LOX Products Accumulate in Roots and Petiole Exudate of GPA-infested Plants

Arabidopsis LOX5 encodes a 9-LOX (Bannenberg et al., 2009), therefore the levels of 9-LOX synthesized 9-HPs (9-hydroperoxy- and 9-hydroxy-fatty acids), 9-hydroxyoctadecadienoic acid (9-HOD), 9-hydroperoxyoctadecadienoic acid (9-HPOD), 9-hydroxyoctadecatrienoic acid (9-HOT) and 9-hydroperoxyoctadecatrienoic acid (9-HPOT) were monitored in roots of WT and *lox5-1* in which shoots were colonized by GPA. It should be noted that hydroperoxides can undergo spontaneous reduction to hydroxides. The levels of all four 9-HPs were higher in roots of GPA-infested WT plants compared to roots of un-infested WT plants (Figure 2.7C). However, in roots of GPA-infested *lox5-1* this increase in 9-HPs was significantly lower, confirming that *LOX5* contributes to the increase in 9-HPs in roots of GPA-infested plants.

Since the *lox5-1* allele impacted time spent in SEP and XP by GPA and also the accumulation of a fecundity promoting activity in petiole exudates (Figure 2.4B; Figure 2.5A), 9-HP levels were also determined in petiole exudates collected from leaves of GPA-infested and as control, un-infested WT and *lox5-1*. The levels of these 9-HPs were elevated in petiole exudates collected from GPA-infested leaves of WT plant (Figure 2.9A). The accumulation of these 9-HPs was however reduced in petiole exudates collected from GPA-infested leaves of *lox5-1*. To determine if one or more of these 9-HPs were capable of promoting fecundity, GPAs were reared on an artificial diet containing either 9-HOD, 9-HPOD, 9-HOT or 9-HPOT. In comparison to control diets, and diet containing ethanol (0.1%) that was used to dissolve the 9-HPs, size of the insect population was larger on 9-HOD and 9-HPOD supplemented diets, thus confirming that a

9-LOX product promotes insect fecundity (Figure 2.9B). 9-HOT and 9-HPOT did not enhance insect fecundity at the concentrations used. Quite the contrary, 9-HOT adversely impacted insect fecundity in this assay. Irrigation of the *lox5-1* plants with 9-HOD facilitated GPA infestation of the shoot (Figure 2.10A) and restored water content in insects collected from these plants (Figure 2.10B), thus confirming that an oxylipin synthesized in the roots by LOX5 facilitates infestation of the shoots by GPA.

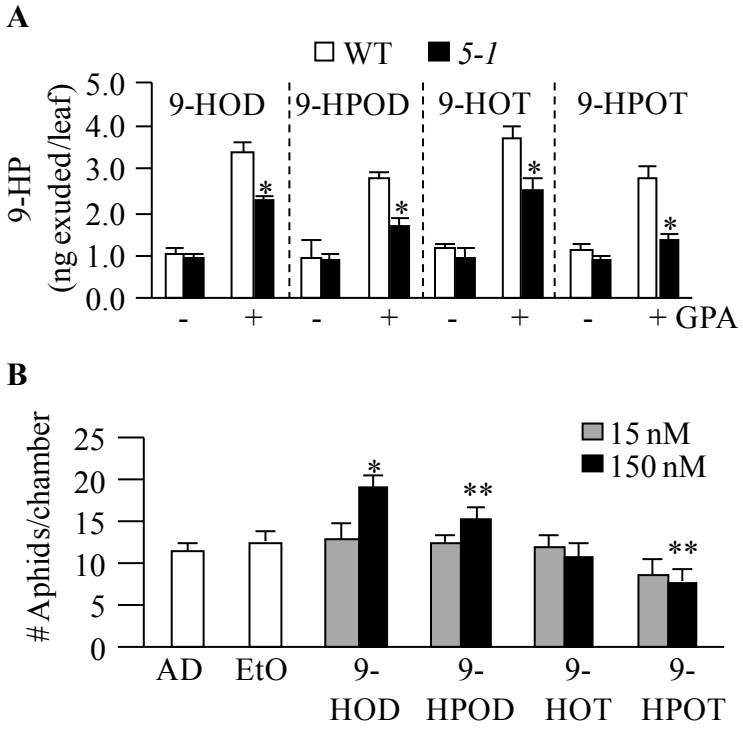

Figure 2.9. 9-LOX-derived oxylipins accumulate in petiole exudates of GPA-infested plants and enhance GPA fecundity. **A.** Profile of 9-LOX-derived 9-HPs in petiole exudates collected from leaves of un-infested (-GPA) and GPA-infested (+GPA) WT and *lox5-1* (*5-1*) plants. The insects were allowed to feed on plants for 48 h prior to harvesting leaves for petiole exudate collection. Data points show means (± SEM) (n=3). An asterisk (*) indicates significant difference from WT at the corresponding time point ($P<0.05$; *t*-test). 9-HOD (9-hydroxyoctadecadienoic acid), 9-HPOD (9-hydroperoxyoctadecadienoic acid), 9-HOT (9-hydroxyoctadecatrienonic acid) and 9-HPOT (9-hydroperoxyoctadecatrienoic acid). **B.** Artificial diet assay with 9-HPs. Comparison of GPA numbers (mean ± SEM) (n=4) on artificial diet supplemented with 9-HOD, 9-HPOD, 9-HOT and 9-HPOT at final concentrations of 15 and 150 nM. Artificial diet (AD) alone and diet supplemented with 0.1% ethanol (EtO), which was used as solvent for the oxylipins, provided the controls. Significant differences from AD and EtO determined by GLM/ANOVA are indicated by asterisks (*, $P<0.05$; **, $P<0.1$).

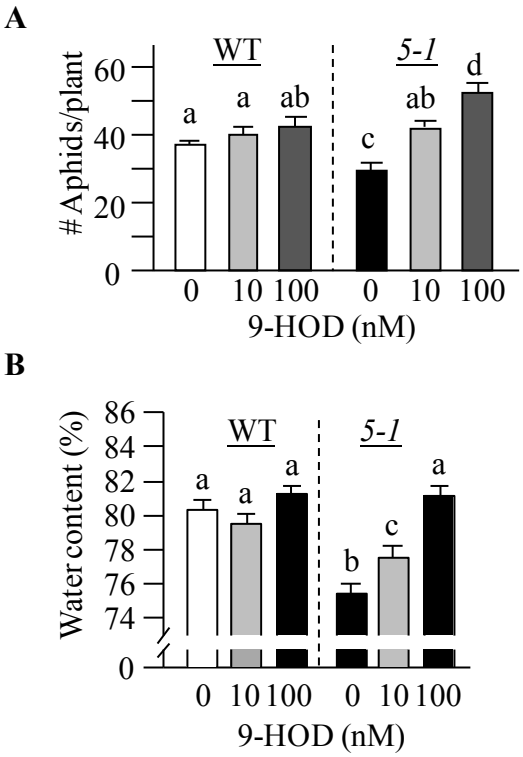

Figure 2.10. LOX5-derived 9-HPs complement *lox5-1* phenotype. **A.** Complementation of the *lox5-1* phenotype by 9-HOD (9-hydroxyoctadecadienoic acid) applied to roots. No-Choice test comparison of GPA numbers (Mean ± SEM) on WT and *lox5-1* (*5-1*) plants that were treated irrigated with 9-HOD (10 and 100 nM). Controls were plants that did not receive 9-HOD (0 nM). **B.** Percentage water content in GPAs reared on *lox5-1* plants treated with 9-HOD. Each bar represents the percentage content of water (mean ± SEM; n=6 groups of 30-40 GPAs) in GPAs reared on WT and *lox5-1* mutant plants that were irrigated with 9-HOD (10 or 100 nM). Controls were WT and *lox5-1* plants that did not receive 9-HOD (0 nM). In **A** and **B**, different letters above the bars indicate values that are significantly different ($P<0.05$; GLM/ANOVA).

2.3.6 GPA Feeding Does Not Affect Salicylic Acid and Jasmonic Acid Signaling in *lox5*

The 9-LOX-derived oxylipin 9-keto-10(*E*),12(*Z*),15(*Z*)-octadecatrienoic acid (9-KOT) modulates expression of JA- and salicylic acid-regulated genes (Vicente et al., 2011). JA signaling is reported to promote defense against insect pests, and SA has been reported to antagonize the activation of JA signaling in plant-insect interactions (Walling, 2008). The expression of the *PR1* (*PATHOGENESIS-RELATED 1*) and *PDF1.2* (*PLANT DEFENSIN 1.2*) genes which are molecular markers for the activation of SA and JA signaling, respectively was

therefore monitored (Figure 2.11). Although expression of *PR1* and *PDF1.2* were elevated in GPA-infested plants, no discernible differences were observed in the temporal pattern and extent of expression of these genes between GPA-infested wild type and *lox5*, suggesting that the *lox5* phenotype is not associated with any obvious changes in the activation of SA and JA signaling.

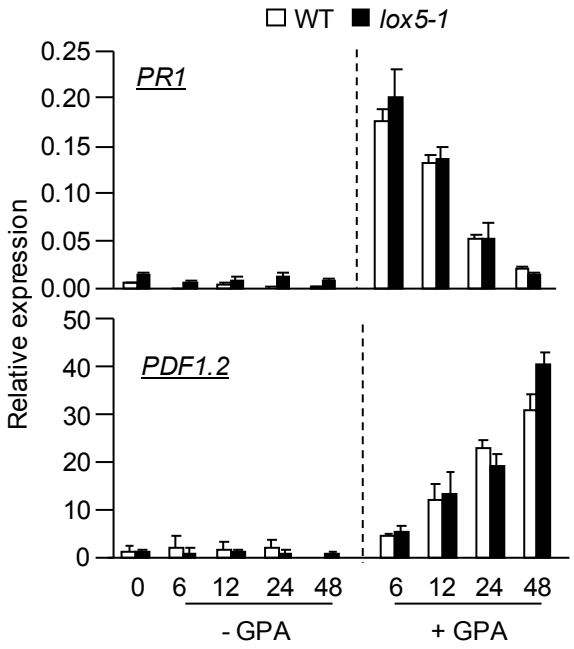

Figure 2.11. *PR1* and *PDF1.2* Expression in WT and *lox5-1* plants. qRT-PCR was performed on RNA from leaves of un-infested and GPA-infested plants harvested at 0, 6, 12, 24 and 48 hours post infestation (hpi). The *EF1α* gene provided the control for qRT-PCR. The expression level of *PR1* and *PDF1.2* relative to expression level of *EF1α* at each time point is presented. Values are presented as means ± SEM (n=3).

2.4 Discussion

Oxylipins participate in plant defense by serving as signals to induce defense responses or by directly functioning as antimicrobial compounds (Blée, 2002; Prost et al., 2005). Results presented here indicate a 9-LOX-derived oxylipin, or product thereof, is a susceptibility factor that facilitates the colonization of *Arabidopsis* by GPA. GPA infestation of the foliage resulted in the up-regulation of *LOX5* expression in roots (Figure 2.7A). This was associated with a concomitant increase in level of 9-HPs in the roots and petiole exudates of GPA-infested plants

(Figure 2.7C and 2.9A). The increase in content of 9-HPS in GPA-infested plants was dependent on *LOX5* (Figure 2.7C and 2.9A). The lower population of GPAs on *lox5* as compared to WT plants implies a role for *LOX5* in plant susceptibility to GPA (Figure 2.2A-C). Furthermore, the expression of LOX5 or a LOX5-GFP fusion from the *35S* promoter and exogenous application of the LOX5-derived oxylipin, 9-HOD, complemented the *lox5-1* deficiency resulting in the promotion of insect infestation (Figure 2.2B and 2.10A). Taken together, this data confirms an important role of a LOX5-synthesized oxylipin(s), or product thereof, in facilitating GPA infestation in *Arabidopsis*.

Infestation by aphids is known to manipulate host physiology leading to alterations in the composition of phloem sap (Sandström et al., 2000; Dinant et al., 2010; Wilson et al., 2011). Results presented here demonstrate that aphids also manipulate host physiology to promote accumulation of an insect fecundity-promoting factor in petiole exudates (Figure 2.4), which is in direct contrast to petiole exudates collected from un-infested *Arabidopsis* leaves that contain an activity that adversely impacted GPA fecundity (Figure 2.4) (Louis et al., 2010a; Louis et al., 2010b). Compared to WT plants, the fecundity-promoting effect was significantly lower for petiole exudates collected from GPA-infested leaves of the *lox5-1* mutant (Figure 2.4), which suggests that *LOX5* is required for this accumulation. The increased fecundity observed when 9-HOD and 9-HPOD were supplemented to the artificial diet further support the involvement of *LOX5* in promoting insect fecundity (Figure 2.9B). Additionally, the levels of 9-HOD and 9-HPOD in petiole exudates from GPA-infested leaves of WT *Arabidopsis* were significantly higher (Figure 2.9A). On the other hand, the levels of these oxylipins were lower in petiole exudates collected from GPA-infested *lox5-1* plants. Collectively these results confirm that GPA fecundity is enhanced by LOX5-synthesized oxylipins or products thereof.

Altered feeding behavior of the GPA on *lox5-1* could contribute to reduced fecundity that was observed. As summarized in Figure 2.12, a factor derived from the *LOX5* gene in *Arabidopsis* facilitates aphid feeding from the sieve elements and thus sap consumption. GPA spent less time in SEP in the absence of *LOX5* (Figure 2.5A; Table 2.1). The osmotic pressure of the sugar rich phloem sap is considerably higher that the aphid hemolymph and the resulting gradient contribute to water loss from the hemolymph leading to dehydration and loss of body water content in GPA (Douglas, 2006). The active ingestion of xylem sap is an important mechanism for rehydration and osmoregulation of haemolymph (Spiller et al., 1990; Pompon et al., 2009); Powell and Hardie, 2002; Douglas, 2006). The diluted composition of xylem sap as compared to phloem sap (Mattson, 1980; Taiz and Zeiger, 2002) enable aphids to osmoregulate their hemolymph and prevent dehydration (Pompon et al., 2009; Pompon et al., 2011). The importance of xylem sap consumption to the insect is evident from studies with bird cherry-oat aphid (*Rhopalosiphum padi*). On wheat plants treated with the neonicotinoid insecticide thiamethoxam, which disrupts XP, the water content in bird cherry-oat aphid was significantly reduced and size of the insect population was reduced (Daniels et al., 2009). These results show that *LOX5* also impacts the ability of the insect to drink from the xylem. By comparison to the WT plant, when on *lox5-1* the GPAs did not exhibit XP and water content in the insects was lower than in insects reared on WT plants (Figure 2.5A and 2.5B). In GPA reared on 9-HOD treated *lox5-1 w*ater content was restored (Figure 2.10B), which confirms the importance of a *LOX5*-derived oxylipin in promoting hydration of GPA on *Arabidopsis*.

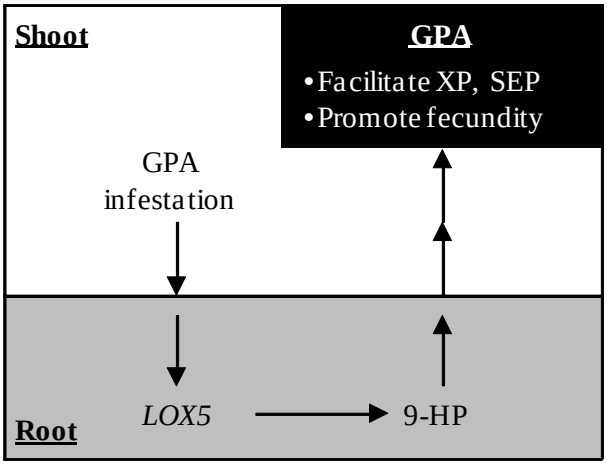

Figure 2.12. Model depicting the role of root-derived LOX5-synthesized oxylipin in *Arabidopsis* interaction with GPA. GPA infestation on *Arabidopsis* shoots induces expression of *LOX5* in roots within 3 h of infestation. It is anticipated that a plant-derived and/or an insect-derived signal originating in the shoot is delivered to the roots leading to the stimulation of *LOX5* expression and increased synthesis of 9-HPs. These 9-HPs are subsequently transported to the shoots, most likely through the vasculature, where they are utilized by GPA as cues that facilitate insect feeding from the sieve elements and water consumption from xylem, and insect fecundity. Alternatively, these 9-HPs may counter and/or attenuate defenses targeting GPA.

Additionally, 9-LOX derived oxylipins or a product thereof, synthesized by LOX5 may directly impact GPA behavior and physiology and thus impact time spent by the insect in SEP and XP, and as a consequence fecundity. The presence of 9-HOD in GPA reared on plants, as compared to GPA reared on an artificial diet (Harmel et al., 2007) and the fact that GPAs lacks a 9-HOD synthesizing activity suggest that GPAs derive 9-HOD from the host plant (Harmel et al., 2007). The fact that 9-HOD and other 9-LOX-derived oxylipins are present in phloem sap provides further confirmation (Harmel et al., 2007). The results presented here show that the content of 9-HOD and other 9-LOX-derived oxylipins increases in petiole exudates collected from GPA-infested leaves, compared to petiole exudates from un-infested leaves of *Arabidopsis* (Figure 2.9A). The observation that GPAs reared on 9-HOD supplemented artificial diet exhibit increased fecundity (Figure 2.9B) suggests that LOX5-derived oxylipins have the potential to directly impact GPA. However, since *LOX5*-synthesized oxylipins also impact physiological

processes in plants (Vellosillo et al., 2007), it is equally plausible that the impact of LOX5-synthesized oxylipins on facilitating GPA infestation is mediated through their influence on molecular and physiological mechanisms in *Arabidopsis*. In plants 9-LOXs are also known to influence plant defense against pathogens (Hwang and Hwang, 2010; López et al., 2011) therefore an antagonistic relationship between LOX5-oxylipins and *Arabidopsis* defenses could promote GPA fecundity and feeding behavior, thus promoting colonization of *Arabidopsis*.

Previous research on plant defense mechanisms to sap-sucking insects like aphids have largely focused on investigating responses in leaves (Giordanengo et al., 2010), and there are no examples of root-derived metabolites influencing aphid feeding behavior on shoots. *LOX5* expression is up-regulated in roots of GPA-infested plants (Figure 2.6; Figure 2.7A). The induction of *LOX5* transcript in roots was associated with 2-fold increase in 9-LOX derived 9-HPs in roots of WT plants, which was not observed in roots of GPA-infested *lox5-1* plants (Figure 2.7A). Further evidence supporting the role for root derived 9-HPs in *Arabidopsis* interaction with GPA was obtained by reciprocal grafting of *Arabidopsis* seedlings. In all graft combinations that were tested, the size of GPA population was largely determined by the genotype of the rootstock (Figure 2.7B; Figure 2.8). In reciprocal grafts between WT and *lox5-1* plants, GPA population size on *lox5/LOX5* chimeras was similar to populations on *LOX5/LOX5* self grafted plants. Correspondingly, *LOX5/lox5* chimeric plants had GPA population sizes that were similar to populations on *lox5/lox5*self grafts (Figure 2.7B). Plant roots are known sites of synthesis of secondary metabolites that enable plants tolerate above ground herbivory (Erb et al., 2008; Erb et al., 2009). For instance, nicotine is synthesized in roots of tobacco plants, where it is loaded into the xylem and translocated to the shoots in response to insect attack (Dawson, 1941; Morita et al., 2009). The induction of *LOX5* expression and accumulation of 9-HPs in roots in

response to foliar herbivory implies the presence of a shoot to root signal (Figure 2.12). Further research is necessary to identify the responsible signal(s) which may be either of plant or insect origin or a combination of both. Plant hormones like auxin and jasmonates have been implicated to play a role in root nicotine production in tobacco (Baldwin et al., 1994; Shi et al., 2006). On the other hand, the signal(s) may be aphid derived, given that aphid saliva that is injected into the plant contains numerous proteins with diverse activities that are capable of eliciting and/or suppressing plant defenses (De Vos and Jander, 2009). In a recent study, 48 candidate effectors were identified in aphid saliva of which Mp10 and Mp42 reduced aphid fecundity by inducing plant defense responses (Bos et al., 2010). Our data suggest that a plant or aphid derived signal(s) induces the accumulation of *LOX5* derived 9-HPs in roots of GPA infested plants.

In summary, the results presented here indicate an important role for root synthesized oxylipins in promoting colonization of foliage by GPA. Plant oxylipins (e.g. jasmonates) are inducers of plant defense against pathogens and insect pests and further the volatile products of the LOX pathways are involved in tritrophic interactions (Wasternack and Hause, 2002). However a phloem-feeding generalist herbivore, *M. persicae*, has adapted to exploit *Arabidopsis* plant oxylipins synthesized in roots and present in vascular sap to facilitate foliar infestation. These results have broader ramifications to agriculture given that GPA is an insect pest of economic importance infesting more than 50 families of plants (Blackman and Eastop, 2000)and being a vector of several plant pathogenic viruses (Brault et al., 2010).

2.5 Methods

2.5.1 Plant and Insect Cultivation

Green peach aphid (GPA; *Myzus persicae* Sülzer) (Kansas State University, Museum of

Entomological and Prairie Arthropod Research, voucher specimen #194) was reared on an equal combination of commercially available of radish (Early scarlet globe) and mustard (Florida broadleaf) which were maintained at 22^{o}C under a 14 h light (100 μE m^{-2} sec^{-1})/ 10 h dark regime. For aphid hydration studies, adult apterous aphids were starved by confining them in a petri dish for 4-6 h at 22^{o}C. *Arabidopsis* was grown at 22^{o}C under 14 h light (100 μE m^{-2} sec^{-1})/ 10 h dark regime in autoclaved compost-peat based planting mixture (Premier Pro Mix-PGX, Procunier, Canada). Plants that were approximately four weeks old were used for all experiments. The *lox1-1* (SALK_059431), *lox5-1* (SALK_044826) and *lox5-3* (SALK_050933) are in the accession Columbia-0 (Col-0). To generate the *lox1lox5* double mutant, pollen from the *lox1-1* plant was used to pollinate *lox5-1* flowers. Segregants that were homozygous for *lox1-1* and *lox5-1* were identified using PCR (see below).

2.5.2 Transgenic Plants

To generate *35S:LOX1* and *35S:LOX5* constructs in which the *LOX1* and *LOX5* coding regions are cloned downstream of the *Cauliflower mosaic virus 35S* promoter, RNA extracted from roots was used to amplify the coding region of *LOX1* and *LOX5* using the following primer combinations: LOX1-C-F and LOX1-C-R (full length) and LOX5-C-F and LOX5-C-R (full length) (Table 2.2). The PCR products were cloned into the pCR®8/GW/TOPO® vector (Invitrogen) and subsequently used in a LR recombination reaction with the destination vectors pMDC32 (Curtis and Grossniklaus, 2003) to yield pMDC32-*35S:LOX1* and pMDC32-*35S:LOX5* plasmids. To generate *35S:LOX1-GFP* and *35S:LOX5-GFP* constructs in which the GFP coding region is fused to the C-terminus (last amino acid) of LOX1 and LOX5, RNA extracted from roots was used to amplify the coding region of *LOX1* and *LOX5* using the following primer

combinations: LOX1-C-F and LOX1-NS-R (full length without stop codon), and LOX5-C-F and LOX5-NS-R (full length without stop codon) (Table 2.2). The PCR products were cloned into the pCR®8/GW/TOPO® vector (Invitrogen) and subsequently used in a LR recombination reaction with the destination vectors pMDC83 (Curtis and Grossniklaus, 2003) to yield pMDC83-*35S:LOX1-GFP* and pMDC83-*35S:LOX5-GFP* plasmids. The pMDC32-*35S:LOX1*, pMDC32-*35S:LOX5*, pMDC83-*35S:LOX1-GFP* and pMDC83-*35S:LOX5-GFP* plasmids were electroporated into *Agrobacterium tumefaciens* strain GV3101 and used to transform *Arabidopsis* by the floral-dip method (Clough and Bent, 1998). Transgenic plants were selected for their resistance to hygromycin. To generate 35S:LOX1 and 35S:LOX1-GFP plants, the pMDC32-*35S:LOX1* and pMDC83-*35S:LOX1-GFP* constructs, respectively, were transformed into the *lox1-1* mutant, while the pMDC32-*35S:LOX5* and pMDC83-*35S:LOX5-GFP* constructs were transformed into the *lox5-1* mutant to generate transgenic 35S:LOX5 and 35S:LOX5-GFP plants, respectively.

2.5.3 No-choice Assays

For no-choice assays, 20 adult apterous (wingless) adult aphids were released on each plant and the total numbers of insects (Adults + nymphs) were counted 2 days later (Pegadaraju et al., 2005).

2.5.4 EPG Recording

Electrical penetration graph (EPG) (Walker, 2000) technique was used to monitor GPA feeding behavior as previously described (Pegadaraju et al., 2007). A total of 12 replicates were

done each with fresh GPA and plants. Data obtained from EPG was analyzed by nonparametric Kruskal-Wallis test ($P < 0.05$).

Table 2.2 Primers used in this study

Primer name	Primer sequence (5'→3')
LBb1.3	ATTTTGCCGATTTCGGAAC
SALK_059431-F	AGCTCCTTGAACCTCACTTCC
SALK_059431-R	GAGACGCTATTTGGAATTCCC
SALK_044826-F	AGGAAGACTTGGAAAAGCAGC
SALK_044826-R	CTGAGTCAGGACCCTTGTCAG
SALK_050933-F	CGACCCGTTATCAAATCCATC
SALK_050933-R	TGATTAGCTCCGGTGTTTCAC
LOX5 qRT-F	CTTGCCTGACATCCTCAAAGAGAGC
LOX5 qRT-R	GGTTCGGGTCGCATAGGTTTTAGTG
PR1 qRT-F	TTCTTCCCTCGAAAGCTCAA
PR1 qRT-R	AAGGCCCACCAGAGTGTATG
PDF1.2 qRT-F	CATGTTTGGCTCCTTCAAG
PDF1.2 qRT-R	CTTGTTCTCTTTGCTGCTTTC
EF1α-F	AGGTCCACCAACCTTGACTG
EF1α-R	GAGACTCGTGGTGCATCTCA
LOX1-C-F	GAATCAAAACTAGTACTTCACCCAA
LOX1-C-R	GAAGCGAGAGTTGTTTCAGATAG
LOX1-C-NS-R	GATAGAGACGCTATTTGGAATTCCCCT
LOX1-S-R	GGCTCGAGTCAGATAGAGACGCTATTTGG
LOX5-C-F	GAAGAAGACTAGTACAATGGAGGAAGAT
LOX5-C-R	GAGAGAGGGTTTATCTTTAGATTGAGAC
LOX5-C-NS-R	GATTGAGACACTGTTCGGGATCCCTTTC
LOX5-S-R	ATAAGAATGCGGCCGCTTAGATTGAGACACTGTTCG

2.5.5 Petiole Exudate Collection

Petiole exudates were collected from *Arabidopsis* leaves using a previously described method (Chaturvedi et al., 2008) with a few modifications. Leaves were cut at the base of their petioles and the petiole immediately dipped in 50% Ethanol followed by 0.0005% Bleach and finally in 1 mM EDTA (pH 8.0) solution. After 20 minutes, a small portion of the petiole base was cut and 6 leaves were immediately placed in 3 ml of 1 mM EDTA (pH 8.0) contained in a well of a 24-well tissue culture plate (ICN Biochemical Inc.; http://www.mpbio.com). High humidity was maintained by placing the entire setup on wet paper towels and under a transparent dome. The leaves were allowed to exude for a period of 12-14 h. After the exudation period, petiole exudates were collected by passing it through a 0.45 µm syringe filters (Fisherbrand® 25 mm Syringe Filter; Catalog # 09-719B; http://www.fishersci.com). Petiole exudates from 6-wells were pooled, lyophilized and resuspened in 2 ml of 1mM EDTA for use in all experiments. Petiole exudates were collected from uninfested (Control; '- GPA') and infested ('+ GPA') plants, 48 h post infestation. For collection of petiole exudates from GPA-infested plants, only leaves that had a minimum of five aphids were used. Petiole exudates from two separate experiments were pooled for oxylipin measurements.

2.5.6 Artificial Diet Assays

An artificial diet (Dadd and Mittler, 1966) was used for all feeding trial bioassays. The composition of the diet is provided in table 2.3. A feeding chamber was constructed as previously described (Louis et al., 2010b). Each feeding chamber contained a total volume of 500 µL that included the artificial diet with or without supplementation with petiole exudates or chemicals. The 9-LOX oxylipins, 9-HOD, 9-HPOD, 9-HOT and 9-HPOT were diluted in ethanol

and added to 440 µL artificial diet to a final volume of 500 µL to a final concentration of 15 and 150 nM. The oxylipins were suspended in the artificial diet by vigorous vortexing for 2 hours. Six adult apterous GPA were placed on the feeding chamber and total aphids (adults + nymphs) were determined 2 days later.

Table 2.3. Composition of artificial diet

Component Name	**Amount (mg)**	**Component Name**	**Amount (mg)**
L-Alanine	100	L-Ascorbic acid	100
L-Arginine	270	D-Biotin	0.1
L-Asparagine	550	D-Pantothenic acid	5
L-Aspartic acid	140	Choline chloride salt	50
L-Cysteine HCL	40	Folic acid	0.5
L-Glutamic acid	140	Myo-inositol	50
L-Glutamine	150	Nictoinic acid	10
L-Glycine	80	L-Pyridoxine	2.5
L-Histidine	80	Riboflavin	0.5
L-Isoleucine	80	L-Thiamine HCL	2.5
L-Leucine	80	Potassium Dihydrogen-orthophosphate	500
L-Lysine HCL	120	Magnesium chloride heptahydrate	200
L-Methionine	40	Cupric-Sodium EDTA salt	0.4
L-Phenylalanine	40	Ferric-Sodium EDTA salt	1.5
L-Proline	80	Manganese di chloride tetrahydrate	0.4
L-Serine	80	Zinc EDTA	0.8
L-Threonine	140	Sucrose	17. 5 gm
L-Tryptophan	80	Sterilized water	100 ml
L-Tyrosine	40	Cholesterol	2.5
L-Valine	80		

2.5.7 Measurement of GPA Body Water Content

Aphids were starved for 14-16 h by placing them in a petri dish without any water. Twenty starved aphids were allowed to feed on *Arabidopsis* for 12 h. To estimate water content, 30-40 adult aphids from two plants were collected and immediately weighed. Dry weights were obtained after drying the aphids at 60^{o}C for 24 h. The water content of the aphids was determined by subtracting the dry weight from the fresh weight of the aphids.

2.5.8 Micrografting

Single-hypocotyl grafts were constructed as previously described (Turnbull et al., 2002). *Arabidopsis* seedlings were germinated on ½ MS plates with 2% agar and 1% sucrose under a 14 h light (100 μE m^{-2})/10 h dark regime at 22^{o}C. One day prior to grafting the 10 d old seedlings were moved to 27^{o}C. The scion and rootstock were cut transversely and aligned precisely under sterile conditions without a grafting collar. The grafted seedlings were placed back at 27^{o}C for 3-4 days to promote graft union and monitored every day and any adventitious roots that were observed were crushed with forceps. After 7-12 d, grafts that were functional were transplanted to soil. No-Choice assays were carried out on plants that were 4-5 weeks old. Genotyping of the rootstock and scion for each graft was done for each graft combination by first collecting root and shoot separately for DNA extraction. The DNA from different individuals was pooled together for genomic PCR analysis to verify the genotype as described.

2.5.9 Confocal Laser Scanning Microscopy

Protoplasts from *35S:LOX1-GFP* and *35S:LOX5-GFP* were isolated as previously reported (Jin et al., 2001) and observed with a Ziess 200M inverted optical microscope fitted

with a CSU-10 Yokogawa Confocal scanner (McBain Instruments) and captured with a digital camera (Hamamatsu). Excitation wavelengths and emission filters were 488 nm/band-pass 505-535 nm for GFP and 488 nm/band-pass 672-712 nm for chloroplast auto-fluorescence. Three-dimensional images were obtained using Imaris 6.2 (BitPlane) software and are presented as stacks of neighboring sections.

2.5.10 Genomic PCR

Primers used in this study are listed in Supplemental Table S2.1. Plants homozygous for *lox1-1* (SALK_059431), *lox5-1* (SALK_044826) and *lox5-3* (SALK_050933) were identified using a multiplex PCR involving primers LBb1.3 (Left border for T-DNA insertion) and the corresponding gene specific primers respectively. PCR using the three primers was performed under the following conditions: 95^{o}C for 5 min, followed by 35 cycles of 95^{o}C for 30 s, 55^{o}C for 45s and 72^{o}C for 2 min, with a final extension of 72^{o}C for 5 min.

2.5.11 Reverse Transcriptase-PCR and Quantitative Real Time-PCR

RNA extraction from leaves and roots and cDNA synthesis was performed as previously described (Pegadaraju et al., 2005). DNA contamination in samples was removed by treatment RNase-free DNase. For each time point and treatment, RNA was collected from two biological replicates, each consisting of leaves pooled from 4 plants. Gene specific primers used for Reverse Transcriptase (RT-PCR) and Quantitative Real Time-PCR (qRT-PCR) are listed in Table S1 and were identified using the AtRTPrimer database (http://atrtprimer.kaist.ac.kr/) (Han and Kim, 2006). The PCR conditions for all RT-PCR reactions were as follows: 95^{o}C for 5 min, followed by 25 cycles of 95^{o}C for 30 s, 58^{o}C for 45 s and 72^{o}C for 45 s, followed by a final

extension step of 72°C for 5 min. Real-time PCR was performed with Sybr® Green PCR Master Mix (Applied Biosystems, Cat No: 4309155) on a Eco qPCR system (Illumina®) using the following amplification protocol: 10 min polymerase activation and denaturation at 95°C, 40 cycles of 95°C for 10s, 58°C for 30s and 72°C for 30s. This was followed by a product melt to confirm a single PCR product. The level of *LOX5* expression was normalized to that of *EF1α* by subtracting the C_t value of *EF1α* from the C_t value of *LOX5*.

2.5.12 Oxylipin Profiling

Lipids were extracted from plant samples as previously described (Göbel et al., 2002). The extracted lipids were resuspended in 100 µl methanol:water (80:20 v/v). Oxylipins in the lipid extract were first purified by reverse phase-HPLC using an Agilent 1100 HPLC coupled to a UV diode array detector. A Nucleosil 120-5 C18 column (4.6 x 150 mm, 5 µm Macherey–Nagel, Bethlehem, PA, USA) with a binary gradient solvent system [solvent A: methanol:water:acetic acid (80:20:0.1 v/v/v) and solvent B: methanol:acetic acid (100:0.1 v/v)] , flow rate of 0.18 ml/ min was used, as previously described (12). Subsequently, the oxylipins were resolved by normal-phase HPLC over a Zorbax Rx-SIL column (2.1x150 mm, 5 µm, Agilent, Waldbronn, Germany) with linear solvent systems (hexane: isopropanol: trifluoroacetic acid 100:1:0.02 v/v/v) and a flow rate of 0.125 ml/min. To determine if the oxylipin products were formed by enzymatic reaction and/or auto-oxidation, chiral phase HPLC (CP) analysis was used to determine the *S* and *R* isomer composition of the oxylipins. Only the *S* isomer was detected, thus confirming that major proportions of the 9-HPs were enzymatically synthesized.

2.5.13 Statistical Analysis

For all assays, *t*-test or analysis of means using the General Linear Model (GLM) was used to separate the means. For the EPG analysis, the mean time spent by the aphids on various activities was analyzed using the non-parametric Kruskal-Wallis test. (Minitab v15).

2.5.14 Accession Numbers

LOX1 (At1g55020), *LOX5* (At3g22400), *lox1-1* (SALK_059431), *lox5-1* (SALK_044826), *lox5-3* (SALK_050933), *EF1α* (At5g60390). *PR1* (At2g14610), *PDF1.2* (At5g44420)

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CHAPTER 3

ROLE OF THE 9-LOX PATHWAY IN SYSTEMIC ACQUIRED RESISTANCE

3.1 Abstract

Plant lipids and lipid carrier proteins have been implicated in systemic acquired resistance**.** The activity of plant 9-lipoxygenases (LOXs) is induced in response to pest and pathogen infection. Here, we utilized *Arabidopsis* mutants, *lox1* and *lox5*, to determine the role of oxylipins synthesized by 9-LOXs in systemic acquired resistance (SAR), an inducible plant defense response that provides enhanced resistance to a broad-spectrum of pathogens throughout the foliage of a plant in response to localized infection by a pathogen. Compared to wild-type *Arabidopsis*, infection by an avirulent (Avr) pathogen, *Pseudomonas syringae* pv *tomato*, did not induce systemic defenses in *lox1* and *lox5* mutants. The response of the 9-LOX mutant plants to primary infections by an avirulent or virulent pathogen was similar to the response of wild-type plants. Infection cell death and hypersensitive response (HR) induced by avirulent pathogen were weaker in the mutant plants. The activity of LOX1 and LOX5 is required for the synthesis and perception of a SAR-inducing activity present in petiole exudates (Pet-ex) collected from wild-type leaves inoculated with avirulent pathogen (Avr-Pet-ex). These findings demonstrate an important role for 9-LOXs in inducing HR and SAR.

3.2 Introduction

Pathogens that are able to successfully colonize and infect plant tissue posses specific adaptations that enable them to overcome the plants constitutive and preformed defenses (Jones and Dangl, 2006). Plants, in turn have evolved to recognize the invasion by means of *R* genes that help recognize specific pathogen effectors and trigger effector-triggered immunity (ETI) that

limits infection. The activation of ETI leads to several physiological changes in the infected cell and in many cases is accompanied by the hypersensitive response (HR), a form of programmed cell death. The rapid death of host cells around the infection site is thought to restrict the spread of biotrophic pathogens and also release signals that promote defenses in surrounding tissues (Heath, 2000; Greenberg and Yao, 2004; Chisholm et al., 2006). The early physiological changes that occur upon pathogen effector recognition include a rapid burst of reactive oxygen species (ROS), ion fluxes and accumulation of salicylic acid (SA) followed by late responses such as accumulation of antimicrobial compounds and the induction of expression of defense genes, such as the *PATHOGENESIS-RELATED* (PR) group of genes, many of which encode proteins with antimicrobial activities (Boller and Felix, 2009). Additionally, ETI results in the development of systemic acquired resistance (SAR), a form of induced resistance that 'primes' the uninfected organs of the plant to respond faster and stronger to subsequent pathogen attack (Durrant and Dong, 2004; Conrath, 2006). SAR is systemic in nature and provides long-lasting resistance to secondary infection against a broad-spectrum of pathogens (Durrant and Dong, 2004). Salicylic acid signaling is critical for manifestation of SAR. However, other hormonal pathways also modulate SAR (Glazebrook, 2005).

3.2.1 Long Distance Signaling in SAR

The induction of SAR requires communication by the pathogen-treated organ with the other uninfested organs. This long-distance communication requires the systemic translocation of a signal. This mobile signal alerts distal uninfected plant parts of an impending infection resulting in the increase in SA levels and expression of a sub-set of *PR* genes in the distal parts. In addition, organs exhibiting SAR are 'primed' to respond faster to subsequent attack by

pathogens. Although, SA is essential for the SAR pathway, several studies have conclusively shown that it is not the mobile signal (Durrant and Dong, 2004). The nature of the long-distance SAR signal and the events occurring after SAR signal perception in the distal tissue are still unclear (Vlot et al., 2008; Shah, 2009). Several metabolites have been proposed as candidate SAR signals. Lipids or lipid-derived molecules have been implicated in this process (Maldonado et al., 2002; Nandi et al., 2004; Chaturvedi et al., 2008). In tobacco plants, a SA derivative, methyl salicylate and an unidentified lipid-derived molecule act as the mobile signal (Park et al., 2007; Liu et al., 2011b). Subsequently, it was shown that MeSA is also important for SAR in *Arabidopsis* and potato (Manosalva et al., 2010). However, MeSA's involvement in SAR in *Arabidopsis* has been questioned by another study. More recently, it was demonstrated that MeSA's requirement in SAR is influenced by light (Liu et al., 2011a). The metabolite, azelaic acid was identified in petiole exudates (Pet-ex) of plants in which SAR was induced suggesting that it may be a mobile signal although millimolar quantities of the compound are required (Jung et al., 2009). Recently, an abietane diterpenoid, dehydroabietinal, was identified in pet-exs of plants treated with an avirulent pathogen as a SAR inducing compound that in a SA-dependant manner in picomolar quantities (Chaturvedi et al., 2012). The identification of several potential signal molecules suggests that plants have evolved several mechanisms by which they can efficiently induce SAR in response to various pathogens.

3.2.2 HR, Its Relationship with Oxylipins and SAR

The development of tissue necrosis or HR that occurs in response to plant recognition of pathogen infection at the invasion site has been traditionally thought to indicate the initiation of SAR (Durrant and Dong, 2004). In addition to limiting pathogen spread, HR mediated cell death

has been suggested to be required for the generation of the long-distance SAR signal (Cameron et al., 1994). However, the generation of SAR in the absence of HR by a low inoculum inoculation by virulent or a high inoculum inoculation by nonpathogenic microbes suggest that HR is not essential for SAR signal generation ((Mishina and Zeier, 2007). Nevertheless, it is unclear whether the molecular signaling events that occur during the process of HR or the actual death of the cell itself contribute to SAR signal generation.

Cell death during HR occurs after the production of ROS which contribute to loss of cell integrity and viability because of their reactivity towards various biological membranes in the cell, proteins and nucleic acids (Morel and Dangl, 1997). Further, secondary oxidative bursts also occur in distal tissue leading to the formation of 'micro-HRs' providing evidence that HR may contribute to SAR (Alvarez et al., 1998). In *Arabidopsis*, the *fad7fad8* mutation which prevents the synthesis of chloroplastic trienonic fatty acids resulted in a reduction of ROS accumulation in leaves that were inoculated with an avirulent pathogen (Yaeno et al., 2004). Further, linolenic acid application activated NADPH oxidase that is responsible for the generation of ROS suggesting an important role for chloroplast lipids in plant defense response and SAR (Yaeno et al., 2004). In addition to oxidative damage by ROS, lipoxygenase (LOX)-mediated fatty acid peroxidation also contributes to loss of membrane integrity and cell death. LOXs are key enzymes in the oxylipin pathway that catalyze the introduction of molecular oxygen to either C-9 (9-LOXs) or C-13 (13-LOXs) to free fatty acids resulting in fatty acid hydroperoxides and their metabolites, collectively called oxylipins (Feussner and Wasternack, 2002). In several plant-pathogen interactions, the occurrence of HR is associated with an increase in 9-LOX activity (Porta and Rocha-Sosa, 2002). For instance, the levels of 9-LOX transcripts and related products increase in tobacco, potato and cotton in response to treatment

with fungal elicitors or infection with pathogens (Rusterucci et al., 1999; Gobel et al., 2001; Göbel et al., 2002; Jalloul et al., 2002; Montillet et al., 2005; Marmey et al., 2007). Genetic evidence in cotton and *Arabidopsis* also imply a role for 9-LOXs in cell death and plant defense responses (Hwang and Hwang, 2010; Vicente et al., 2011). However, in potato the transgenic suppression of a 9-LOX gene did not affect the extent and timing of necrosis in response to *P. syringae* pv. *maculicola* (*Psm*) infection due to compensation by oxidative damage and increase in the levels of 13-LOX-derived oxylipins (Gobel et al., 2003). This suggests that although lipid peroxidation is an important feature of HR, the source of the fatty acid peroxides may not be as important. Nevertheless, the involvement of 9-LOXs in HR-mediated cell death and resulting resistance in several plant species suggest that 9-LOX gene activity is highly conserved. An additional role for 9-LOX fatty acid hydroperoxides is in production of a range of defense products and in defense signaling (Blée, 2002; Howe and Schilmiller, 2002; López et al., 2011; Vicente et al., 2011).

Additionally, 9-LOX fatty acid hydroperoxides are thought to contribute to plant defenses by acting as signal molecules modulating plant responses through interaction with hormones involved in defense. The 9-LOX product 9-HOT, induces the deposition of callose and production of reactive oxygen species (ROS) which are characteristic stress responses that occur during to pathogen infection (Vellosillo et al., 2007). Additionally, *noxy2* (*non-responding to oxylipins 2*), a mutant that is insensitive to 9-HOT displayed enhanced susceptibility to *Pseudomonas* infection (Vellosillo et al., 2007). Identification of two *noxy* mutants, *noxy6* and *noxy22* as constitutive ethylene (ET) mutants suggest a negative interaction of ET with 9-HOT signaling (López et al., 2011). This observation further suggests that 9-LOX and ET pathways exert tight control over oxidative stress during pathogen infection enabling the plant to survive

and achieve full resistance. A different 9-LOX product, 9-KOT, produced by the dehydration of 9-HPOT has also been implicated in plant defense against bacteria (Vicente et al., 2011). Levels of 9-KOT accumulate in response to the bacterial infection and *lox1dox1* mutants that are unable to synthesize 9-KOT display local susceptibility and are partially impaired in systemic acquired resistance (SAR). A subset of genes that are upregulated in response to infection by both virulent and avirulent pathogens, in oxidative and osmotic stress and also by the plant hormones, SA and abscisic acid (ABA) are also induced by 9-KOT treatment (Vicente et al., 2011).

Oxylipins that are regulators of plant defenses are also antimicrobial in nature suggesting that these oxylipins play a dual role in plant defense. In a comprehensive study by Prost et al. (2005), the antimicrobial properties of 43 naturally occurring oxylipins against 13 plant pathogens including bacteria, oomycetes and fungi was surveyed in vitro. Oxylipins that were derived from the 9-/13-LOX pathways including 9-HOD, 9-HPOD, 9-HOT, 9-HPOT, 9-KOT, 13-HOT and 13-HPOT were among the most active oxylipins displaying antimicrobial properties were oxylipins derived via the 9-/13-LOX pathway including 9-HPOD, 9-HPOT, 9-HOD, 9-HOT and 9-KOT (Granér et al., 2003; Prost et al., 2005; Vicente et al., 2011)

In this study, we utilized the *Arabidopsis-P. syringae* pathosystem to determine the role of 9-LOXs in SAR. We found that in *Arabidopsis* 9-LOX mutants, *lox1* and *lox5*, the activation of systemic defenses is absent and SAR is compromised. Resistance to local infection by avirulent and virulent pathogens is however not affected. Further, the 9-LOX mutants display a delayed HR and are deficient in the generation and perception of the mobile SAR signal. While this work was in progress, another group recently demonstrated that in *Arabidopsis* a 9-lipoxygenase, LOX1 and an α-Dioxygenase (α-DOX) are redundantly involved in SAR (Vicente et al., 2011).

3.3 Results

3.3.1 Hypersensitive Response is Delayed in 9-LOX Mutants

LOX1 expression was induced as early as 6 hours post inoculation (hpi) in avirulent pathogen-inoculated leaves of wild type (WT) *Arabidopsis* accession Columbia-0 (Col) plants (Figure 3.1). By comparison, *LOX5* expression was transiently induced, with maximal expression observed at 6 hpi (Figure 3.1).

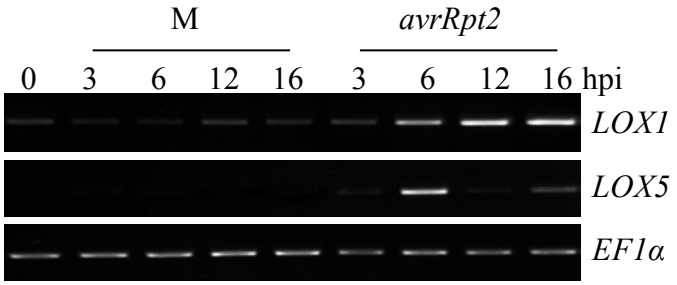
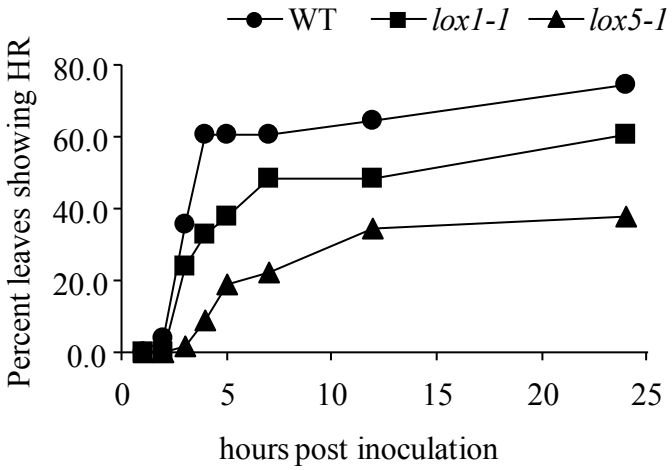


Figure 3.1. Expression of *LOX1* and *LOX5* in response to infection by avirulent pathogen. RT-PCR analysis of *LOX1* and *LOX5* expression in leaves of WT plants that were inoculated with 10 mM $MgCl_2$ (M) or the avirulent pathogen, *Pst* (Avr). Leaves were collected for RNA extraction at 0, 3, 6, 12 and 16 hours post inoculation (hpi). *EF1α* provided the control for RT-PCR.

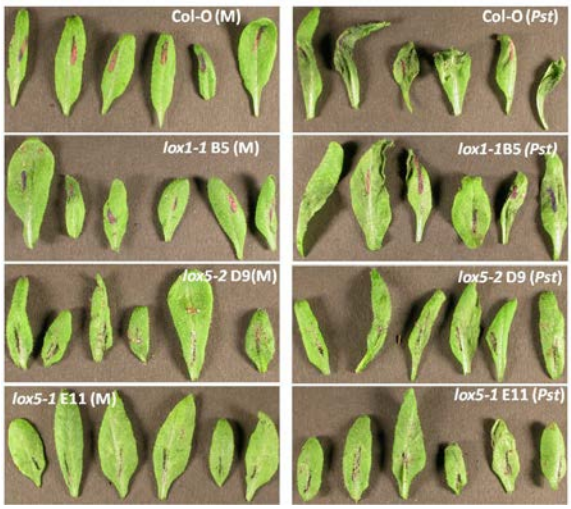
In plants, infection by avirulent pathogens results in the development of HR at the site of infection (Durrant and Dong, 2004) and the 9-LOX pathway plays an important role in this process. In *Arabidopsis* leaves that are inoculated with an avirulent pathogen, *P. syringae* pv *tomato* (*Pst*) DC3000 with the *avrRpt2* avirulence gene, the collapse of tissue indicates HR (Figure 3.2A). Analysis of *Pst*-induced tissue collapse of leaf tissue in WT and 9-LOX mutants showed significant differences. Over a 24 h period following bacterial infection, the percentage of leaves that showed total collapse was lower in *lox1-1* and *lox5-1* as compared to WT (Figure 3.2 A, B). The extent of cell death that occurs during HR was monitored microscopically by autofluorescence, a measure of accumulation of phenolics, and measuring the extent of electrolyte leakage. As shown in Figure 3.2C and 3.2D, bacterial infection by avirulent *Pst*

resulted in large areas of autofluorescence in WT plants. By comparison, in the *lox1-1* and *lox5-1* mutants, the extent of autofluorescence was reduced. Similarly, electrolyte leakage was significantly lower in the mutants compared to the WT plants (Figure 3.2 C, D). Together, these data suggest that the magnitude of HR due to infection by an avirulent pathogen is lower in the 9-LOX mutants.

A



B



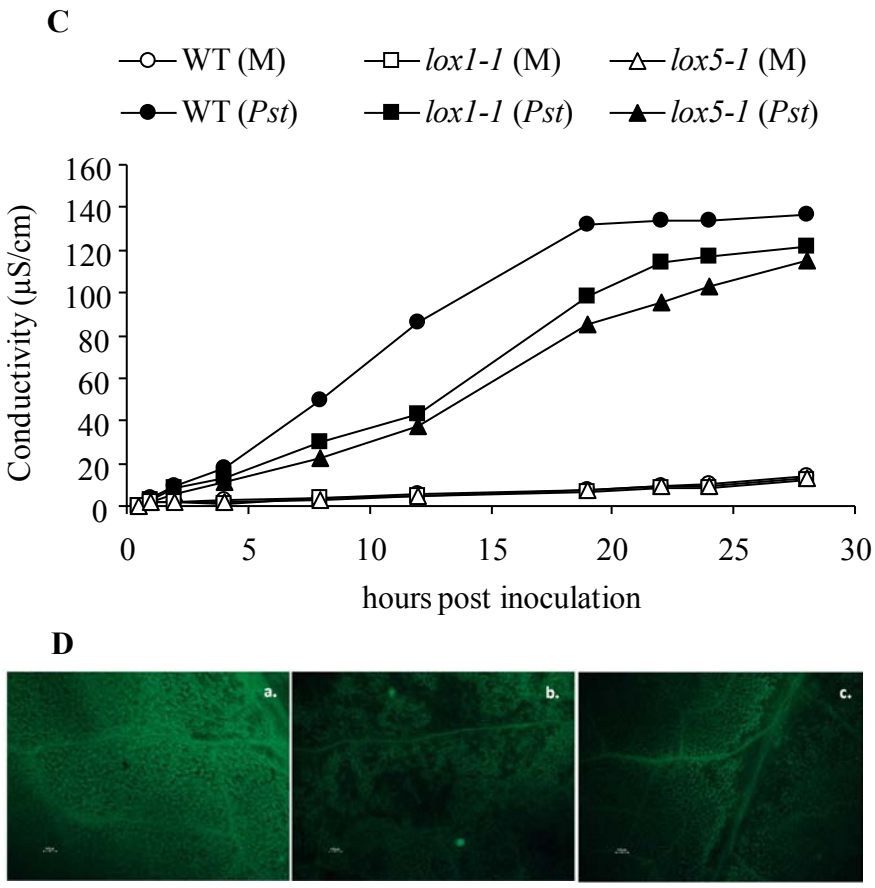


Figure 3.2 Hypersensitive response is delayed in 9-LOX mutants. **A.** Percentage of leaves showing total collapse at different time points after infiltration of *Pst* (10^5 CFU ml^{-1}) in wild-type accession Col-0 (WT), *lox1-1* and *lox5-1* mutants. **B.** Representative sample of WT, *lox1-1* and *lox5-1* leaves that were infiltrated with 10 mM $MgCl_2$ (M) or *Pst*. **C.** Electrolyte leakage from leaf tissue of WT, *lox1-1* and *lox5-1* inoculated with 10 mM MgCl2 (M) or *Pst*. The experiment was repeated twice and data from representative experiment is presented. **D.** Cell death was visualized by observing for autofluorescence in leaves 24h after infiltration with *Pst* DC3000 *avrRpt2*. **a.** Wild-type accession Col-0, **b.** *lox1-1* and **c.** *lox5-1*. In A, B, C and D, leaves from 4wk old plants were infiltrated with *P. syringae* pv *tomato* DC3000 (*Pst*) containing the *avrRpt2* gene and HR response was monitored over a 24h period.

3.3.2 Basal Resistance Against Bacterial Pathogen is not Impacted in 9-LOX Mutants

To determine if *LOX1* and *LOX5* are required for controlling growth of avirulent bacterial pathogen, growth of *Pst avrRpt2* strain was compared at 72 hpi in WT, *lox1-1* and *lox5-1* plants. As shown in Figure 3.3A, bacterial numbers were comparable between WT and the *lox* mutant

plants, indicating that *LOX1* and *LOX5* are not important for basal resistance against avirulent *Pst*. Similarly, growth of the virulent *P. syringae* pv *maculicola* (*Psm*) strain was not impacted in the *lox1* and *lox5* mutants (Figure 3.3B).

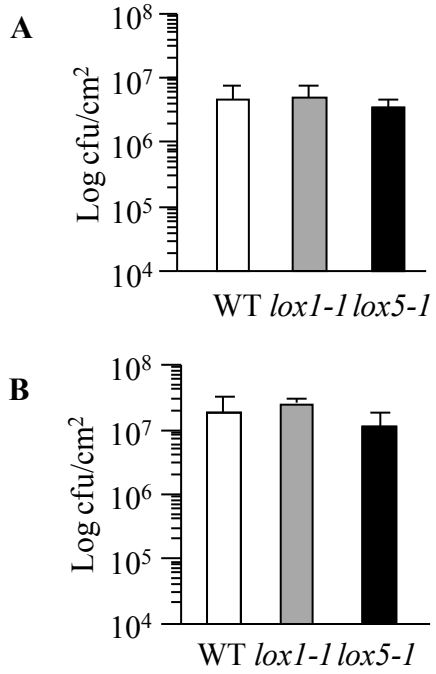


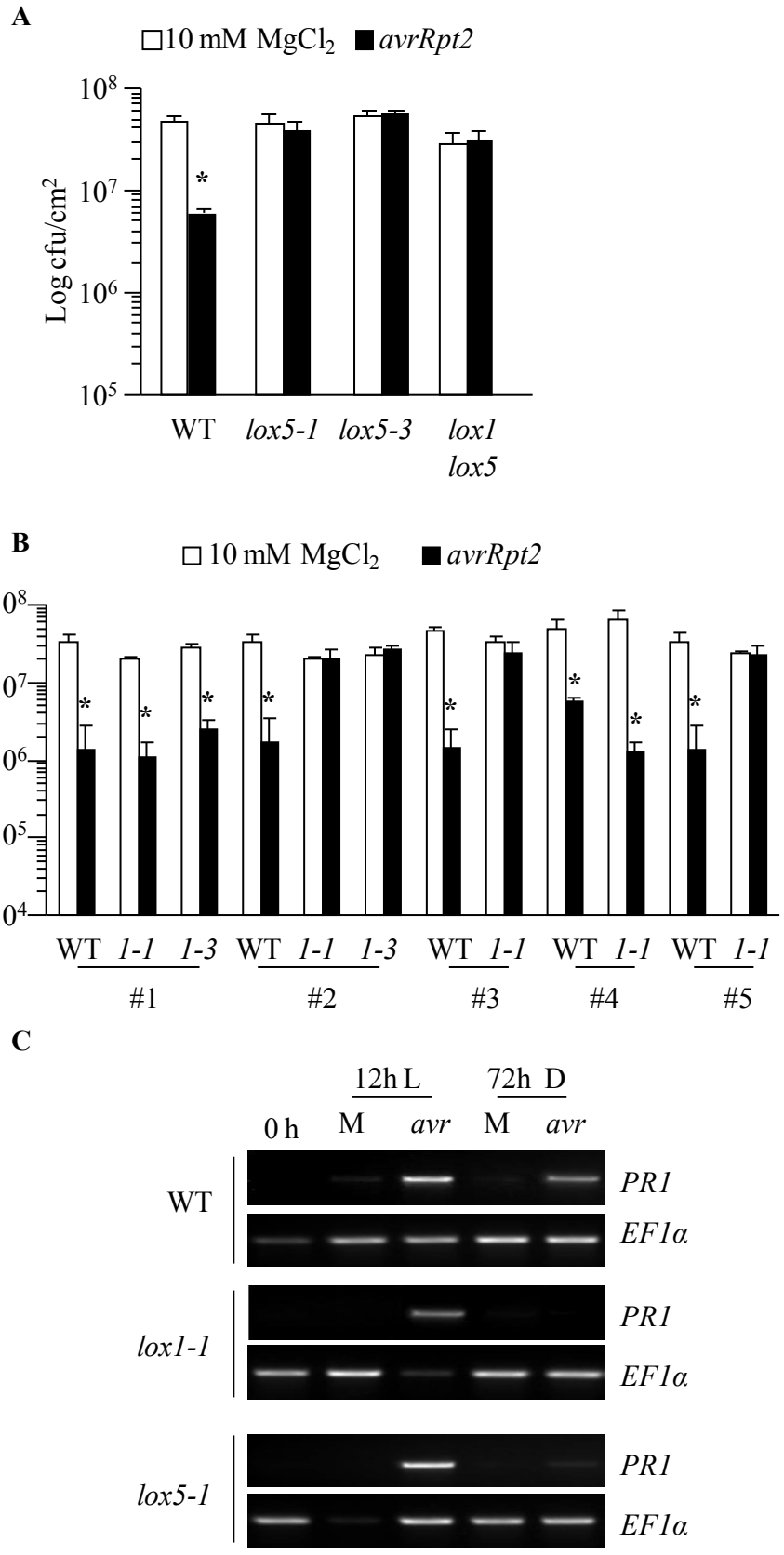
Figure 3.3. Basal resistance to avirulent and virulent pathogen is not altered in 9-LOX mutants. **A.** Growth of avirulent pathogen, *P. syringae* pv. *tomato* DC3000 carrying the *avrRpt2* gene (*Pst*) in wild-type accession Col-0 (WT), *lox1-1* and *lox5-1* mutants. B**.** Growth of a virulent strain of *Pst* lacking the *avrRpt2* gene (P61) in wild-type accession Col-0 (WT), *lox1-1* and *lox5-1* mutants. In A and B, three-four leaves were infiltrated with *bacterial* strain indicated and bacterial growth was monitored 3 days post-inoculation (dpi). Each bar represents the average bacterial growth in 15 leaf discs ± standard error. The experiments were repeated twice with similar results.

3.3.3 Systemic Acquired Resistance is Compromised in *Arabidopsis* 9-LOX Mutants

To determine if the 9-LOX pathway contributes to SAR, SAR conferred enhanced resistance against the virulent bacterial pathogen *Psm* was studied in the *lox1-1*, *lox1-3*, *and lox5-1*and *lox5-3* mutant plants. SAR was induced by the infiltration of 3-4 fully-expanded lower leaves (1º inoculation) of each plant with a suspension in 10 mM $MgCl_2$ of an avirulent pathogen, *Pst* and 72 h later 3-4 distal leaves were challenged with a virulent strain, *Psm* (2º

inoculation). The growth of *Psm* was monitored in the 2º inoculated leaves 72 hours post inoculation (hpi). As a control, the growth of *Psm* was also monitored in distal leaves of plants that were pre-treated with 10 mM $MgCl_2$ as control. As expected, the growth of *Psm* was lower in wild type (WT) accession Columbia-0 (Col) plants that received a *Pst* 1º inoculation implying the activation of SAR (Figure 3.4A). By comparison, *Psm* numbers were comparable between the 10 mM $MgCl_2$ and *Pst* 1º inoculated *lox5-1* and *lox5-3* plants(Figure 3.4A) , implying that biologically-induced SAR was compromised in these 9-LOX mutants. In case of the *lox1-1* and *lox1-3* mutant plants, SAR was attenuated in three out of five experiments (Figure 3.4B). In the other two experiments, significant SAR activity was observed, suggesting that the contribution of *LOX1* in SAR is conditioned by other factors (Figure 3.4B), most likely environmental factors. For example, humidity and light are known to impact the strength of SAR.

The induction of SAR in response to *Pst* infection is associated with the increased accumulation of *PR1* transcripts in the local and distal uninfected tissue. Although, *PR1* transcripts accumulated to WT levels in *lox1-1* and *lox5-1*, the induction of SAR-conferred *PR1* transcript accumulation was attenuated in the distal leaves of *Pst*-treated *lox1-1* and *lox5-1* mutants (Figure 3.4C). The ectopic expression of *LOX1* and *LOX5* or *LOX1-GFP* and *LOX5-GFP* fusions from the *Cauliflower mosaic virus 35S* promoter restored the induction of systemic defense and SAR-mediated enhanced resistance to *Psm* (Figure 3.4D). Compared to the *lox1-1* and *lox5-1* single mutants, SAR was comparably compromised in the 9-LOX double mutant, *lox1-1 lox5-1* plants (Figure 3.4A). Taken together, the data confirm that 9-LOX activity is required for the activation of SAR.


A
□10 mM MgCl2 ■ avrRpt2
Log cfu/cm2
10^8
10^7
10^6
10^5
*
WT
lox5-1
lox5-3
lox1 lox5
B
□ 10 mM MgCl2 ■ avrRpt2
Log cfu/cm2
10^8
10^7
10^6
10^5
10^4
WT 1-1 1-3 WT 1-1 1-3 WT 1-1 WT 1-1 WT 1-1
#1
#2
#3
#4
#5
C
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72h D
0 h
M
avr
M
avr
WT
lox1-1
lox5-1
PR1
EF1α

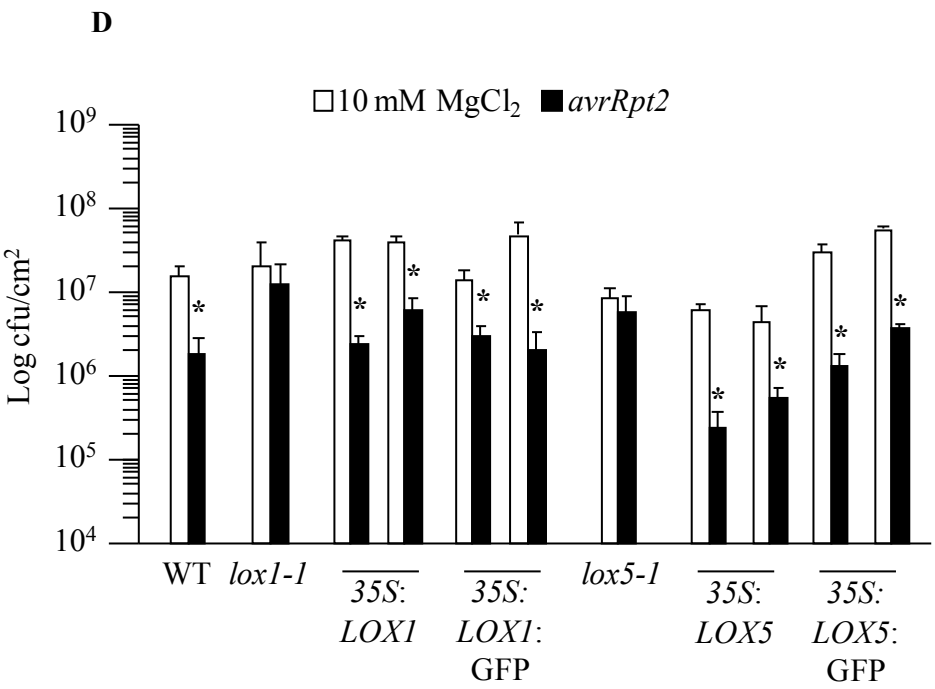

Figure 3.4. 9-LOXes are required for SAR. **A.** Growth of the virulent pathogen *P. syringae* pv *maculicola* (*Psm*) in wild-type (WT) accession Col-0, *lox1-1*, *lox5-1* and *lox1 lox5* double mutant plants. **B.** Growth of virulent *Psm* in WT, *lox1-1* and *lox1-3* in five different experiments where SAR was not attenuated in 2 out of 5 experiments with *lox1* mutant plants. **B.** Growth of virulent *Psm* in WT and *lox1-1* showing a second phenotype. **C.** Reverse transcription-PCR analysis of *PR1* expression in the local (L) and upper/distal leaves (D) of WT, *lox1-1* and *lox5-1* mutants, at 12 and 72 h post inoculation with 10 mM $MgCl_2$ (M) or the avirulent pathogen, *Pst*. *EF1α* provided the control for RT-PCR. **D.** Growth of virulent *Psm* in WT, *lox1-1*, *lox5-1*, two independent lines of *35S:LOX1*, *35S:LOX1-GFP*, *35S:LOX5* and *35S:LOX5-GFP* transgenic lines. In A, B and D, three-four lower leaves were infiltrated with *P. syringae* pv. *tomato* DC3000 carrying the *avrRpt2* virulence gene (*Pst*). Plants that were infiltrated with 10 mM $MgCl_2$ provided controls. Three days later, three-four upper leaves were infiltrated with *Psm* and bacterial numbers monitored 3 days post-inoculation (dpi). Each bar represents the average *Psm* count in 15 leaf discs ± standard error of mean. The experiments in A were repeated three times and the experiment in D was repeated two times and data from a representative experiment presented. Error bars represent standard errors. Asterisk indicates significant difference ($P < 0.05$) from corresponding mock control by *t*-test.

3.3.4 9-LOXs are Required for SAR signal(s) Perception and Generation

The delayed HR and compromised SAR suggest that 9-LOX mutants are either unable to generate or perceive the SAR signal(s). The systemic nature of SAR suggests that phloem acts as a conduit for the movement of the SAR signal(s). To determine if *lox1-1* and *lox5-1* are involved

in the generation or perception of the SAR signal(s), petiole exudates (pet-exs), which are enriched in phloem sap, were collected from WT and mutant plants that were treated with either 10 mM $MgCl_2$ (M-Pet-ex) or *Pst* (Avr-Pet-ex). Petiole exudates from *Pst*-treated WT plants was previously shown to induce systemic expression of *PR1* and a enhanced resistance against *Psm* when infiltrated into *Arabidopsis* plants as compared to M-Pet-ex (Maldonado et al., 2002; Chaturvedi et al., 2008). In agreement with previous studies, the infiltration of Avr-Pet-ex from WT plants compared to M-Pet-ex was able to enhance resistance to *Psm* in the upper leaves of WT plants (Figure 3.5).

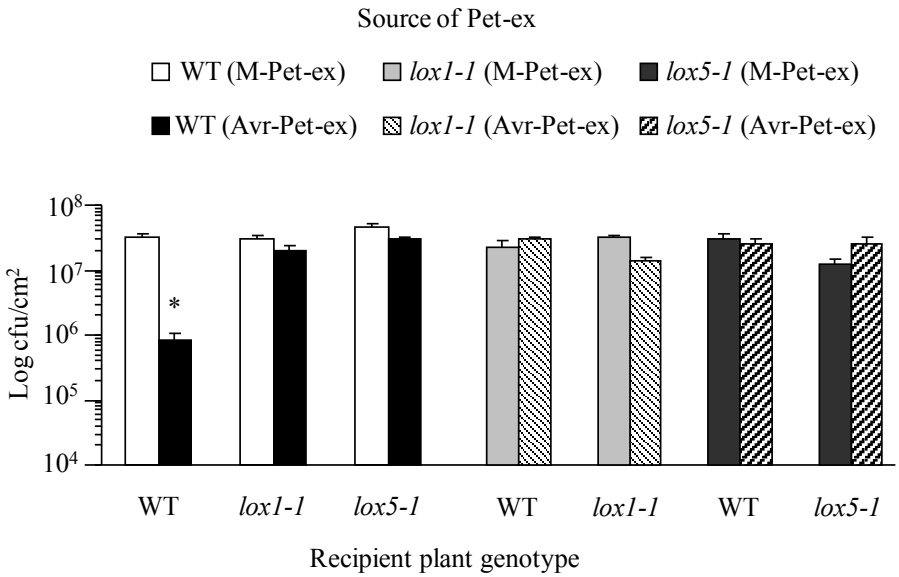

Figure 3.5. SAR signal perception and generation requires 9-LOX function. Petiole exudates were collected from WT, *lox1-1* and *lox5-1* mutants that were treated with 10 mM $MgCl_2$ (M-Pet-ex) and the avirulent pathogen, *Pst* (Avr-Pet-ex). These Pet-exs were infiltrated into three-four lower leaves of WT and 9-LOX mutants. Three days later, three-four upper leaves were challenged with *P. syringae* pv. *maculicola* (*Psm*) and bacterial growth was determined 3 days post-inoculation. Each bar represents the average *Psm* growth in 15 leaf discs ± standard error. The experiment was repeated three times and data from one experiment is presented. Asterisk indicates significant difference ($P < 0.05$) from corresponding mock control by *t*-test.

However, a similar induction of SAR associated enhanced resistance against *Psm* was not observed when WT Avr-Pet-ex was infiltrated into lower leaves *lox1-1* and *lox5-1* (Figure 3.5)

demonstrating that *lox1-1* and *lox5-1* are unable to perceive the SAR signal(s) that is present in WT Avr-Pet-ex. To determine if *lox1-1* and *lox5-1* are able to generate the signal(s) that is necessary for SAR induction, Avr-Pet-ex collected from *lox1-1* and *lox5-1* mutants was applied to 3-4 lower fully expanded leaves of WT plants to determine if it would promote resistance against *Psm* in the upper leaves. Plants similarly treated with M-Pet-ex collected from *lox1-1* and *lox5-1* plants provided the controls. As shown in Figure 3.6, Avr-Pet-exs from *lox1-1* and *lox5-1* mutants were unable to promote SAR in the WT plant. *Psm* numbers in plants treated with M-Pet-ex and Avr-Pet-ex collected from *lox1-1* and *lox5-1* plants were comparable to those in plants treated with M-Pet-ex collected from WT plants (Figure 3.5). Together, these data suggest that the *lox1* and *lox5* mutants are defective in the generation and perception of the long-distance SAR signal(s).

3.3.5 Effect of Azelaic Acid on SAR in 9-LOX Mutants

Azelaic acid, a C9 dicarboxylic acid, has been implicated as SAR signals (Jung et al., 2009). To determine if azelaic acid can induce systemic defenses in the 9-LOX mutants, the compound was infiltrated into lower leaves of WT, *lox1-1* and *lox5-1* mutant plants and the growth of *Psm* was monitored in the distal leaves. Plants that were treated with 5 mM MES which was used to dissolve azelaic acid provided the controls. As expected and shown in Figure 3.6, the infiltration of azelaic acid into the lower leaves was able to enhance resistance to *Psm* in the distal leaves of the WT plants. In the 9-LOX mutants however, infiltration of azelaic acid in the lower leaves did not induce systemic defenses and *Psm* growth in the distal leaves was similar to the levels observed in plants that were treated with 5 mM MES (Figure 3.6). This data suggests that *lox1-1* and *lox5-1* are unable to perceive and/or respond to azelaic acid.

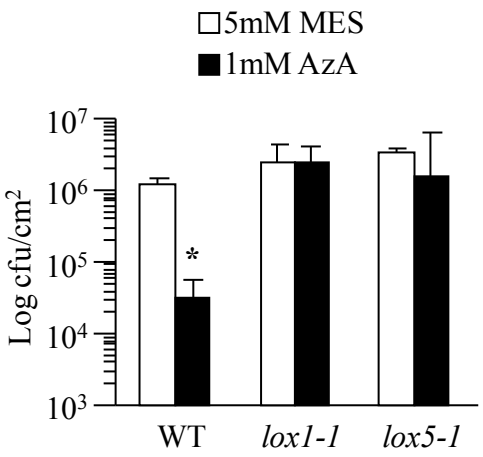

Figure 3 6 SAR Response of 9-LOX mutants to azelaic acid treatment. Azelaic acid (AzA) was infiltrated in three-four lower leaves of WT, *lox1-1* and *lox5-1* mutants at 1 mM concentration. Plants that were treated with 5 mM MES that was used as solvent for AzA provided the controls. Three days after chemical treatment, three-four upper leaves were challenged with *P. syringae* pv. *maculicola* (*Psm*) and bacterial growth was determined 3 days post-inoculation. Each bar represents the average *Psm* growth in 15 leaf discs ± standard error of mean. The experiment was repeated two times and data from one experiment is presented. Asterisk indicates significant difference ($P < 0.05$) from corresponding mock control by *t*-test.

3.4 Discussion

In this study, the data presented indicate a role for *Arabidopsis* 9-LOXs, *LOX1* and *LOX5*, in contributing to the activation of systemic acquired resistance (Figure 3.4). The induction of *LOX1* and *LOX5* in response to infection by avirulent pathogen suggests that 9-LOXs are important for the plant defense response (Figure 3.1). However, mutations of LOX1 and LOX5 did not affect the ability to mount sufficient local defenses to avirulent and virulent pathogens (Figure 3.3). *LOX1* and *LOX5* were found to be crucial for the plant to be able to stimulate HR in response to infection by avirulent pathogens (Figure 3.2). The delay in onset of HR suggests that timely HR is required for the activation of SAR. The inability of petiole exudates from *Pst*-treated (Avr-Pet-ex) *lox1* and *lox5* to activate SAR in WT plants demonstrates that 9-LOX activity is required for the production of a SAR-inducing activity that is present in Avr-Pet-ex of WT plants (Figure 3.5). Further, the inability of Avr-Pet-ex from WT plants to

activate SAR when applied onto *lox1* and *lox5* implies that 9-LOX activity is also essential in distal uninfected tissue for the activation of systemic defenses (Figure 3.5). Together, these results suggest that compromised SAR in 9-LOX mutants stems from the inability of the mutants to initiate timely HR and also respond to SAR-inducing factors.

LOX1 and *LOX5* are 9-LOXs that are induced during HR of *Arabidopsis* leaves during infection by an avirulent pathogen, *Pst* suggest an essential role for 9-LOXs in establishing HR. Cell death observed during HR has been suggested to be in part the result of lipid peroxidation that is initiated by LOX activity. The accumulation of LOXs has been shown to be associated with leaf cell death in *Arabidopsis* (Montillet et al., 2002; Hwang and Hwang, 2010). In accordance with these studies, mutations in the 9-LOX pathway reduced the plants ability to mount HR during an incompatible interaction with an avirulent pathogen. At the same time, reduced UV-visible fluorescence was detected in 9-LOX mutants suggesting the lack of accumulation of phenolic compounds associated with cell death confirming a role of 9-LOX-derived oxylipins in cell death during HR. However, the inability of 9-LOX mutants to mount a strong HR response did not affect the mutants' ability to limit growth of *Pst* and *Psm*, in agreement with previous reports (Vicente et al., 2011). Contrary to Hwang and Hwang, 2010 and Vicente et al., 2011, enhanced susceptibility to *Psm* was not observed in the 9-LOX mutants. However, the data here suggests that compromised SAR observed in 9-LOX mutants is not the result of enhanced susceptibility to either avirulent or virulent pathogens.

Several studies have shown the involvement of lipid-derived molecule(s) as critical factors in the induction of SAR (Nandi et al., 2004; Chaturvedi et al., 2008; Jung et al., 2009; Chanda et al., 2011). Results presented here show that mutation of 9-LOXs in *Arabidopsis* results in the inability of the mutants to initiate SAR implying a role for 9-LOX derived

oxylipins in this process. The presence of 9-LOX derived oxylipins in phloem sap (Harmel et al., 2007; Nalam et al., 2012) suggests that these compounds can potentially act as mobile signals. The protecting action of 9-LOX derived oxylipins against bacterial pathogens is restricted to local tissues (Vicente et al., 2011) suggesting that they do not act as mobile signals that induce systemic resistance. However, further experimentation is required to conclusively determine this possibility.

The induction of systemic resistance in response to local infection by an avirulent pathogen requires the generation of a signal in the inoculated tissue which must then be transported systemically via the vasculature, generally the phloem. In the past few years several, phloem-mobile signals including a methylated derivative of SA (MeSA), an abietane diterpenoid dehydroabietinal (DA), jasmonic acid (JA), a lipid derived signal, a lipid transfer protein and a dicarboxylic acid azelaic acid (AzA) have all been implicated as the mobile signal(s) (Vlot et al., 2008; Shah, 2009). The observation that unlike WT Avr-Pet-ex, Avr-Pet-ex from *lox1* and *lox5* was unable to initiate SAR and that Avr-Pet-ex from *lox1*and *lox5* is unable to initiate systemic resistance in WT plants (Figure 3.5) provides clues to the lack of induction of systemic resistance in *lox1* and *lox5*. This data suggests that *lox1* and *lox5* are unable to produce the SAR promoting activity is present in Avr-Pet-ex from WT plants. The measurement of known SAR signals such as MeSA, DA, JA and AzA in Avr-Pet-ex from *lox1* and *lox5* will provide an insight into the signal(s) that are absent. Although, not essential, the magnitude of HR has been suggested to contribute to the induction of SAR (Cameron et al., 1994). The inability of 9-LOX mutants to initiate SAR and the incidence of reduced HR in these mutants suggests that the manifestation of HR is necessary for the generation of a long distance SAR signal during incompatible interactions. Further the attenuation of *PR1* expression, a biomarker for SA accumulation, in the

distal leaves of *lox1* and *lox5* during SAR suggests a possible role for 9-LOXs in influencing MeSA and SA responses. Oxylipins derived from the 9-LOX pathway have also been implicated in modulating plant defense response by interaction with the plant defense hormones such as ethylene and abscisic acid (López et al., 2011; Vicente et al., 2011). The lack of induction of systemic resistance in 9-LOX mutants can therefore be due to various possibilities and further analysis is necessary.

Among the various SAR signals, a dicarboxylic acid, azelaic acid bears structural similarity to 9-Oxo-nonanic acid, a 9-LOX derivative. However, the biosynthetic pathway for azelaic acid is as yet unknown. The synthesis of 9-Oxo-nonanic occurs due to the action of hydroperoxide lyase on 9-hydroperoxytrienonic acid (9-HPOT) (Feussner and Wasternack, 2002). Although, WT Avr-Pet-ex contain azelaic acid (Jung et al., 2009), the failure of WT Avr-Pet-ex to initiate SAR in *lox1* and *lox5* raises the possibility that the 9-LOX mutants are unable to respond to azelaic acid and initiate SAR. Indeed, infiltration with azelaic acid was unable to activate SAR in *lox1* and *lox5* (Figure 3.6) providing additional evidence for the role of 9-LOXs in the synthesis of azelaic acid. However, further experiments involving the measurement of azelaic acid in the Avr-Pet-exs of *lox1* and *lox5* and the application of 9-Oxo-nonanic acid are needed to confirm this possibility. In summary, the results presented here demonstrated that 9-LOX activity is required for the activation of the SAR response in *Arabidopsis*.

3.5 Methods

3.5.1 Plant and Pathogen Cultivation

Arabidopsis was grown at 22^{o}C under 14 h light (100 μE m^{-2} sec^{-1})/ 10 h dark regime in autoclaved compost-peat based planting mixture (Premier Pro Mix-PGX, Procunier, Canada).

Plants that were approximately 28-32 days old were used for all experiments. *Pseudomonas syringae* pv. *maculicola* (*Psm*) ES4326 was cultured at 28^{o}C on King's B medium (King et al., 1954) containing streptomycin (100 mg/L). *P. s. tomato* DC3000 (*Pst)* containing *avrRpt2* and *Pst* without *avrRpt2* (P61) were also culture in King's B media with 25 mg/L rifampicin and 50 mg/L kanamycin. An overnight culture was used for all experiments.

3.5.2 Bacterial Inoculations

Bacterial inoculations were performed with a needle-less syringe, by infiltrating the bacterial suspension into the abaxial side of the leaves. The bacterial strains used in the study were *Pst* DC3000 with *avrRpt2* (*Pst*, avirulent), *Pst* DC3000 (P61, virulent) and *Psm* (virulent). For quantification of bacterial growth, 3-4 fully expanded leaves were infiltrated with a bacterial suspension of 10^5 colony forming units (CFU) ml^{-1}. For analyses of systemic acquired resistance (SAR), 3-4 lower fully expanded leaves were infiltrated with a suspension (10^7 CFU ml^{-1}) in 10 mM $MgCl_2$ of the avirulent pathogen, *Pst* DC3000 with *avrRpt2*. Plants that were infiltrated with 10 mM $MgCl_2$ provided the mock controls. The plants were covered with a transparent dome for 16h to maintain high humidity after which the dome was removed. Three days later, 3-4 upper leaves were challenged with a suspension (10^5 CFU per ml, OD 0.00025) of the virulent pathogen *Psm* ES4326. The *Psm* challenged leaves were harvested 3 days post inoculation (dpi) and a cork borer was used to punch out leaf discs (area = 0.28 cm^2), which were ground in 10 mM $MgCl_2$ and a serial 10-fold dilutions were plated on King's medium containing streptomycin (100 mg per ml). The plates were then incubated at 28^{o}C for 2 days before counting the bacterial colonies. A total of 15 leaf discs (3 replications of five leaf discs in each sample) were analyzed per treatment.

3.5.3 Measurement of Electrolyte Leakage

Leaves 4 week old *Arabidopsis* plants were infiltrated with a suspension (10^7 CFU ml^{-1}) in 10 mM $MgCl_2$ of the avirulent pathogen, *Pst* DC3000 with *avrRpt2* to determine electrolyte leakage. Leaf discs (diameter = 0.7 cm) were removed from plants at respective time points and washed for 10 min in distilled water and transferred to 10 ml of distilled water. Conductance was then measured with a conductivity meter (Model Con 510, Oakton).

3.5.4 Reverse Transcriptase-PCR

RNA extraction from leaves and roots and cDNA synthesis was performed as previously described (Pegadaraju et al., 2005). DNA contamination in samples was removed by treatment RNase-free DNase. For each time point and treatment, RNA was collected from two biological replicates, each consisting of leaves pooled from 4 plants. Gene specific primers used for Reverse Transcriptase (RT-PCR) are listed in Table 4.1. The PCR conditions for all RT-PCR reactions were as follows: 95^{o}C for 5 min, followed by 25 cycles of 95^{o}C for 30 s, 58^{o}C for 45 s and 72^{o}C for 45 s, followed by a final extension step of 72^{o}C for 5 min.

3.5.5 Petiole Exudate Collection

Petiole exudates (Pet-ex) were collected from *Arabidopsis* leaves using a previously described method (Chaturvedi et al., 2008). A bacterial suspension of *Pst* DC3000 *avrRpt2* (10^7 CFU ml^{-1}) or 10 mM $MgCl_2$ were infiltrated into the abaxial surface of leaves. The plants were placed under a transparent plastic dome to maintain high humidity. Eight hours after infiltration, leaves were cut at the base of their petioles and the petiole immediately dipped in 50% Ethanol

followed by 0.0005% Bleach and finally in 1 mM EDTA (pH 8.0) solution. After 10 minutes, a small portion of the petiole base was cut and 6 leaves were immediately placed in 3 ml of 1 mM EDTA (pH 8.0) contained in a well of a 24-well tissue culture plate (ICN Biochemical Inc.; http://www.mpbio.com). High humidity was maintained by placing the entire setup on wet paper towels and under a transparent dome. The leaves were allowed to exude for a period of 48 h. For each treatment 100-120 leaves were used. The petiole exudates (Pet-exs) from multiple wells were pooled. The pet-exs were diluted 2-fold in sterile water. The biological activity of the pet-exs was determined in a SAR experiment as above.

3.5.6 Chemical Treatment

Azelaic acid was dissolved in 5 mM MES [2-(N-morpholino)ethanesulfonic acid] buffer (pH 5.6) and infiltrated into leaves with a needless syringe. The biological activity of the chemical treatments was determined in a SAR experiment as above.

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CHAPTER 4

Arabidopsis 9-LOX CONTRIBUTE TO HOST SUSCEPTIBILITY TO *Fusarium graminearum* INFECTION

4.1 Abstract

Fusarium head blight (FHB) of wheat, caused by *Fusarium graminearum* (*Fg*) Schwabe, is a common and devastating disease in many wheat-growing regions around the world. Losses due to FHB in some years have reached $1 billion in the United States. The ability of *Fg* to infect and colonize *Arabidopsis* provides an excellent model pathosystem to identify host signaling mechanisms that contribute to this interaction. Previously, it was shown that a complex interaction between salicylic acid (JA) and jasmonic acid (JA) signaling modulates host response to this interaction. SA signaling contributed to resistance. However, JA signaling had a dual role, contributing to host susceptibility during the early stages of infection by attenuating SA signaling, and working in concert with SA in host defense during the later stages of *Fg* infection. Results presented here indicate that *Arabidopsis* 9-lipoxygenases (9-LOXs) contribute to plant susceptibility to *Fg*. Loss of *LOX1* and *LOX5* function resulted in enhanced resistance to *Fg* in the *lox1* and *lox5* mutant plants. This increase in resistance was in part due to the faster activation of salicylic acid (SA)-mediated defenses and suppression of jasmonic acid (JA) responses in *Fg* -infected *lox1* and *lox5* mutants, compared to the wild type plant. Similarly in wheat, RNA-interference-mediated silencing of a wheat lipoxygenase, TaLpx-1, resulted in enhanced FHB resistance, suggesting conservation of LOX function in susceptibility of *Arabidopsis* and wheat to *Fg*.

4.2 Introduction

4.2.1 *Fusarium graminearum*

The genus *Fusarium* comprises of ascomycetes fungi of which several are phytopathogenic. Several economically important plants have at least one *Fusarium*-associated disease. The types of disease caused include various kinds of root rots, seedling blights, foot rots and head blight (Parry et al., 1995; Leslie et al., 2006). Of special importance is Fusarium head blight (FHB) of wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*), caused by a closely related complex of *Fusarium* species with *Fusarium graminearum* Schwabe (abbreviated hereafter as *Fg*) being the most prevalent in the major wheat growing regions around the world. FHB incidence in the field can be observed as premature bleaching of spikes which gives them a scabby appearance. In that past, severe epidemics of FHB have resulted in annual losses ranging from $300 million to $3 billion in the US alone (Johnson et al., 2003; Bai and Shaner, 2004). The economic losses caused by FHB are exacerbated by the severely reduced quality of the harvested grain which are not only shrunken and shriveled but also contaminated with mycotoxins, for example, deoxynivalenol, which render the grain unsafe for consumption by humans and animals (Bai and Shaner, 1994; McMullen et al., 1997a; Bai and Shaner, 2004). Wheat cultivars highly resistant to the disease or tolerant to the toxin are currently not available. Furthermore, the use of fungicides for controlling the disease is limited by cost, difficulty in efficient application to wheat heads and an incomplete understanding of factors that influence disease development (McMullen et al., 1997b; Pirgozliev et al., 2003). The increased incidence of FHB in the wheat and barley growing regions around the world has led to an intense research effort to understand the disease process to aid the development of resistance.

The fungus overwinters on infected crop residue (i.e. debris of previously harvested

wheat, barley or maize) as saprophytic mycelia. Although, *Fg* reproduce sexually via ascospores or asexually via macro conidia, the primary inoculum of virulent *Fg* consists of ascospores present in mature perithecia (Trail et al., 2002). In addition to the amount of inoculum present on crop residue in the field, the environment plays a crucial role in determining the severity of FHB outbreaks. Under warm, moist conditions during the spring, mature perithecia break open releasing sticky ascospores (Markell and Francl, 2003) that are dispersed by wind, rain splash and even insects (Parry et al., 1995). The monocyclic nature of FHB can therefore be attributed to the relatively short period of crop susceptibility that occurs during spike emergence and favorable environmental conditions.

The inflorescence of wheat, also known as a spike, is most susceptible to FHB infection. A wheat spike consists of several spikelets which contain two to four potentially fertile florets. The floret has two sheathing structures or glumes, the outer lemma and inner palea that enclose the stamens and carpel forming the caryopsis. After fertilization and seed development, each grain in a floret is enclosed by the lemma and palea that provide protection to the mature grain. The period of highest susceptibility to fungal infection occurs during a 3-5 day period corresponding to flowering or anthesis, when the spike emerges from the flag leaf sheath (Sutton, 1982). Once the ascospores adhere on to the spike surface, they germinate on the exterior surfaces of the florets and glumes. Upon germination of spores, the ensuing fungal hyphae rapidly elongate to form a complex mycelial network. The hyphae penetrate host tissue through natural openings, such as the stomates, or by direct penetration at other susceptible sites within the inflorescence (Bushnell et al., 2003; Lewandowski et al., 2006). Hydrolases secreted by *Fg* are thought to facilitate in the penetration of wheat tissue (Kang and Buchenauer, 2000; Bushnell et al., 2003). In wheat, anthers provide the initial path for FHB infection and higher anther

extrusion correlated with resistance (Strange and Smith, 1971; Skinnes et al., 2008). Once penetration of the host is achieved, fungal hyphae grow subcuticularly and intracellularly in the wheat spike for up to 2 d after initial infection (Pritsch et al., 2000; Jansen et al., 2005). Although, the infection exhibits a necrotrophic phase, during which *Fg* kills the host cells to obtain nutrients, during the later stages of infection, the initial intracellular growth is thought to constitute a biotrophic phase, during which *Fg* grows intracellularly and obtains nutrients from living cells (Boddu et al., 2006; Ding et al., 2011). After the successful establishment of infection, *Fg* is thought to switch to necrotrophic phase during which hyphae systemically infect adjacent florets and proceed to infect the rest of the spike (Jansen et al., 2005). During this phase of fungal growth, hyphae grow in the apoplast causing major perturbations in the plant cell ultimately killing it. The mycotoxin, DON, contributes to the spread of fungus by causing cell damage through promotion of hydrogen peroxide production and prevention of protein synthesis (Bushnell et al., 2005; Desmond et al., 2008; Bushnell et al., 2010). The vascular bundle comprising of the phloem and xylem, that supply developing grains with nutrients, acts as a conduit for fungal hyphae to rapidly spread from spikelet to spikelet. The clogging of the vascular bundle coupled with the production of mycotoxins and plant cell hydrolyzing enzymes prevents grain development and also produce dark purple to black necrotic lesions on the surface of the florets and glumes.

4.2.2 Plant Defense against *Fusarium graminearum*

In the absence of monogenic gene-for gene resistance to *Fg*, breeders have looked to ‘exotic’ or ‘wild’ relatives of cultivated wheat as sources for resistance genes. Numerous studies have shown that natural resistance to FHB in wheat is quantitative in nature and mapping studies

have identified 52 quantitative trait loci (QTL) that contribute to FHB resistance (Buerstmayr et al., 2009). Although, large genetic variation for FHB resistance is available in the wheat gene pool, the challenge for wheat breeders has been the introgression of these QTL which are often associated with undesirable characteristics such as spike shattering and susceptibility to other pests and pathogens (Rudd et al., 2001). The major goal of breeders has therefore been the development of regionally adapted commercial wheat cultivars that combine high yield and quality with resistance to diseases and pests including FHB. Although, this approach has aided the development of FHB resistant cultivars, the level of resistance provided is insufficient to prevent FHB epidemics.

Two major categories of resistance have been proposed in wheat (Schroeder and Christensen, 1963). The ability of certain resistant lines to resist initial infection is termed type I resistance, whereas the ability to prevent fungal spread from initially infected spikelets to the rest of the spike and thus contribute to systemic resistance is classified as type II resistance. Three other categories of resistance to FHB have been proposed (Mesterhazy, 1995), which include resistance to kernel infection (type III), resistance to DON accumulation (type IV) and finally, tolerance (type V). These categories are not as widely accepted as type I and II resistance because of the difficulties associated with their determination (Bai and Shaner, 2004). In wheat resistance to FHB is thought to be a complex, quantitative trait involving a complex and interacting network of pathways (Bai and Shaner, 2004; Walter et al., 2010).

Type II resistance has been more extensively investigated in wheat since it is more stable and less affected by non-genetic factors as compared to type I resistance (Bai and Shaner, 2004). In wheat plants with type II resistance, fungal movement from infected spikes via the vascular bundles either delayed or prevented (Ribichich et al., 2000). The *Fhb1* locus has been associated

with conferring robust type II resistance that is stable and consistent across environments. The source of the *Fhb1* locus is a Chinese spring wheat cultivar, Sumai 3. Although, the mechanism of *Fhb1*-mediated resistance is unknown, several factors including physical and chemical barriers to resist fungal spread, timing of induced plant defenses and detoxification of DON have been implicated in contributing to type II resistance (Walter et al., 2010; Bischof et al., 2011).

The economic impact of FHB has led to research focused on unraveling host molecular mechanisms associated with resistance. A combination of transcriptomic, proteomic and metabolomic approaches is shedding light on the complexity of the interaction between *Fg* and the host plant (Walter et al., 2010; Bischof et al., 2011; Kazan et al., 2011).

Analysis of host gene expression at the transcriptional level in response to *Fg* infection reveals significant overlap between the classes of host genes induced with many other plant-microbe interactions. The expression of a large set of genes involved in cell death, cell wall modifications, toxin (DON) metabolism and hormone homeostasis is altered in response to *F. graminearum* infection (Boddu et al., 2006; Bernardo et al., 2007; Golkari et al., 2009; Jia et al., 2009). In order to determine the mechanism of resistance associated with *Fhb1* QTL, near-isogenic wheat lines (NILs) that differ by the *Fhb1* locus were used in microarray studies (Golkari et al., 2009; Jia et al., 2009). Interestingly, although the expression of a large set of genes is altered in response to *Fg*-infection, only a small subset of genes displayed differential expression between the NILs. Jia et al. (2009) identified 14 genes whereas Golkari et al. (2009) identified 25 gene transcripts whose expression was significantly up-regulated in the presence of *Fhb1* locus. Analysis of the candidates may provide further insights into the mechanism of resistance conferred by the *Fhb1* locus. It should be noted however that although the *Fhb1* locus

was the same in both the studies, the parents that were used to generate the NILs were different suggesting a possible role for other genotypic factors.

In wheat-*Fg* interaction, defense signaling cascades mediated by the hormones SA, JA and ET have been observed to be induced in several microarray studies (Kruger et al., 2002; Kong et al., 2005; Bernardo et al., 2007; Li and Yen, 2008). In wheat, expression of the gene encoding Pathogenesis-Related 1 protein (*PR1*), whose expression is promoted by SA, is induced in *Fg*-infected spikes (Pritsch et al., 2001) suggesting a role for SA-mediated defenses in host defense against *Fg* in wheat. Further, the constitutive expression in wheat of the *Arabidopsis NON-EXPRESSOR OF PATHOGENESIS-RELATED GENES1* (At*NPR1*), a key regulator of SA-mediated responses in *Arabidopsis*, resulted in increased FHB resistance (Makandar et al., 2006). Recently, it was shown that the FHB disease severity is higher in transgenic *NahG*-expressing wheat in which SA accumulation is diminished due to its conversion to catechol by the NahG-encoded SA hydroxylase (Makandar et al., 2012). Thus, SA-mediated defenses contribute to basal resistance to *Fg*. The role of the JA pathway in wheat defense against FHB appears to be more complex. JA applied early during infection suppressed *AtNPR1*-expression determined resistance against FHB. However, when applied later during infection, JA promoted FHB resistance in wheat (Makandar et al., 2012). Consistent with this observation, RNAi mediated silencing of the wheat ethylene insensitive 2 (*EIN2*) gene resulted in increased FHB resistance. By utilizing an integrated proteomic and transcriptomic approach in combination with measurements of SA and JA in a FHB-resistant line and its susceptible mutant, Ding et al. (2011) were able to demonstrate a biphasic signaling event that occurs during FHB-resistance. During the early stages of infection (6 hours after inoculation, hai), SA signaling pathways are activated followed by the activation of JA signaling around 12 hai and the activation ET signaling

occurring between the two phases. In the susceptible mutant, the activation of SA signaling is delayed further highlighting the importance of SA in basal resistance to FHB in wheat (Ding et al., 2011). The study provides an integrated view of the coordinated and ordered activation of SA and JA/ET signaling pathways that is necessary for the development of resistance in *Fg*-infected wheat.

4.2.3 Lipoxygenases in Cereals

In cereal crops, LOXs from barley have been studied in more detail than in wheat. Three LOX genes have been reported for barley of which *LOX-1* (*LoxA*) encodes a 9-LOX and *LOX-2* (*LoxC*) encodes a 13-LOX (Doderer et al., 1992; Van Mechelen et al., 1999). The product related to *LOX-3* (*LoxB*) has not been identified so far. Expression analysis revealed that these LOXs are mainly expressed in grains and germinating seedlings (Schmitt and Van Mechelen, 1997). In common wheat three LOX loci, *Lpx-1, 2* and *3* have been reported (Hart and Langston, 1977; Hessler et al., 2002; Wan et al., 2002; Carrera et al., 2007). Based on sequence similarities with barley *LOX* genes, the *Lpx-1* locus has been associated with the barley *LoxA* gene (Hessler et al., 2002) whereas the *Lpx-2* and *Lpx-3* loci correspond to barley *LoxC* and *LoxB* respectively (Carrera et al., 2007). Wheat is a hexaploid with three genomes, A, B and D, therefore the genes for each loci present on each genome are named *Lpx-A1, B1* and *D1* for the *TaLpx-1* locus, *Lpx-A2, B2* and *D2* for the *TaLpx-2* locus and *Lpx-A3, B3* and *D3* for the *TaLpx-3* locus. Although, the full-length sequences of three wheat *LOX* genes (*TaLOX1*, *TaLOX2* and *TaLOX3*) were recently cloned and found to correspond to *Lpx-D1, Lpx-D2* and *Lpx-A3* respectively (Feng et al., 2010; Feng et al., 2011), the biochemical characterization of wheat *Lpx* genes is still lacking.

Wheat LOX isoforms have been mainly isolated from endosperm where they play an

important role in influencing the quality of wheat flour and its end products, pasta and bread (Hsieh and McDonald, 1984). The bright color of the final pasta product is the result of accumulation of natural carotenoid yellow pigments and their oxidative degradation by LOX whereas high LOX activity negatively impacts bread quality. High LOX activity during milling for pasta and bread processing, results in the excessive oxidative degradation of carotenoids resulting in loss of quality and nutritional value (Borrelli et al., 1999; Leenhardt et al., 2006). High carotenoid content in wheat flour enhances the quality and nutritional value of pasta since carotenoids are involved in reducing oxidative damage by scavenging peroxyradicals (Bast et al., 1996). The development of wheat cultivars with low LOX activity and higher yellow pigment has therefore been an important breeding objective.

4.2.4 9-LOX in Cereal Defense/Susceptibility to Disease

The importance of the 9-LOX pathway in defense and susceptibility to diseases in cereal crops has been shown in several studies in maize. A maize mutant for *ZmLOX3* which encodes a 9-LOX displays reduced diseases severity to a variety of fungal pathogens, *Fusarium verticilloides*, *Colletotrichum graminicola* and *Cochliobolus heterosporus* (Gao et al., 2007; Gao et al., 2009). However, the same knock-out mutant is also susceptible to infection by maize seed pathogens, *Aspergillus flavus* and *A. nidulans* (Gao et al., 2009) suggesting that the role of the 9-LOX pathway in contributing to maize resistance or susceptibility is pathogen specific.

Several lines of evidence implicate a role for LOXs in wheat interaction with pests and pathogens. The application of BTH [benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester] a synthetic analog of salicylic acid (SA) provided resistance against several fungal pathogens (Gorlach et al., 1996; Makandar et al., 2012) and the onset of resistance was tightly correlated

with the induction of various genes called *WCI* (Wheat Chemically Induced) which include a putative lipoxygenase-encoding gene *WCI-2* (Gorlach et al., 1996). LOX activity was stimulated in wheat leaves in response to wounding, treatment with elicitors from the rust fungus, *Puccinia graminis* and methyl jasmonate (MJ) (Bohland et al., 1997; Mauch et al., 1997). The production of endogenous elicitors, oligogalactouronides, during infection by powdery mildew, *Blumeria graminis* f. sp. *tritici* resulted in increased LOX activity which contributes to resistance. (Randoux et al., 2010). However, in wheat interaction with a hemibiotrophic pathogen *Mycosphaerella graminicola,* that causes Septoria leaf blotch the induction of defense responses was associated with down regulation of a putative wheat LOX suggesting that repression of LOX activity can also contribute to resistance (Ray et al., 2003). In addition, wheat LOXs also appear to play important roles during insect infestation. *WCI-2*, was strongly up-regulated during Hessian fly, *Mayetiola destructor* (Say), larval feeding on wheat plants (Sardesai et al., 2005). Wheat plants that are resistant to infestation by the Russian wheat aphid, *Diuraphis noxia,* displayed a rapid induction of genes involved in oxylipin metabolism (Smith et al., 2010). With respect to wheat-*Fg* interaction, microarray-based expression profiling indicated genes of oxylipin metabolism, including several LOXs, were differentially expressed in spikes infected with *Fg* (Kruger et al., 2002; Li and Yen, 2008). However, it is still unclear whether the induction/repression of these genes in response to *Fg*-infection contributes to host resistance or susceptibility. The lack of availability of wheat mutants in the LOX pathway makes it difficult to further characterize their role in wheat-*Fg* interaction.

4.2.5 *Arabidopsis* as a Model System for Studying Plant Defense and Susceptibility to *Fg*

The discovery that *Fg* can infect, colonize and cause disease in *Arabidopsis* has led to its

utilization as model pathosystem to study plant resistance/susceptibility mechanisms in response to *Fg* infection. The inoculation of *Arabidopsis* inflorescence results in fungal growth and disease development on the emerging siliques (Urban et al., 2002; Cuzick et al., 2008). However, the floral assay prevents the further study of changes in gene expression that occur in response to *Fg* infection. A second assay method involved the placement of fungal spores on either unwounded or wounded leaf surfaces (Chen et al., 2006). In this case, although the spores germinated and the hyphae branched, leaf penetration was poor (Chen et al., 2006). An efficient infection procedure is needed to overcome the drawbacks of the above mentioned methods.

In *Arabidopsis,* SA is required for resistance to *Fg*. The *Arabidopsis npr1* mutant, which is insensitive to SA and the *sid2* mutant, which is deficient in SA production, and the NahG plants, which are deficient in SA, display enhanced susceptibility to Fg than the WT plant. By contrast, the *ssi2* mutant, which contains high endogenous levels of SA exhibited enhanced basal resistance to Fg. In agreement for a role for SA in defense against *Fg*, exogenous application of SA enhanced resistance to *Fg* (Cuzick et al., 2008; Makandar et al., 2010). By contrast to SA, JA was shown to have a dichotomous role in *Arabidopsis*-*Fg* interaction. During the early stages of infection, JA suppressed the activation of SA signaling and thus contributed to disease susceptibility. The *jar1* mutant, which is unable to synthesize JA-Ile, the biologically active form of jasmonate, exhibited heightened resistance to *Fg* and pretreatment with MeJA attenuated basal resistance against *Fg* and also the *AtNPR1* over-expression-conferred enhanced resistance against *Fg* in *Arabidopsis* (Makandar et al., 2010). However, the *npr1jar1* double mutant, which is doubly defective in SA and JA signaling was more susceptible to *Fg* than the *npr1* single mutant plant, suggesting that in the absence of SA, JA likely has a role in defense, as well. Indeed, JA applied later during infection, after SA signaling had been activated, promoted

resistance against *Fg*. Gene expression and physiological studies indicated that during the early stages of infection JA promoted disease susceptibility by attenuating SA accumulation and the activation of SA signaling, thus contributing to susceptibility (Makandar et al., 2010). Thus the involvement of SA and JA in *Arabidopsis*-*Fg* interaction is similar to the role of these signaling molecules in wheat-*Fg* interaction. Similar to JA signaling, the ET signaling pathway appears to contribute to disease susceptibility in *Arabidopsis*. Disease severity was lower in *Arabidopsis* ET-insensitive mutants whereas ET-overproducing mutants showed enhanced susceptibility (Cuzick et al., 2008; Chen et al., 2009a).

A better understanding of the complex nature of interaction between Fg interaction with wheat is far from complete. The utilization of *Arabidopsis* as model pathosystem has greatly aided in this process. Identification of the key components involved in host-Fg interaction will provide the means to combat this disease in a more efficient and sustainable manner. In this study, we have utilized the *Arabidopsis*-*Fg* model system to determine the role of the 9-LOXs in host-pathogen interaction. We provide evidence that the 9-LOXs contribute to host susceptibility by suppressing SA-mediated defense responses. We further demonstrate that RNA interference (RNAi)-mediated silencing of putative wheat LOXs contributes to enhance resistance to FHB in wheat.

4.3 Results

4.3.1 *Fg* Successfully Colonizes *Arabidopsis thaliana*

In order to overcome the drawbacks of previous inoculation methods (Urban et al., 2002; Chen et al., 2006; Cuzick et al., 2008), an assay was developed in which *Fg* is introduced directly into the apoplastic region of *Arabidopsis* leaves (Makandar et al., 2006; Makandar et al., 2010). A needless syringe was used to infiltrate a suspension of *Fg* into the abaxial side of leaves

of 4-week old WT (Col-0) *Arabidopsis* plants. This method of inoculation resulted in successful infection and disease (Figure 4.1A-C). The presence of *Fg* and hyphal growth could be observed in *Arabidopsis* leaves 5 days post inoculation (dpi) (Figure 4.1A). The inoculated leaves exhibited disease symptoms in the form of chlorosis of leaf tissue. Based on the percentage of leaf area showing chlorosis, leaves were categorized into four classes (<25, 25 to 50, 50 to 75 and >75% chlorosis) (Figure 4.1B). In order to determine if the level of chlorosis observed is a direct result of *Fg* growth, the presence of *Fg* was monitored in the leaves using PCR of the fungal *Tri5* gene (Figure 4.1C). The level of chlorosis observed correlated to the amount of fungal DNA present (Fig. 4.1C), with leaves showing >75% chlorosis having the largest amount of fungal DNA. Taken together, this data provides evidence that leaf infiltration of *Arabidopsis* with *Fg* results in successful colonization and disease development.

4.3.2 *LOX1* and *LOX5* Expression is Induced in Response to *Fg* Infection

The basal level of expression of *LOX1* is relatively higher than *LOX5* in leaves during plant growth and development as indicated in a survey of publicly available microarray databases (http://www.geneinvestigator.com). *Fg* infection resulted in the induction of *LOX1* expression at 12 hours post inoculation (hpi), with a temporal increase in infected leaf tissue at the time points observed (Figure 4.2A, upper panel). The expression of *LOX5* in response to *Fg* infection on the other hand shows a peak at 6 hpi and then declined to basal levels at 12, 24 and 48 hpi (Figure 4.2A, lower panel). The expression of *LOX1* and *LOX5* in response to *Fg* infection was also examined by means of promoter β-glucuronidase (GUS) constructs (Vellosillo et al., 2007). GUS staining was observed for both *LOX1* (Figure 4.2B, I and II, 0 and 48 hpi) and *LOX5* (Figure 4.2B, III and IV, 0, 12 hpi) in response to *Fg* infection.

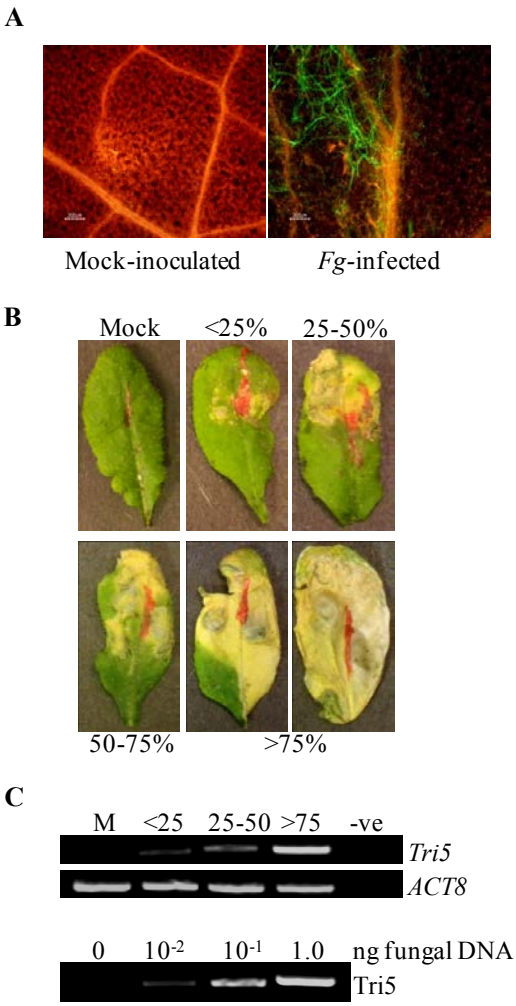


Figure 4.1. *Fg* infection of *Arabidopsis* leaves. **A.** Colonization of *Arabidopsis* accession Col-0 leaves by *Fg*. Plant cells fluoresce red due to binding of Toluidine blue-O and fungal hyphae fluoresce green due to binding of the chitin specific WGA- Alexa Fluor 488. A mock-inoculated leaf provided the negative control for fluorescence emitted from fungal mass. **B.** Disease symptoms in *Fg*–infected leaves. Diseased leaves are grouped into four classes (<25, 25 to 50, 50 to 75, and >75% chlorosis) based on the percentage of leaf area showing chlorosis relative to the mock-inoculated leaf. **C.** Top panel: polymerase chain reaction (PCR) analysis of *Fg Tri5* gene to monitor fungal growth in leaves exhibiting different levels of chlorosis. PCR analysis for the *Arabidopsis* ACT8 gene provided a positive control for DNA quality, DNA extracted from mock (M)-inoculated leaves provided a negative control for *Tri5* amplification, and PCR reactions lacking plant and fungal DNA (-ve) provided a negative control for PCR. PCR conducted with fungal DNA isolated from *Fg* cultivated on synthetic medium was used for determining the relative abundance of fungal DNA in leaf samples. Leaves infiltrated with a suspension lacking fungus provided the mock control. These experiments were conducted two times. Reprinted from Makandar, R., Nalam, V., Chaturvedi, R., Jeannotte, R., Sparks, A.A and Shah, J. (2010) Involvement of salicylate and jasmonate signaling pathways in *Arabidopsis* interaction with *Fusarium graminearum*. Mol. Plant-Microbe. Interact. 23:861-870.

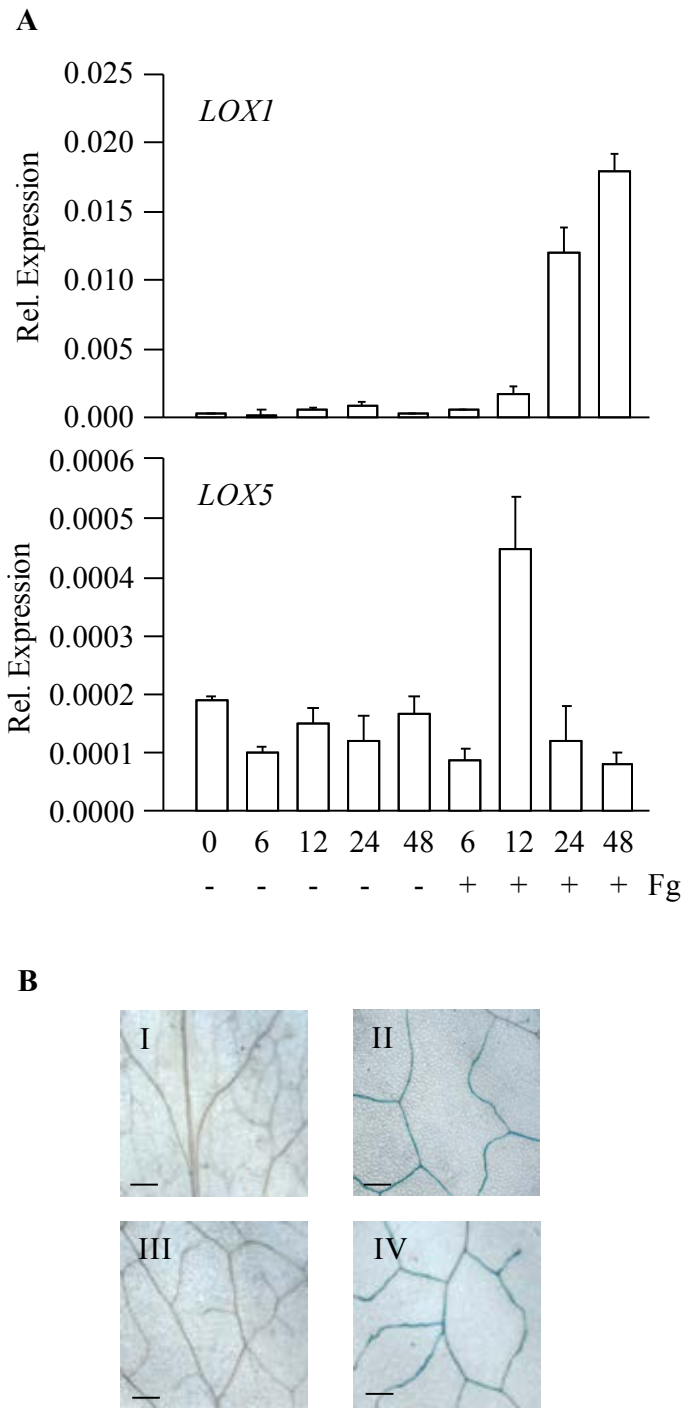


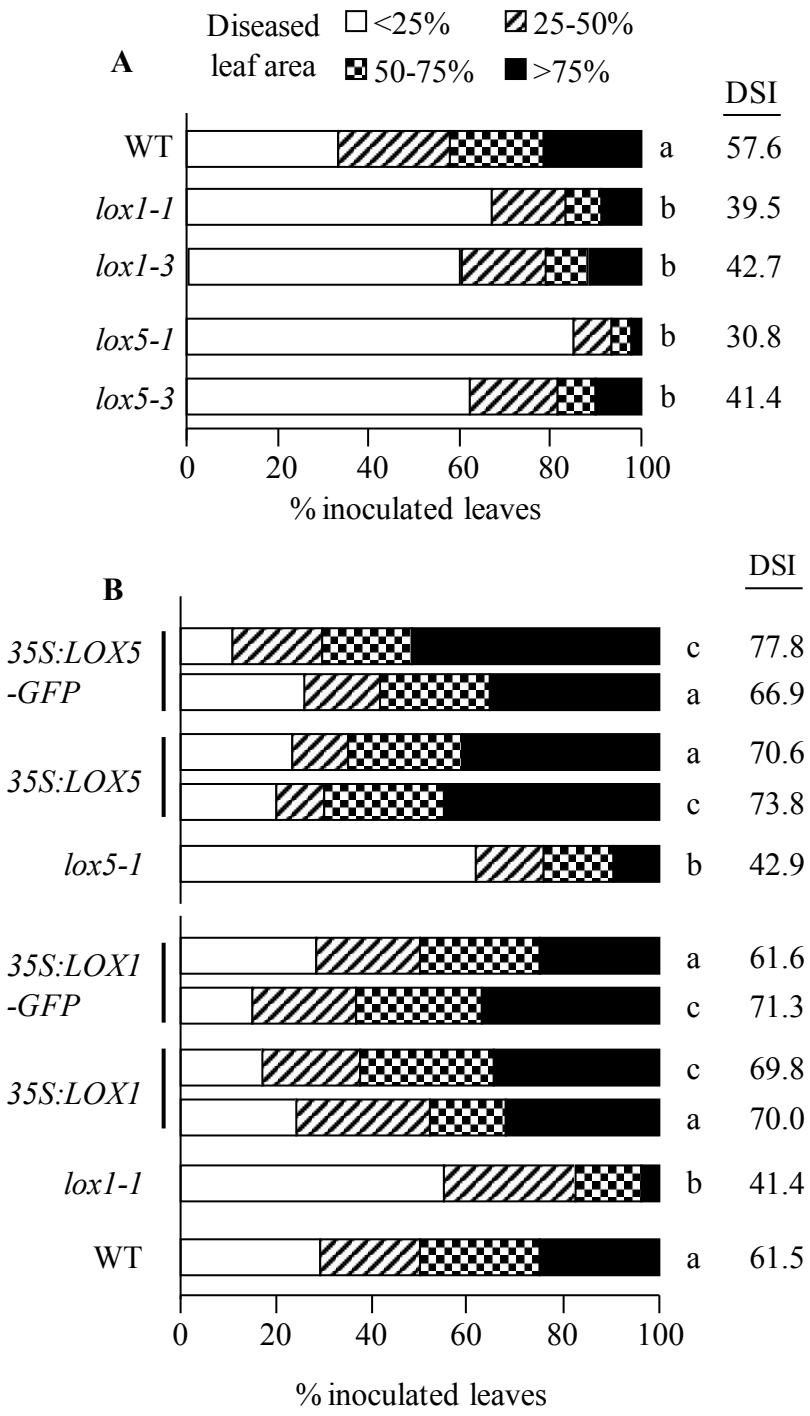
Figure 4.2. *LOX1* and *LOX5* expression in response *F. graminearum* infection. **A.** *LOX1* (upper panel) and *LOX5* (lower panel) expression in leaves of un-infected (- *Fg*)and *Fg*-infected (+ *Fg*) WT plants. qRT-PCR was performed on RNA from leaves of un-infected and *Fg*-infected plants harvested at 0, 6, 12, 24 and 48 hours post inoculation (hpi). The expression level of *LOX1* and *LOX5* relative to expression level of *EF1α* at each time point is presented (n=3). **B.** GUS activity in leaves of un-infected and *Fg*-infected transgenic lines containing promoter GUS constructs of *pLOX1:GUS* (I , 0 hpi and II, 48 hpi) and *pLOX5:GUS* (III, 0 hpi and IV, 12 hpi). Bar = 100 µm.

In plants harboring the *pLOX1:GUS* and *pLOX5:GUS* constructs, GUS activity was observed only in the minor veins of the infected leaves (Figure 4.2B). These results indicate that *Arabidopsis* 9-LOX genes are induced in response to *Fg* infection, and further that *LOX1* and *LOX5* are expressed at elevated levels primarily in the minor veins of the infected leaves.

4.3.3 *Arabidopsis* 9-LOXes Contribute to Severity of *Fg* Infection

Previous reports have implicated a role for 9-LOXs and their metabolites in plant defense responses to pests and pathogens (Gao et al., 2007; Vellosillo et al., 2007; Gao et al., 2008; Gao et al., 2009; Hwang and Hwang, 2010; López et al., 2011). As shown in Figure 4.3A, fungal disease severity was significantly lower on 9-LOX mutants; *lox1-1*, *lox1-3*, *lox5-1* and *lox5-3*, which contain T-DNA insertions within the coding sequence (Figure 2.3A-C).

Since *Fg* infects the spikes or floral tissues of wheat and barley, the response of 9-LOX mutants to *Fg* infection of *Arabidopsis* inflorescence was determined as previously described (Urban et al., 2002). Compared with WT plants, the *Fusarium-Arabidopsis* disease score was lower for *lox1-1*, *lox1-3*, *lox5-1* and *lox5-3* (Table 4.1), reflecting the heightened resistance that was also observed in the leaf infection assays (Figure 4.3A). Ectopic expression of *LOX1* and *LOX5* or a *LOX1-GFP* and *LOX5-GFP* fusion from the *Cauliflower mosaic virus 35S* promoter attenuated the *lox1-1* and *lox5-1* conferred enhanced resistance to *Fg* (Figure 4.3B). In comparison to the 9-LOX single mutants' *lox1-1* and *lox5-1*, the *lox1-1lox5-1* double mutant displayed *Fg* disease severity comparable to individual 9-LOX mutants (Figure 4.3C). Taken together, the data suggests that *LOX1* and *LOX5* have redundant roles in *Arabidopsis-Fg* interaction.


A
Diseased leaf area
<25%
25-50%
50-75%
>75%
DSI
WT
lox1-1
lox1-3
lox5-1
lox5-3
a
b
b
b
b
57.6
39.5
42.7
30.8
41.4
0
20
40
60
80
100
% inoculated leaves
B
DSI
35S:LOX5 -GFP
35S:LOX5
lox5-1
35S:LOX1 -GFP
35S:LOX1
lox1-1
WT
c
a
a
c
b
a
c
a
b
a
77.8
66.9
70.6
73.8
42.9
61.6
71.3
69.8
70.0
41.4
61.5
0
20
40
60
80
100
% inoculated leaves

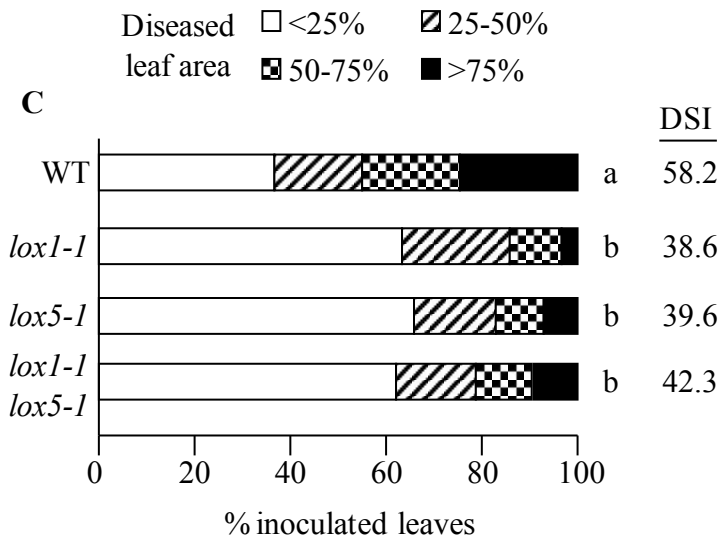


Figure 4.3. *F. graminearum* disease severity on 9-LOX mutants is lower. **A.** Disease severity in wild-type (WT) *Arabidopsis* accession Col-0 and the *lox1-1, lox5-1* and *lox5-3* mutant plants at 4 days post inoculation (dpi). **B.** *F. graminearum* disease severity in leaves of WT and two independent *35S:LOX1* and *35S:LOX1-GFP* transgenic lines in *lox1-1* background and *35S:LOX5* and *35S:LOX5-GFP* transgenic lines in *lox5-1* background. **C.** *F. graminearum* disease severity in leaves of WT (Col-0), *lox1-1*, *lox5-1* and *lox1-1lox5-1* double mutant. Percentage of inoculated leaves exhibiting necrosis covering <25, 25-50, 50-75 and >75% of the leaf area was determined for each genotype. For each genotype, ~ 90 leaves were infiltrated with fungal inoculum. Disease severity Index (DSI) from each genotype was computed based on leaf symptoms. Different letters next to the bars indicate values that are significantly different ($P < 0.05$) from each other by GLM/ANOVA. The experiments were repeated three times with similar results.

4.3.4 Salicylic Acid-Mediated Defenses are Activated Faster in *Fg* Infected in 9-LOX Mutants

Previous research had highlighted the importance of SA signaling in *Arabidopsis* and Wheat-*Fg* interaction and its requirement for basal resistance to *Fg* in these plant species (Makandar et al., 2006; Savitch et al., 2007; Cuzick et al., 2008; Makandar et al., 2010; Ding et al., 2011; Makandar et al., 2012). To determine whether SA-mediated defenses are involved in the enhanced resistance observed in the 9-LOX mutants, expression of the SA-inducible *PR1* gene was monitored in *Fg*-inoculated leaves of WT, *lox1-1* and *lox5-1* plants. As expected, in the WT plant, fungal-infection resulted in increased expression of *PR1* compared with mock-

inoculated leaves (Figure 4.4A). Basal expression of *PR1* transcript in uninfested WT and the *lox1-1* and *lox5-1* mutants was comparable, thus indicating that SA signaling is not constitutively active in the 9-LOX mutants.

Table 4.1 *F. graminearum* disease on *Arabidopsis* inflorescence

Genotype	*Fusarium-Arabidopsis* disease Score
WT (Col-0)	4.1 ± 0.6
lox1-1	2.7 ± 0.3
lox1-3	2.9 ± 0.4
lox5-1	1.5 ± 0.6
lox5-3	1.2 ± 0.3

[a] *Fusarium-Arabidopsis* disease score was evaluated 7 days post inoculation. The experiment was repeated twice with similar results

However, in response to *Fg* infection, *PR1* transcript accumulated to significantly higher levels in the *lox1-1* and *lox5-1* mutants than in the WT plant (Figure 4.4A). The extent of *Fg* resistance in the *lox1-1* and *lox5-1* mutants was comparable to that observed in SA-treated WT plants (Figure 4.4B and C), and pretreatment with SA did not result in further increase in disease resistance in the *lox1-1* and *lox5-1* mutants, further suggesting that hyperactivation of SA signaling likely contributes to the heightened resistance observed in the 9-LOX mutants.

4.3.5 Enhanced SA Accumulation in 9-LOX Mutants

Compared to mock-inoculated leaves, fungal-inoculation induced accumulation of SA in WT plants within 12 hours followed by attenuation at 24 hours post inoculation (hpi). The attenuation of SA content observed in WT plants was not observed in *lox1-1* and *lox5-1*, where SA content was 1.5 fold higher at 24 hpi (Figure. 4.5A).

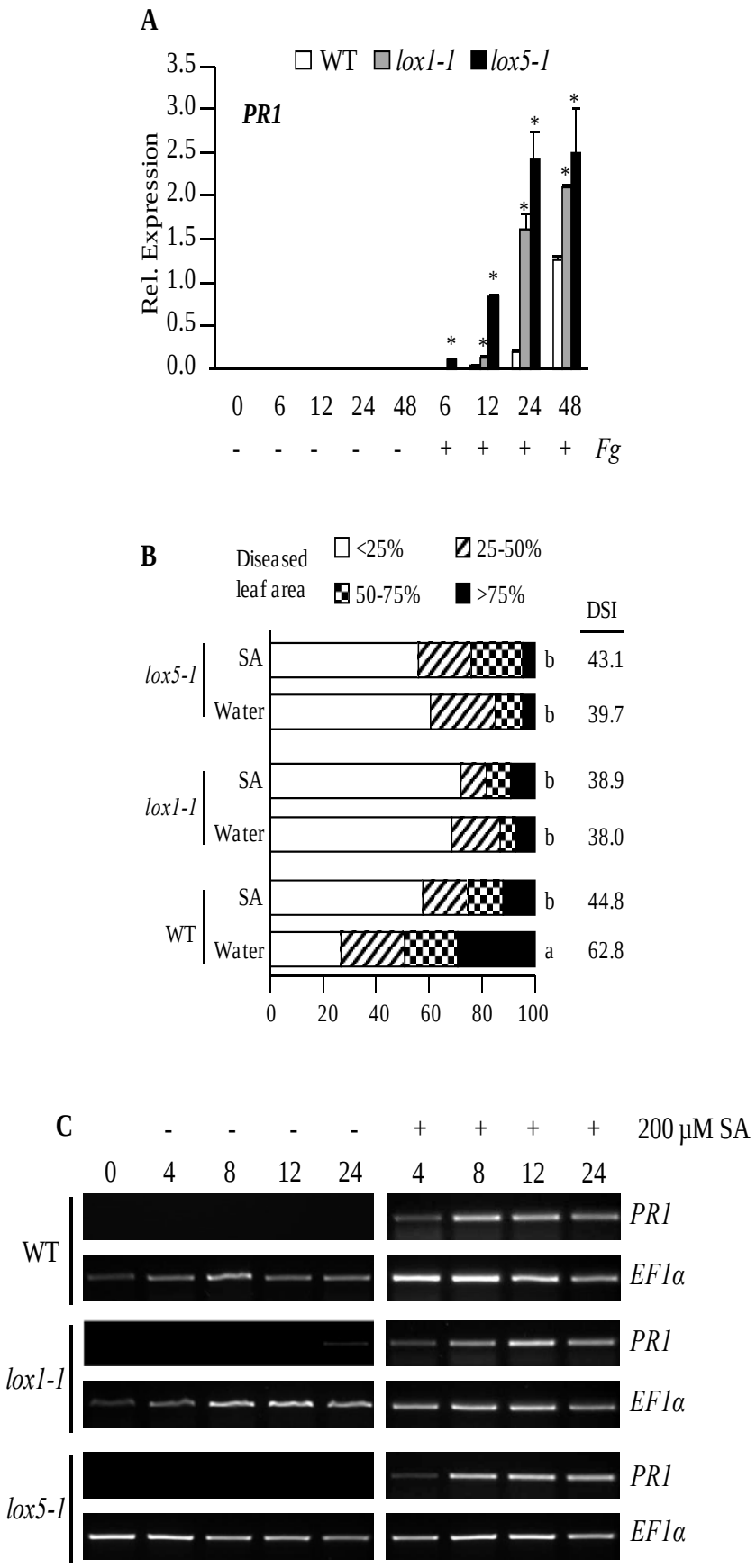


Figure 4.4. Salicylic acid (SA)-mediated defenses are hyper-activated in 9-LOX mutants in response to *Fg* infection. A. *PR1* expression in leaves of un-infected (- *Fg*) and *Fg*-infected (+ *Fg*) WT, *lox1-1* and *lox5-1* plants. qRT-PCR was performed on RNA from leaves of un-infected and *Fg*-infected plants harvested at 0, 6, 12, 24 and 48 hours post inoculation (hpi). The expression level of *PR1* relative to expression level of *EF1α* at each time point is presented (n=3). Asterisks represent values that are significantly different (P <0.05) from the corresponding WT control. B. *Fg* disease severity on leaves of WT (Col-0), *lox1-1* and *lox5-1* mutant plants that were previously treated with 200 µM solution of SA. Plants pretreated with water provided controls for the experiment. Disease severity Index (DSI) from each genotype was computed based on leaf symptoms. Different letters next to the bars indicate values that are significantly different (P < 0.05) from each other by GLM/ANOVA. The experiment was repeated twice with similar results. C. *PR1* expression in leaves of mock- and SA-treated WT, *lox1-1* and *lox5-1* plants. Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed on RNA from leaves of mock- and SA-treated plants harvested at 0, 4, 8 and 24 hours after treatment. *EF1α* provided a positive control for RNA quality.

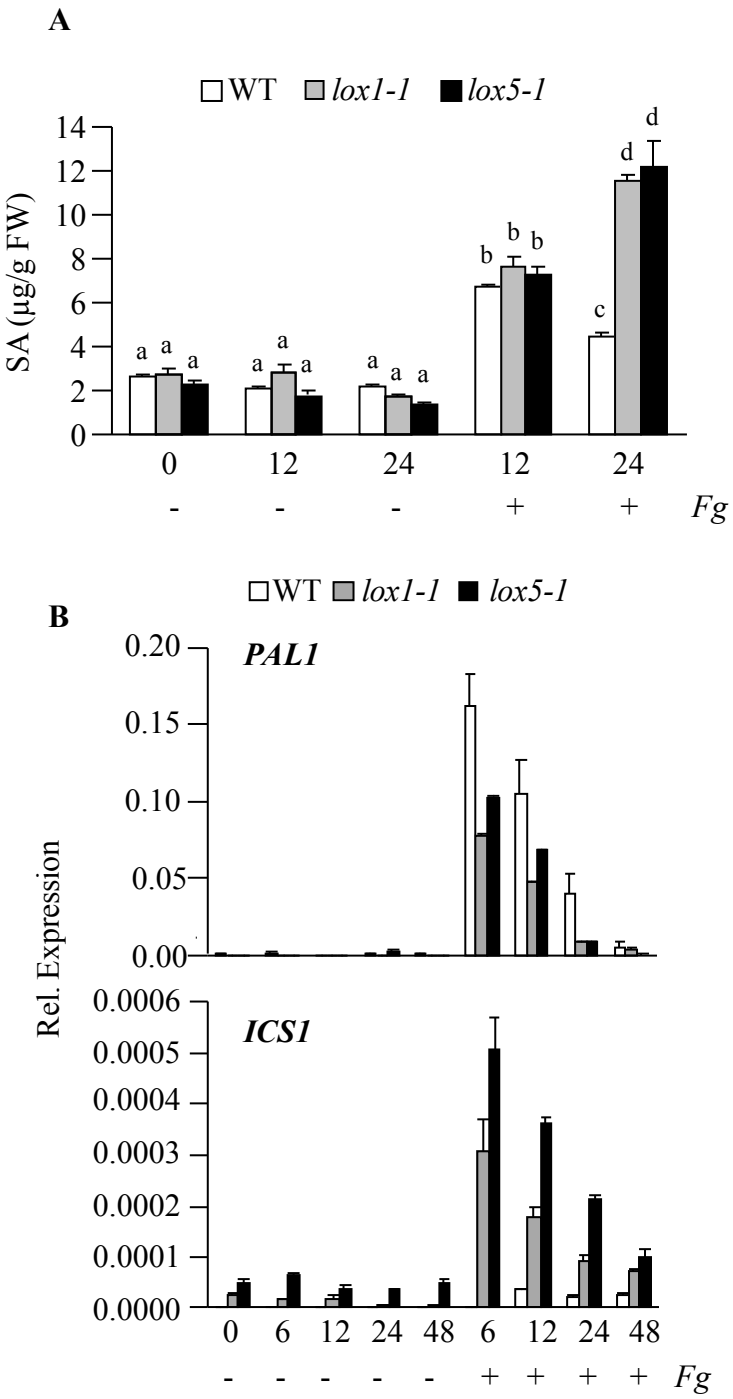


Figure 4. 5. Enhanced accumulation of SA content in 9-LOX mutants contributes to resistance to *Fg* **A.** SA content in leaves of un-infected (- *Fg*) and *Fg*-infected (+ *Fg*) WT, *lox1-1* and *lox5-1* plants. Leaves for SA quantification were harvested 12 and 24 hpi. Each bar represents the average SA content ± standard error (SEM) from three replications per treatment. Different letters next to the bars indicate values that are significantly different (P <0.05) from each other. The experiment was repeated twice. **B.** *PAL1* (upper panel) and *SID2* (lower panel) expression in leaves of un-infected (- *Fg*) and *Fg*-infected (+ *Fg*) WT plants. qRT-PCR was performed on RNA from leaves of un-infected and *Fg*-infected plants harvested at 0, 6, 12, 24 and 48 hours post inoculation (hpi). The expression level of *PAL1* and *SID2* relative to expression level of *EF1α* at each time point is presented (n=3).

In *Arabidopsis*, the PAL (phenylalanine ammonia lyase) and ICS (isochorismate synthase) pathways contribute to SA biosynthesis (Chen et al., 2009b). To determine if one or both of the pathways are activated in response to *Fg* infection, the expression of *PAL1* and *ICS1* which are

key regulators of the respective pathways was followed in a time course experiment (Figure. 4.5B). The expression of *PAL1* was induced by *Fg* infection within 6 hpi in WT and 9-LOX mutants, followed by a reduction in expression levels at the time points observed (Figure 4.5B, upper panel). However, the *PAL1* transcript accumulated to significantly lower amounts in fungal-inoculated leaves of *lox1-1* and *lox5-1* as compared to leaves of fungal-inoculated WT plants. On the other hand, the accumulation of *ICS1* transcripts did not increase in fungal-inoculated leaves of WT to the levels observed in fungal-inoculated leaves of *lox1* and *lox5* (Figure 4.5B, lower panel).

In the fungal-inoculated leaves of *lox1-1* and *lox5-1* however, *ICS1* transcripts accumulated to the highest level within 6 hpi followed by a gradual decrease in transcript levels (Figure 4.5B, lower panel). *ICS1* transcript accumulation was also significantly lower in *Fg*-inoculated leaves of *lox1-1* as compared to *lox5-1*.

4.3.6 Jasmonic Acid Signaling is Suppressed in 9-LOX Mutants

In response to *Fg* infection, *PDF1.2* transcripts, a molecular marker for JA signaling showed a transient increase in WT plants (Figure 4.6A). *PDF1.2* expression showed a biphasic response with peaks at 12 and 48 hpi, with highest levels at 48 hpi in Fg-inoculated WT plants. By comparison, the increase in PDF1.2 expression was slower in the Fg-inoculated lox1-*1* and *lox5-1* plants (Figure 4.6A). In the 9-LOX mutants, *PDF1.2* transcripts show a gradual increase from 6 to 48 hpi with significantly lower expression as compared to fungal-inoculated WT plants at 12 and 48 hpi-(Figure 4.6A). Given the antagonistic nature of cross-talk between SA and JA signaling pathways (Kunkel and Brooks, 2002), it was hypothesized that induction of JA signaling would suppress the hyper-activation of SA-signaling in 9-LOX mutants. Indeed, pretreatment with exogenous methyl jasmonate (MJ) attenuated SA-mediated defenses in 9-LOX

mutants. Compared to water and ethanol treated plants enhanced *Fg* disease severity was observed in *lox1-1* and *lox5-1* similar to WT plants (Figure 4.6B). Taken together, these data suggest that hyper-activation of SA-signaling and defenses observed in 9-LOX mutants in response to *Fg*-inoculation suppresses JA-signaling and can be overcome by MJ pretreatment. Alternatively, the slower induction of *PDF1.2* could be due to the pathogen not growing as rapidly on the 9-LOX mutants, and not necessarily due to a direct impact of the mutations in *lox1* and *lox5* on JA signaling.

4.3.7 Identification of Wheat Lipoxygenases

In order to determine if LOXs play a role in wheat interaction with *Fg*, three putative wheat LOXs, *TaLpx-1*, *TaLpx-2* and *TaLpx-3* were cloned from the wheat cultivar, Bobwhite (see Materials and Methods for details). The three cDNAs all possessed intact ORFs. Analysis of the deduced amino acid sequence revealed that all three putative TaLpx proteins posses a lipoxygenase domain (PFAM00305). In addition, *TaLpx-2* and *TaLpx-3* possess a PLAT/LH2 beta-barrel domain that is found in a variety of membrane or lipid associated proteins, in the N-terminal region, that was absent in *TaLpx-1* (Figure 4.7). The *TaLpx* proteins share less than 74% amino acid sequence identity between them indicating that the three cDNAs isolated, encode for three different LOXs (Table 4.2). *TaLpx1* is 93% identical to barley *HvLoxA*, which encodes a 9-LOX and *TaLpx2* is 95% identical to barley *HvLoxC*, which encodes a 13-LOX, whereas *TaLpx-3* is 96% identical to barley *HvLoxB* (Table 4.2, Figure 4.7).

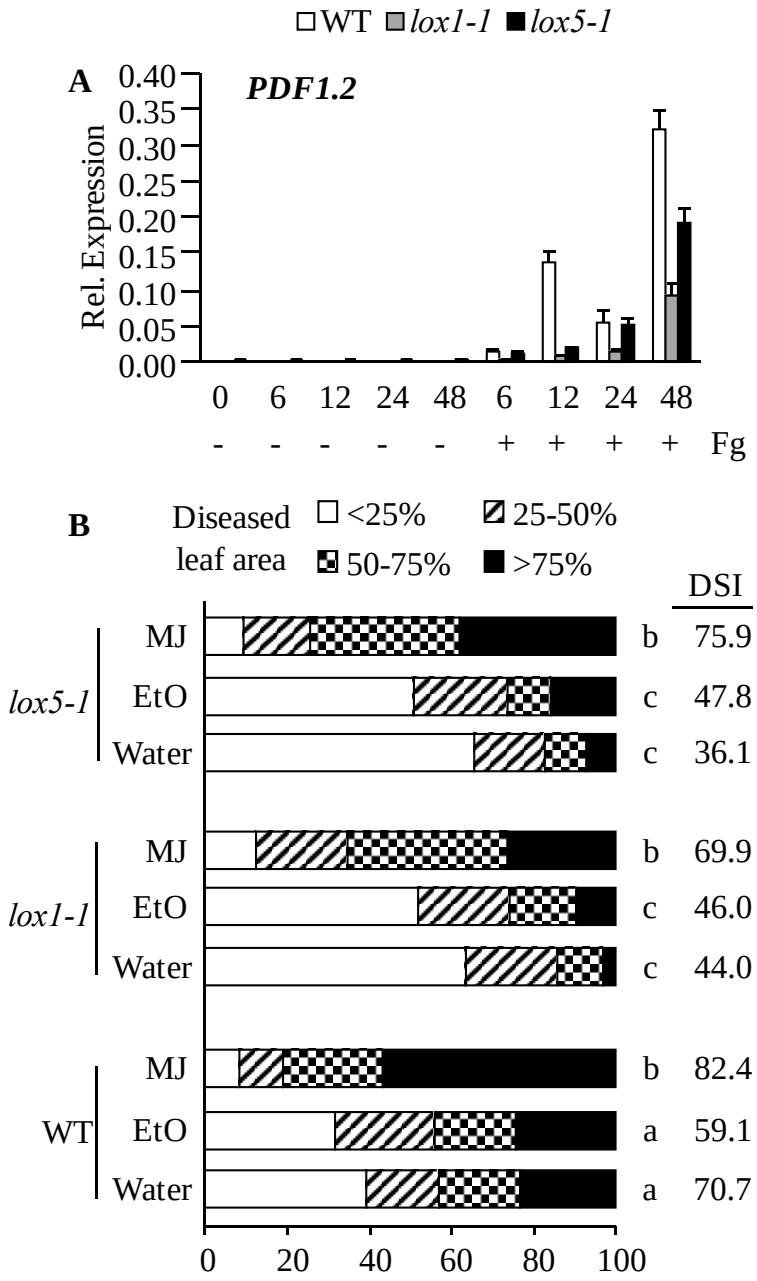


Figure 4. 6. Jasmonic acid signaling is suppressed in 9-LOX mutants.
A. *PDF1.2*expression in leaves of un-infected (- *Fg*) and *Fg*-infected (+ *Fg*) WT, *lox1-1* and *lox5-1* plants. qRT-PCR was performed on RNA from leaves of un-infected and *Fg*-infected plants harvested at 0, 6, 12, 24 and 48 hours post inoculation (hpi). The expression level of *PDF1.2* relative to expression level of *EF1α* at each time point is presented (n=3). B. *Fg*-disease severity in leaves of WT (Col-0), *lox1-1* and *lox5-1* plants that were exposed to methyl-JA (MJ) vapors for 24 h prior to inoculation with *Fg*. Plants exposed to water and ethanol (EtO), which was used as a solvent for MJ, provided the controls. Disease severity Index (DSI) from each genotype was computed based on leaf symptoms. Different letters next to the bars indicate values that are significantly different ($P < 0.05$) from each other by GLM/ANOVA. The experiment was repeated two times with similar results.

Table 4.2. Comparisons between wheat and barley LOXs

		TaLpx-1	*TaLpx-2*	*TaLpx-3*	*HvLoxA*	*HvLoxB*	*HvLoxC*
TaLpx-1	Max Iden						
	% Sim						
TaLpx-2	Max Iden	55					
	% Sim	70					
TaLpx-3	Max Iden	60	61				
	% Sim	71	74				
HvLoxA	Max Iden	91	75	73			
	% Sim	93	85	84			
HvLoxB	Max Iden	68	60	92	76		
	% Sim	80	73	96	86		
HvLoxC	Max Iden	62	92	61	77	61	
	% Sim	72	95	75	85	75	

Max Iden – percent identities and % Sim – percent similarities as determined by multiple sequence alignment (http://blast.ncbi.nlm.nih.gov)

4.3.8 LOX Activity of TaLpx-1

Given that Arabidopsis 9-LOXs contribute to host susceptibility to Fg infection and TaLpx-1 is homologous to a barley 9-LOX, HvLoxA, the enzyme activity of TaLpx-1 was determined. The full length cDNA for TaLpx-1 was cloned in an Escherichia coli expression vector. Recombinant TaLpx-1 was detected on Coomassie Brilliant Blue-stained SDS-PAGE gels as a single polypeptide with a size of 73.5 kDa (Figure 4.8A). The positional specificity of TaLpx-1 was determined by incubating whole cell lysate with linoleic or linolenic acid as substrate and the resulting products were analyzed by GC-MS as TMS-derivatives (BSTFA (Thermo scientific, PA, USA).

A

```
TaLpx-1     MGSSHHHHHH SSGLVPRGSH MASMTSDFLG YSIKAITQGI LPAVRTYVDT
HvLOXA      ---------- ---------- ---------- ---------- ----------

TPGEFDSFQD IINLYEGGIK LPNVPALEEL RKQFPLQLIK DLLPVGGDSL LKLPVPHIIQ
---------- ---------- ---------- ---------- ---------- ----------

ADQQAWRTDE EFSREVLAGV NPVMITRLTE FPPKSSLDPS KFGDHTSTVT AAHIEKNLEG
---------- ---------- ---------- ---------- ---------- ----------

LTVQQAPESN RLYILDHHDR FMPFLIDVNN LPGNFIYATR TLFFLRGDGR LTPLAIELSE
---------- ---------- ---------- ---------- ---------- ----------

PVIQGGLTTA KSKVYTPVPS GSVEGWVWEF AKAYAAVNDS GWHQLVSHWL NTHAVMEPFV
---------- ---------- ---------- --ARARAEFG TRHQLVSHWL NTHAVMEPFV
                                             *   *●    *
                                             ●●

ISTNRQLSVT HPVHKLLSPH YRDTMTINAL ARQTLINAGG IFEMTVFPGK FALGMSSVVY
ISTNRHLSVT HPVHKLLSPH YRDTMTINAL ARQTLINAGG IFEMTVFPGK FALGMSAVVY
                       ▲      ▲ ●   ●●     ●

KDWKFTEQGL PDDLIKRGMA VEDPSSPYKV RLLVSDYPYA ADGLAIWHAI EQYVSEYLAI
KDWKFTEQGL PDDLIKRGMA VEDPSSPYKV RLLVSDYPYA ADGLAIWHAI EQYVSEYLAI

YYPNDGVVQG DVELQAWWKE VREVGHGDLK VAPWWPRMQA VGELAKACTT IIWIGSALHA
YYPNDGVLQG DTEVQAWWKE TREVGHGDLK DAPWWPKMQS VPELAKACTT IIWIGSALHA
                                                          *

AVNFGQYPYA GFLPNRPTVS RRRMPEPGTE QYAELERDPE RAFIHTITSQ IQTIIGISLL
AVNFGQYPYA GFLPNRPTVS RRRMPEPGTE EYAELERDPE RAFIHTITSQ IQTIIGVSLL
  *   *

EVLSKHSSDE LYLGQRDTPE WTSDPKALEV FKRFSERLVE IESKVVGMNH DPQLLNRNGP
EVLSKHSSDE LYLGQRDTPE WTSDPKALEV FKRFSDRLVE IESKVVGMNH DPELKNRNGP
  *
  ●

AKFPYMLLYP NTSDHKGAAA GLTAKGIPNS ISI  623
AKFPYMLLYP NTSDHKGAAA GLTAKGIPNS ISI  361
                                  *
```

B

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TaLpx-2     MFGVGGIVSD LTGGLRGPHL KGSVVLMRKN ALDFNDFGAT VMDGVTELLG
HvLOXC      MLGVGGIVSD LTGGIRGAHL KGSVVLMRKN ALDFNDFGAH VMDGVTELLG

RGVTCQLISS THVDHNNGGR GKVGAEANLE QWLLPTNLPF ITTGENKFAV TFDWSVDKLG
RGVTCQLISS TNVDHNNGGR GKVGAEANLE QWLLPTNLPF ITTGENKFAV TFDWSVDKLG

VPGAIIVKNN HAAEFFLKTI TLDNVPGRGT VVFVANSWVY PQAKYRYNRV FFANDTYLPH
VPGAIIVKNN HASEFFLKTI TLDNVPGRGT IVFVANSWVY PQAKYRYNRV FFANDTYLPH

QMPAALKPYR DDELRNLRGD DQQGPYEDHD RVYRYDVYND LGDTRDVLGG SKDLPYPRRC
QMPAALKPYR DDELRNLRGD DQQGPYLDHD RVYRYDVYND LGDSRDVLGG SKDLPYPRRC

RTGRKPSATK PDHESRLLPL VGNVYVPRDE LFGHLKQSDF LGYTLKALVD GIIPAIRTYV
RTGRKPSDSK PDHESRLLLL VQNVYVLRDE LFGHLKQSDL LGYTLKGWLD GIILAIRTYV
                                          ▲       ▲  ▲

DLSPGEFDSF ADILKLYEGG IKLPNIPALE EVRKRFPLQL VKDLIPMGGD FLLKLPKPQI
DLSPGEFDSF ADILKLYEGG IKLPNIPALE EVRKRFPLQL VKDLIPKGGD FLLKLPKPEI

IKADEKAWMT DEEFAREMLA GVNPMMIKRL TEFPPKSTLD PSKYGDHTST ITEAHIGRSL
IKVDQKAWMT DEEFAREMLA GVNPMMIKRL TEFPPKSTLD PSKYGDHTST MTEEHVAKSL

EGLTVEQALA DNRLYIVDQH DNLMPFLVDI NNLDGSFVYA TRTLLFLQGN GTLAPVAIEL
EGLTVQQALA GNRLYIVDQH DNLMPFLIDI NNLDASFVYA TRTLLFLRGD GTLAPVAIEL

SSPLIQGDLT TAKSTVYTPQ HAGVEGWIWQ LAKAYASVND YGWHQLISHW LNTHAVMEPF
SSPLIQGELT TAKSAVYTPQ HAGVEGWIWQ LAKAYASVND YGWHQLISHW LNTHAVMEPF
                                            *   *●    *
                                            ●●

VIATNRQLSV THPVYKLLHP HYRDTMNINA RARGLLINAG GVIEMTVFPR KHAMPMSSMV
VIATNRQLSV THPVYKLLHP HYRDTMNINA RARGLLINAG GVIEMTVFPH KHAMPMSSMV
                          ▲       ▲●   ●●     ●

YKNWNFTEQA LPDDLIKRGM AVEDPSSPHK VRLLIEDYPY AADGLAVWHA IEQWVTEYLT
YKHWNFTEQA LPADLIKRGM AVEDASSPHK VRLLIKDYPY ATDGLAVWDA IEQWVSDYLT

IYYPDDGVLQ GDVELQAWWK EVREVGHGDL KDAAWWPKMH TVAELIKACA TIIWTGSALH
IYYPNDGVLQ GDVELQAWWK EVREVGHGDL KDAAWWPKMQ TVAELIKACA TIIWTGSALH
                                                              *

AAVNFGQYPY SGYHPNKPSA SRRPMPAPGS EEYALLERDP EKAFILTITN QFQALVGISL
AAVNFGQYPY SGYHPNKPSA SRRPMPVQGS EEYAELERDP EKAFIRTITS QFHALVGISL
    *  *

MEILSKHSSD EVYLGQHDTP AWTSDAKAQE AFRRFGARLE GIEKQVVAMN GDPXAQNRTG
MEILSKHSSD EVYLGQHDTP AWTSDAKALE AFKRFGAKLE GIEKQVVAMN SDPQLKNRTG
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C

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TaLpx-3     MLLHGLVDRL TGKNKQAWKE GKIRGTAVLV KSDVLNLGDF HASLLDGVHD
HvLOXB      MLLHGLVDRL TGKNKEAWKE GKIRGTAVLV KSDVLDLGDF HASLLDGVHK

ILGKDDGVIF HLVSATAPDP QNPRRGKVGK PAHLEEMVVT MKSKAAGESV FKVTFEWDDS
ILGKDDGVSF RLVSATAPDP QNGSRGKVGK PAHLEEMVVT MKSTAAGESV FKVTFEWDDS

QGIPGAVLVR NTYRSEYLLK TLTLHGVPGK GTVVFVANSW IYPNVDRVFF ANDTYLPSKM
QGVPGAVIVR NTYRSEYLLK TLTLHGVPGK GTVVFVANSW IYPNVDRLFF ANDTYLPSKM

PALLVQYRQD ELNNLRGDGT TGKYEEWDRV YRYDYYNDLG EPDKGHPRPV LGGTQELPYP
PALLVQYRQD ELNNLRGDDT TGEYKEADRV YRYDYYNDLG EPDNDNPRPV LGGTQELPYP

RRCRTGRPPT KTDPRSESRI PQYKIQEALN IYVPRDERFG HLKLSDFLGY SLKAITEAIL
RRCRTGRPPT ETDPRSESRI PKYKIKEALN IYVPRDERFG HLKLSDFLGY SLKAITEAIL
                                            ▲        ▲   ▲

PIIRTYVDTT PKEFDSFQDI YDLYDGLLKV PDNQHLKELK KKIPLQFIKS LLPVAGDDLL
PITRTYVDST PKEFDSFQDI YNLYDGLLKV PDNQHLKELK NKS-LQFIKK SLAVAGDDSK

NLPLPHVIRS NDYAWRSDEE FAREMLAGVN PVCIKRLTEF PVKSTLDPSV YGDQSSTITE
-LALPHVIKS DQYAWRSDEE FAREMLAAVD PVCIRRLTKF PVKKYLDPSV YGDQSSTITE

DQIQQNLEDG LTVKQAMEKN RLFILDHHDN FMPFLDRINK LEGNYIYASR TLLFLKADGT
DQIQLNLEDG LTVRQAMDKK RLFILDHHDN FMPFLDRINK LEGNYIYASR TLLFLKADGT

LKPLAIELSL PHPDGIQHGA KSTVYLPADI DSGVDGQIWQ LAKAYASVDD SAWHQLISHW
LKPLAIELSQ PHPDGIQHGA KSTVYLPADI NSGVDGQIWQ LAKAYASVDD SAWHQLISHW
                                                      *   *●
                                                     ●●

LNTHAVIEPF VIATNRQLSV VHPVHKLLSP HYRDTLNINA LARTTLINAG GVFEMTVFPE
LNTHAVIEPF VIATNRQLSV VHPVHKLLSP HYRDTLNINA LARTTLINAG GVFELTVFPG
                                    ▲       ▲●   ●●     ●

KYALEMSSIV YKNWKLTEQG LPDDLVKRGM AVPDSSSPYG VRLLIKDYPY AVDGLVIWWA
QYALEMSAVV YKNWKLTEQG LPDDLVKRGM AVPDESSPYG IRLLIKDYPY AVDGLVIWWA

IERWVNEYLA IYYPNDGVLR ADKELEEWWK EVREVGHGDL KDADWWPKMV TVQELAKTCT
IERWVNEYLA IYYPNDGVLR ADKELEEWWK EVREVGHGDL KDADWWPKMV TVQELAKTCT

TIIWVASALH AAVNFGQYPY AGYLPNRPTV SRRKMPEEGE EEYKQLQKGG KEADKVFIHT
TIIWVASALH AAVNFGQYPY AGYLPNRPTV SRRPMPKEGD EEYEQLKEGG EAADMVFIHT
         *    *  *

ITSQFQTILG ITLIEILSKH SSDEVYLGQR DTPEWTSDAK ALEAFKRFGT RLMEIEKRIL
ITSQFQTILG ITLIEILSKH SSDEVYLGQR DTPEWTSDAK ALEAFKRFGS RLVEIEKRIL
                *
                ●

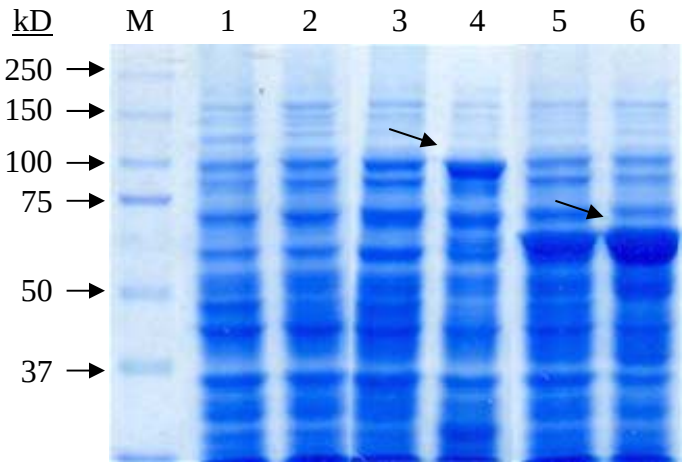
DMNKDPALKN RNGPVKMPYM LLYPNTSDAG GEKGLGLTAM GIPNSVSI  878
DMNKDPALKN RNGPVKMPYM LLYPNTSDAN GEKALGLTAM GIPNSVSI  876
                                                  *
```

Figure 4. 7. Comparisons of the deduced amino acid sequences of wheat and barley LOXs. **A.** Alignment of TaLpx-1 with HvLoxA. **B.** Alignment of TaLpx-2 with HvLoxC. **C.** Alignment of TaLpx-3 with HvLoxB. The sequences underlined in blue represents the PLAT/LH2 beta-barrel and sequences underlined in red represent the lipoxygenase domain (PFAM00305). The residues required for forming the substrate entry site (arrowheads), iron coordination and second-coordination sphere (asterisk) or constructing the oxygen access channel (filled circles) are deduced based on crystal structure of LOX1_SOYBN (Swiss-Prot Accession number P08170).

4.3.9 Wheat LOXs Contribute to Host Susceptibility to FHB

The differential expression of several wheat LOXs during infection by *Fg* (Kruger et al., 2002; Li and Yen, 2008) and the expression of wheat expressed sequence tags (ESTs) corresponding to *TaLpx* loci during spike development (Figure 4.9) suggests a role for LOXs in wheat-*Fg* interaction. To determine if either of the TaLpx genes have a role in wheat-Fg interaction, the FHB response of transgenic wheat plants in which *TaLpx* genes were silenced by RNA interference (RNAi) (Methods for details on construction and screening). Table 4.3 lists the number of independent RNAi transgenic lines for each *TaLpx* gene and the generation screened for FHB response. Since the T_2 and T_3 progeny show segregation for the presence of the transgene, the extent of silencing of *TaLpx* gene was determined by reverse-transcriptase (RT-PCR) in the individuals of the T_2 or T_3 progeny of the various RNAi-transgenics (Table 4.3; Figure 4.10).

A



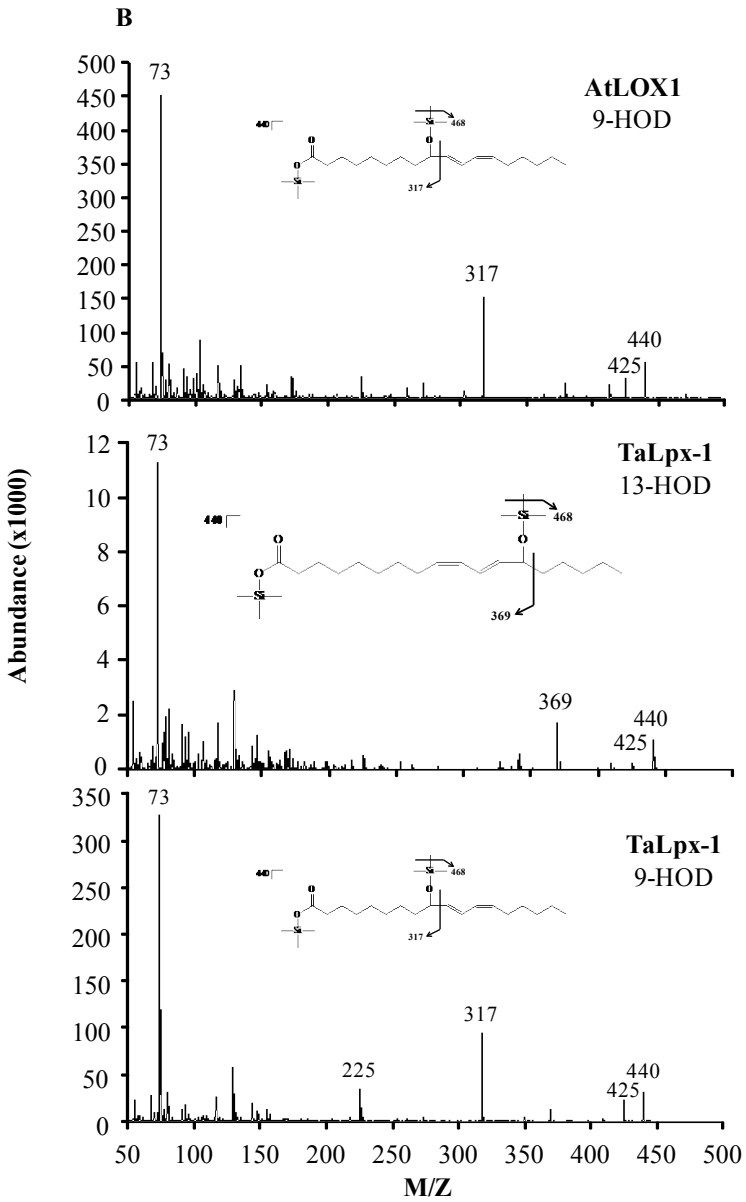


Figure 4. 8. Cloning and characterization of TaLpx-1. **A.** SDS-PAGE of recombinant TaLpx-1 and *Arabidopsis* LOX1 (AtLOX1) proteins. Total protein extracts were analyzed on 10% SDS-PAGE and stained with Coomassie Brilliant Blue. Lane M, Molecular marker (kDa); lane 1-6 , uninduced and induced cell lysate of *E. coli* BL21 cell extracts expressing AtLOX1 and TaLpx-1 or an empty vector as control; lane 1 – uninduced empty vector; lane 2 – induced empty vector; lane 3 – uninduced AtLOX1; lane 4 – induced AtLOX1; lane 5 – uninduced TaLpx-1; lane 6 – induced TaLpx-1. Arrows indicate TaLpx-1 (73.5 kDa) and AtLOX1 (98 kDa). **B.** TaLpx-1 and AtLOX1 products analyzed on GC-MS. Mass fingerprints indicating fragmentation patterns for 9-HOD and 13-HOD are shown.

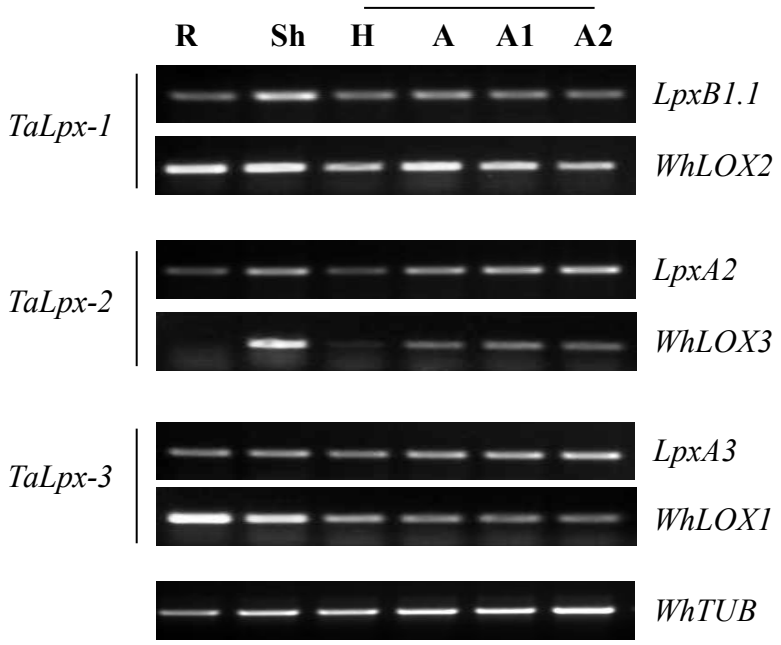


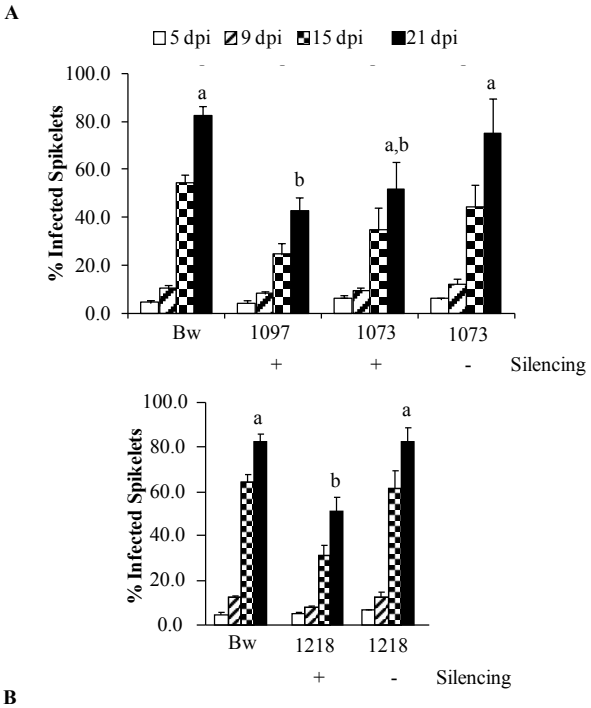
Figure 4. 9. Tissue specific expression of wheat lipoxygenases. Reverse-transcriptase (RT) PCR showing the expression of wheat expressed sequenced tags (ESTs) corresponding to TaLpx-1, TaLpx-2 and *TaLpx-3*. Tissue for RNA was collected from root (R) and shoots (Sh) of 5d-old seedlings, and from spikes at various points during spike development. H - At Heading; A - at Anthesis; A1 - 1 week post anthesis; A2 - 2 weeks post anthesis. Wheat Tubulin (WhTUB) provided the control for RNA quality.

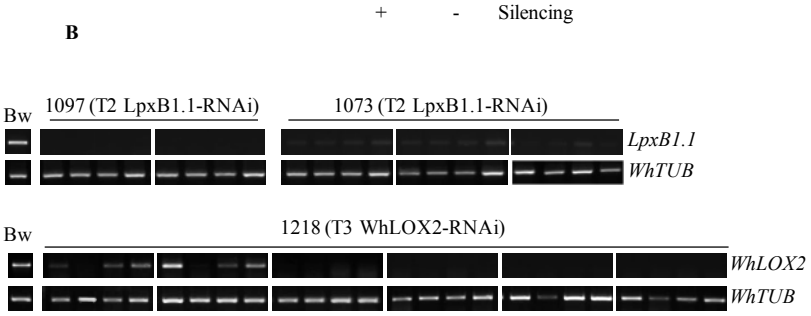
TaLpx-1-silenced T_2 and T_3 progeny of transgenic lines in which the wheat ESTs LpxB1.1 and WhLOX2 were used to silence *TaLpx-1* displayed enhanced resistance to FHB (Figure 4.10 A, B). By contrast, the T_2 and T_3 segregants that lacked the RNA- transgene and thus did not exhibit silencing of *TaLpx-1* exhibited disease severity that was comparable to that in non-transgenic Bobwhite control (Figure 4.10 A, B). Enhanced resistance to FHB was also observed in the T_2 and T_3 progeny in two out of three independent transgenic lines for WhLOX3 and in one line for LpxA2 in whom *TaLpx-2* expression is suppressed (Figure 4.10 C and D). Furthermore, in the T2 and T3 progeny where the expression of *TaLpx-3* is suppressed, three independent lines for LpxA3 and one line for WhLOX1 show enhanced resistance to FHB incidence whereas the segregants which lack the transgene did showed a FHB incidence similar to Bobwhite control

(Figure 4.10 E and F). Taken together, these data suggest that the role of wheat LOXs in host interaction with *Fg* is similar to the role of LOXs in *Arabidopsis*.

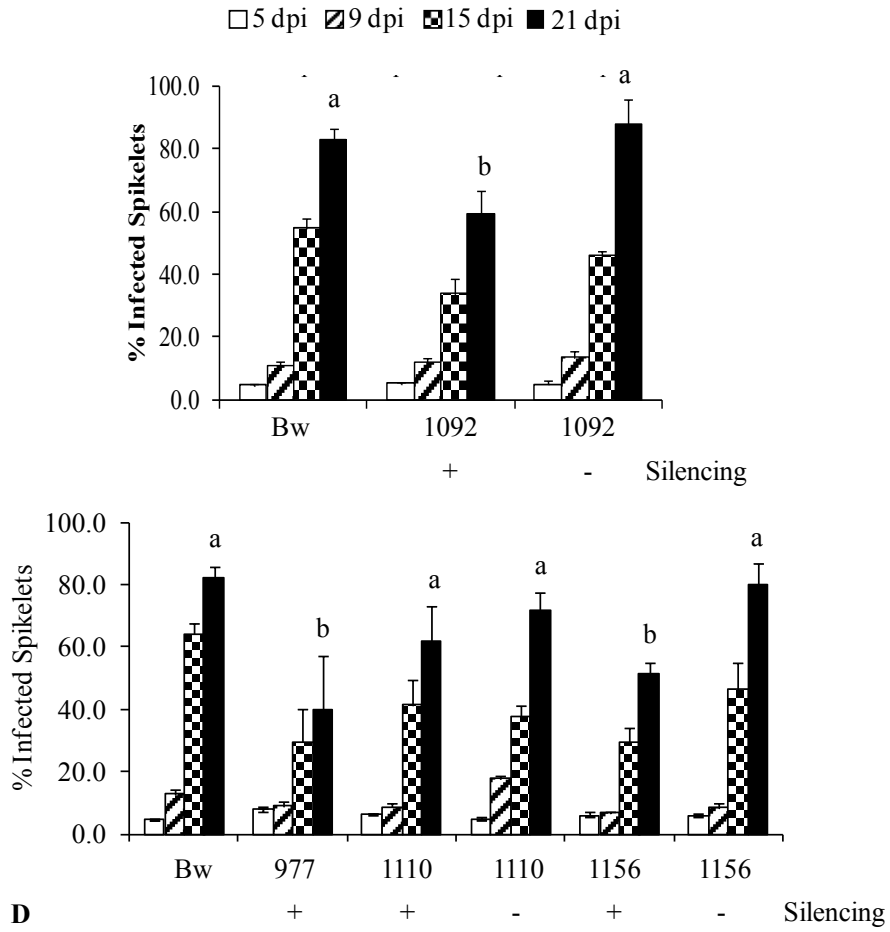
Table 4.3**.** Wheat ESTs, number of independent RNAi transgenics and the generation screened for FHB response

Target Gene	Wheat EST used to generate RNAi construct	No. of independent transgenic lines	Generation screened for FHB response
TaLpx-1	LpxB1.1 (DQ474240)	2	T2
	WhLOX2 (AK332064)	1	T3
TaLpx-2	LpxA2 (DQ448002)	1	T2
	WhLOX3 (AK333416)	3	T3
TaLpx-3	LpxA3 (DQ474244)	3	T2
	WhLOX1 (HQ913602)	1	T3

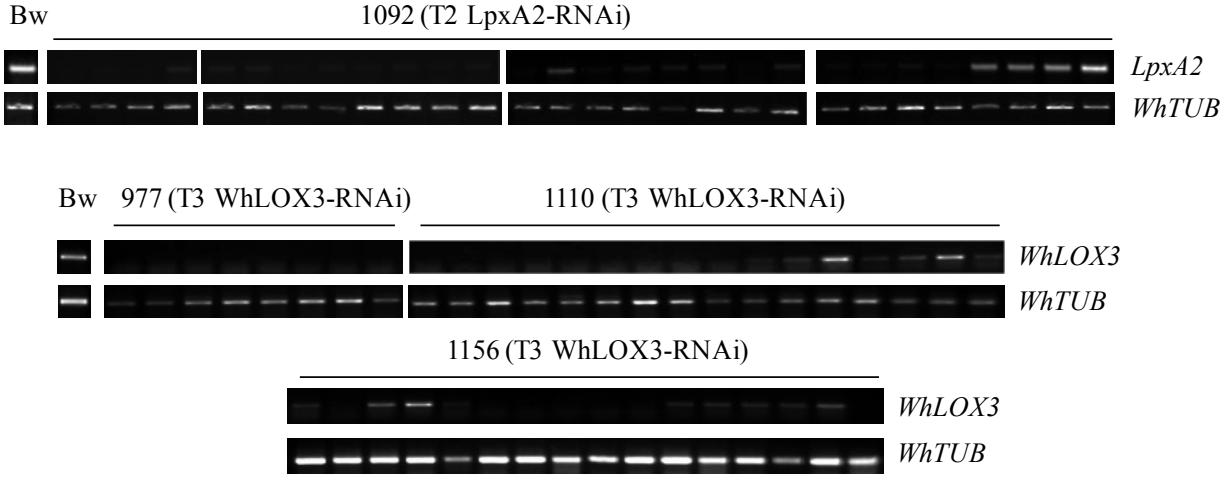




C


□5 dpi ▨9 dpi ▩15 dpi ■21 dpi
% Infected Spikelets
100.0
80.0
60.0
40.0
20.0
0.0
a
b
a
Bw
1092
1092
+
-
Silencing
% Infected Spikelets
100.0
80.0
60.0
40.0
20.0
0.0
a
b
a
a
b
a
Bw
977
1110
1110
1156
1156
+
+
-
+
-
Silencing

D


Bw
1092 (T2 LpxA2-RNAi)
LpxA2
WhTUB
Bw
977 (T3 WhLOX3-RNAi)
1110 (T3 WhLOX3-RNAi)
WhLOX3
WhTUB
1156 (T3 WhLOX3-RNAi)
WhLOX3
WhTUB

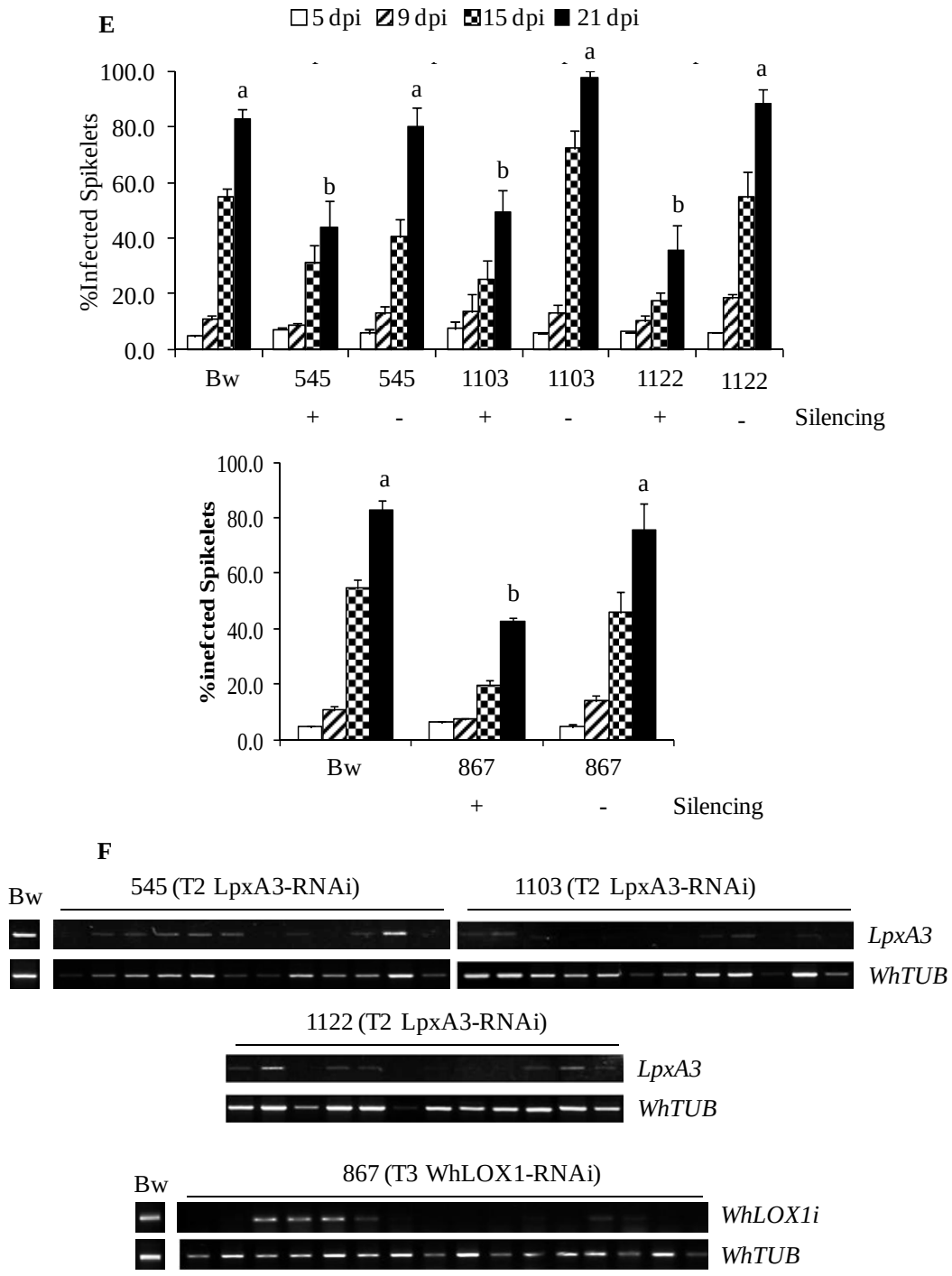


Figure 4.10. Silencing of wheat LOXs enhances resistance to FHB.
A. FHB severity in TaLpx-1 RNAi lines. B. RT-PCR analysis of LpxB1.1 and WhLOX2 RNAi-transgenics C. FHB severity in TaLpx-2 RNAi lines D. RT-PCR analysis of LpxA2 and WhLOX3 RNAi-transgenics. E. FHB severity in TaLpx-2 RNAi lines. F. RT-PCR analysis of LpxA3 and WhLOX1 RNAi-transgenics. FHB disease rating was monitored in Bobwhite, RNAi-silenced (+) and non-silenced (-) wheat. Disease incidence was recorded at 5, 9, 15 and 21 says post inoculation (dpi) as the percentage of infected spikelets in each spike. Bars represent means ± standard error. Different letters above bars indicate values at 21 dpi that are significantly different ($P < 0.05$) as determined by GLM/ANOVA.

4.4 Discussion

Results presented here indicate that *Fg*, which is primarily a floral pathogen of cereal plants, is able to infect and colonize *Arabidopsis*. In order to overcome the physical barrier of the leaf surface, a leaf infiltration assay was developed in this study (Figure 4.1). In combination with the inflorescence infection assay reported by Urban et al., 2002, the *Arabidopsis-Fg* model pathosystem provides an excellent system to identify host factors that contribute to susceptibility and resistance to *Fg*. The enhanced resistance to *Fg* infection in *Arabidopsis* 9-LOX mutants in both leaf and inflorescence infection assays indicates a role for the 9-LOX pathway in contributing to host susceptibility (Figure 4.3A; Table 4.1). Additionally, the enhanced resistance to FHB observed in transgenic wheat where LOXs are silenced using RNA interference (Figure 4.10) suggests conservation of LOX function in *Arabidopsis* and wheat interaction with Fg and highlights the usefulness of the *Arabidopsis-Fg* model pathosystem in identifying host factors involved in the interaction.

The reduced *Fg* disease severity observed in the *Arabidopsis lox1-1* and *lox5-1* mutants correlated with increased accumulation of SA at 24 hpi and stronger expression of the SA-inducible *PR1* gene (Figure 4.4A; Figure 4.5A). The basal levels of SA and *PR1* transcript levels were similar in both WT and 9-LOX mutants (Figure 4.4A; Figure 4.5A) suggesting that the enhanced resistance against *Fg* in the *lox1-1* and *lox5-1* mutants is likely due to stronger induction of SA biosynthesis and signaling in response to *Fg* infection. The observation that exogenous application of SA does not further enhance resistance to *Fg* in *lox1-1* and *lox5-1* (Figure 4.4B) suggests that the higher accumulation of SA in *Fg* inoculated *lox1-1* and *lox5-1* mutants is sufficient to enhance maximal levels of resistance against *Fg*. In *Arabidopsis* and wheat, SA plays a critical role in basal resistance to FHB (Makandar et al., 2010; Makandar et

al., 2012). Therefore, we hypothesize that in *Arabidopsis* 9-LOX function suppresses SA accumulation and thus contributes to overall susceptibility to the pathogen. Further support for this premise comes from studies on a 9-LOX mutant in maize where constitutive overexpression of not only SA-dependant genes but also JA-responsive and biosynthetic genes was observed in the roots of the mutant due to the fact that the 9-LOX gene was mainly expressed in roots (Gao et al., 2008). In *Arabidopsis* also, *LOX5* expression has been shown to occur in roots during development (Vellosillo et al., 2007) and infestation by the green peach aphid, *Myzus persicae* (Figure 2.5A) whereas *LOX1* is mainly induced in leaves during pathogen or pest infection (Figure 4.1; Figure 3.2A; Figure 2.5A). Therefore, a careful analysis of the expression patterns of *LOX1* and *LOX5* in response to *Fg* infection in *Arabidopsis* is necessary to determine their contributions to the suppression of SA-signaling. Since mutations in either LOX1 or LOX5 was sufficient to enhance resistance against *Fg* to comparable levels and resistance was not further enhanced in the *lox1-1 lox5-1* mutant, we suggest that LOX1 and LOX5 activities function in concert to provide a threshold amount of a factor that suppresses activation of SA signaling in *Fg*-infected plants. Mutations in either of these genes results in levels of this factor that are below the threshold required for inhibiting SA accumulation. This likely also explains why disease severity was comparable between the *lox1-1 lox5-1* double mutant and the single mutant plants.

SA and JA are important plant hormones that are involved in the activation of defenses against biotrophic and necrotrophic pathogens respectively (Glazebrook, 2005). The hemi-biotrophic mode of *Fg* infection however suggests a complex interaction between the two pathways. Indeed in *Arabidopsis* and wheat, SA-signaling is critical for basal resistance to *Fg* whereas JA-signaling plays a dichotomous role by constraining SA-signaling during the early

stages and contributing to resistance during the later stages of *Fg* infection (Makandar et al., 2010; Ding et al., 2011; Makandar et al., 2012). The antagonistic mode of interaction between the two signaling pathways has been documented (Kunkel and Brooks, 2002). Although, a synergistic mode of action of low levels of SA and JA on defense gene expression has also been demonstrated in *Arabidopsis* (Mur et al., 2006). Recently, it has also been suggested that the 9-LOX mediated pathway exhibits a suppressor activity on the biosynthesis of both SA and JA in maize (Gao et al., 2008). However, the data presented shows a delayed and lower expression of JA-inducible *PDF1.2* transcripts in *Fg*-infected *Arabidopsis* 9-LOX mutants (Figure 4.6) suggesting that unlike in maize, the 9-LOX mediated pathway exhibits a suppressor activity on only SA and not JA-signaling. It is possible that the 9-LOX products promote JA signaling in *Fg*-inoculated WT plants, which in turn suppresses SA signaling. In the *lox1* and *lox5* mutants, the activation of JA signaling is adversely impacted as indicated by the slower onset of *PDF1.2* expression in response to *Fg* infection (Figure 4.6). This deficiency in the activation of JA signaling in the *lox1-1* and *lox5-1* mutants likely results in the stronger activation of SA signaling leading to enhanced resistance to Fg in *lox1* and *lox5*. Indeed, pretreatment with MJ resulted in breakdown of *lox1* and *lox5* mutation-conferred enhanced resistance to *Fg*. It is also possible that the impact of 9-LOXs on SA signaling is independent of JA. The lower and slower expression of *PDF1.2* in *Fg*-inoculated *lox1-1* and *lox5-1* could be due to lowered fungal growth and spread on this mutant, and not due to a more direct impact of 9-LOX products on JA signaling.

SA biosynthesis in *Arabidopsis* occurs via the phenylalanine ammonia lyase (PAL) and the isochorismate synthase (ICS) pathways (Chen et al., 2009b). Analysis of the transcript levels of the *PAL1* and *ICS2* transcripts in response to *Fg* infection revealed that in WT plants, *PAL1*

accumulates to higher levels than *ICS2* (Figure 4.5B) resulting in only partial resistance. However, in the 9-LOX mutants the induction of *PAL1* was lower as compared to WT whereas the induction of *SID2* was higher (Figure 4.5B). This data in conjunction with the report that the *sid2* mutant is susceptible to *Fg* (Makandar et al., 2010) suggests that the major contributor to SA accumulation during *Fg* infection in *Arabidopsis* is the ICS pathway. However, in order to obtain conclusive evidence SA biosynthetic mutants are being generated in the 9-LOX mutant background. Further, the use of *PAL* inhibitors will help determine the contribution of the PAL pathway to SA biosynthesis during this interaction.

The close phylogenetic relationship between wheat and barley has enabled the isolation of wheat LOX genes. Based on sequence similarities with barley LOX genes, in common wheat three major LOX loci have been reported (Hart and Langston, 1977; Hessler et al., 2002; Wan et al., 2002; Carrera et al., 2007; Feng et al., 2010; Feng et al., 2011). Using a bioinformatic approach, the full length cDNAs of three wheat LOX genes, *TaLpx-1*, *TaLpx-2* and *TaLpx-3* were isolated. The deduced amino acid sequences reveal that *TaLpx-1* is homologous to a barley 9-LOX *HvLoxA*, *TaLpx-2* to a barley 13-LOX *HvLoxC*, and *TaLpx-3* is homologous to an as yet uncharacterized barley LOX *HvLoxB* (Table 4.2). *TaLpx-1* which shares strong homology to *HvLoxA* was chosen for further biochemical characterization (Figure 4.8A and B). The molecular weight of TaLPX-1, which lacks the PLAT/LH2 domain that is commonly observed in other plant LOXs, was 73.5 kDa (Figure 4.7A). TaLPX-1 preferred linoleic acid as a substrate as compared to linolenic acid. Analysis of TaLPX-1 products revealed the presence of both 9- and 13-hydroperoxides suggesting that TaLPX-1 shows dual oxygenation activity however the abundance of 9-hydroperoxides was fivefold higher as compared to 13-hydroperoxides (Figure 4.8B). LOX genes that show dual specificity have been reported in rice and maize among cereal

crops (Kim et al., 2003; Wang et al., 2008) and in several other plant species (Hughes et al., 2001; Palmieri-Thiers et al., 2009; Liu and Han, 2010). However, it should be noted that the positional specificity of LOXs *in vivo* may be determined in a more complex manner involving the physic-chemical state of the substrate, pH and/or temperature (Liavonchanka and Feussner, 2006).

In order to determine whether *TaLpx* genes contribute to wheat susceptibility to *Fg* as observed in the *Arabidopsis-Fg* interaction, transgenic wheat lines were developed where dsRNA-expressing constructs were introduced to silence *TaLpx* genes. The down-regulation of *TaLpx-1* resulted in reduced FHB severity (Figure 4.10). This result confirms that 9-LOXs also contribute to host susceptibility to *Fg* in wheat. The reduced severity to FHB observed in TaLpx-1-silenced lines and in plants silenced for expression of *TaLpx-2* and *TaLpx-3* (Figure 4.10) warrants further careful analysis. At present we cannot rule out the possibility that the dsRNA-expressing constructs for one *TaLpx* gene results in the suppression of other *TaLpx* homeologs, as well. Further analysis is also needed to verify the mechanism of enhanced resistance to FHB observed in the RNAi transgenics. The development of transgenic lines with reduced LOX activity is also desirable for the high carotenoid pigment content in products such as pasta and bread (Borrelli et al., 1999; Humphries et al., 2004; Leenhardt et al., 2006; Carrera et al., 2007). As a result, the RNAi transgenic wheat developed here can contribute to significant improvements in wheat quality.

In summary, the present study has revealed that the *Arabidopsis-Fg* model pathosystem provides a powerful tool for the identification of factors that contribute to host susceptibility that can be extrapolated with wheat. In *Arabidopsis-Fg* interaction, 9-LOX activity contributes to host susceptibility by suppressing SA-mediated defense responses. Further, the reduced disease

severity to FHB in wheat RNAi lines with suppression of *TaLpx* expression revealed that LOX activity is conserved in wheat and *Arabidopsis* interaction with *Fg*.

4.5 Methods

4.5.1 Plant Material and Growth Conditions

Arabidopsis was grown at 22^{o}C under 14 h light (100 µE m^{-2} sec^{-1})/ 10 h dark regime in autoclaved compost-peat based planting mixture (Premier Pro Mix-PGX, Procunier, Canada). Plants that were approximately four weeks old were used for all experiments. The *lox1-1* (SALK_059431), *lox1-3* (SALK_012188), *lox5-1* (SALK_044826) and *lox5-3* (SALK_050933) are in the accession Columbia-0 (Col-0). The *lox1-1lox5-1* double mutant and transgenic plants that were used have been described previously described (Chapter 2).

Wheat was grown in a greenhouse with a temperature regime of 21^{o}C and 18^{o}C during the day and night respectively under 14 h light/10 h dark. The planting mixture was sterilized by autoclaving (Premier Pro Mix-BX; Premier Tech Horticulture, http://www.pthorticulture.com/). Three plants were grown in a gallon-capacity pot.

4.5.2 Fungal Cultivation and Plant Infections

F. graminearum isolate Z-3639 was cultured for 7 d on half-strength potato dextrose agar (Difco Laboratories, Detroit, MI, U.S.A.) at 22^{o}C. Fungal hyphae was washed off the plate using a brush in 10 ml of sterile distilled water and filtered through four layers of sterile cheese cloth. The fungal suspension was infiltrated into the abaxial surface of *Arabidopsis* leaves with a needle-less syringe. Four-five leaves per plant were infiltrated and covered with a clear plastic dome for 48 h to maintain high humidity after which the dome was removed to allow for symptom development. Disease severity was recorded 4 days post inoculation (4 dpi). The

percentage of inoculated leaves exhibiting chlorosis covering <25% (category I), 25-50% (category II), 50-75% (category III) and >75% (category IV) of leaf area were determined for each genotype. A minimum of 60 leaves from 15 plants of each genotype were analyzed for each experiment. Disease severity index was calculated by the formula $100\ \Sigma\ (I\ n_I)\ /\ (N\ k)$ (Dong et al., 2008), where I is the disease severity score (1 for category I, 2 for category II, 3 for category III and 4 for category IV), n_I is the number of leaves with each score, N is the total number of leaves and k is the highest score (in this case it is 4). *Fusarium-Arabidopsis* Disease value for *F. graminearum* infection of *Arabidopsis* inflorescence was done as previously published (Urban et al., 2002). Fungal inoculations of wheat plants were carried out as previously described (Makandar et al., 2012). Disease was evaluated as percentage of infected spikelets at 5, 9, 15 and 21 days post inoculation (dpi). The significance of the data was tested by *t*-test and ANOVA, using the GLM procedure available in Minitab Version 15 (Minitab Inc., Pennsylvania) as indicated.

4.5.3 Microscopic Analysis of *F. graminearum* in *Arabidopsis* Leaves

Fg inoculated leaves were cut at the base and cleared with isopropanol. Cleared leaves were vacuum infiltrated (2 h at 25 mm Hg) with a solution containing WGA-Alexa Fluor 488 (Invitrogen, Carlsbad, CA, U.S.A.) and Toluidine Blue-O supplemented with bovine serum albumin (New England Biolabs, Ipswich, MA, U.S.A.) in phosphate-buffered saline (PBS, pH 7.4), each at 10 µg/ml. The leaves were then washed with PBS and mounted on glass slides with 30% glycerol. The leaves were visualized under UV light with a Nikon Eclipse E600 (Lewisville, TX, U.S.A.) epifluorescent microscope equipped with a Sony (Park Ridge, NJ, U.S.A) camera and a triple excitation filter (excitation filter: 395 to 410, 490 to 505, and 560 to 580; emission filter: 450 to 470, 515 to 545, and 600 to 650). The excitation wavelengths used

for WGA-Alexa Fluor 488 and Toluidine Blue-O were between 395 to 410 and 490 to 505 nm, respectively, with capture between 450 to 470 and 515 to 545 nm, respectively.

4.5.4 GUS Staining

Staining for GUS activity in leaves was performed as previously described (Malamy and Benfey, 1997).

4.5.5 RNA Isolation and Analysis

RNA extraction from *Arabidopsis* leaves and wheat tissue and cDNA synthesis was performed as previously described (Pegadaraju, 2005; Makandar et al., 2012). DNA contamination in samples was removed by treatment RNase-free DNase. For each time point and treatment, RNA was collected from three biological replicates, each consisting of leaves/spikes pooled from four plants. Gene specific primers used for reverse transcriptase (RT-PCR) and quantitative real time-PCR (qRT-PCR) are listed in Table 4.4 and were identified using the AtRTPrimer database (http://atrtprimer.kaist.ac.kr/) (Han and Kim, 2006). The PCR conditions for all RT-PCR reactions were as follows: 95ºC for 5 min, followed by 25 cycles of 95ºC for 30s, 58 ºC for 45s and 72 ºC for 45s, followed by a final extension step of 72 ºC for 5 min.

Real-time PCR was performed with Sybr® Green PCR Master Mix (Applied Biosystems, Cat No: 4309155) on an Eco qPCR system (Illumina®) using the following amplification protocol: 10 min polymerase activation and denaturation at 95ºC, 40 cycles of 95ºC for 10s, 58ºC for 30s and 72^{o}C for 30s. This was followed by a product melt to confirm a single PCR product. The level of gene expression was normalized to that of *EF1α* by subtracting the C_T value of *EF1α* from the C_T value of the gene of interest.

Table 4.4. Primers used in the study

Primer	Forward (5'→3')	Reverse (5'→3')	Product Size
FgTri5	AGCGACTACAGGCTTCCCTC	AAACCATCCAGTTCTCCATCTG	486
AtACT8	ATGAAGATTAAGGTCGTGGCA	TCCGAGTTTGAAGAGGCTAC	450
AtLOX1	CTGAGCTTGCCTCATCCTAATGGAG	ACCACCACCATTGATCAAGATTTGC	303
AtLOX5	CTTGCCTGACATCCTCAAAGAGAGC	GGTTCGGGTCGCATAGGTTTTAGTG	319
AtEF1α	AGGTCCACCAACCTTGACTG	GAGACTCGTGGTGCATCTCA	253
AtPR1	TTCTTCCCTCGAAAGCTCAA	AAGGCCCACCAGAGTGTATG	174
AtPDF1.2	CTTGTTCTCTTTGCTGCTTTC	CATGTTTGGCTCCTTCAAG	145
AtPAL1	AAGTGGCTGCGATCTCAACTATTGG	ATGTGTGGCTTGTTTCTTTCGTGCT	252
AtSID2	TGGCGAGGAGAGTGAATTTGCAG	GCTCGGCCCATTAACAAATTAAAGC	327
WhTub	GACATCAACATTCAGAGCACCATC	ATCTGTGCCTTGACCGTATCAGG	409
AtLOX1 - Clone	CACCCATATGATGTTCGGAGAACTTAGGG	GGCTCGAGTCAGATAGAGACGCTATTTGG	2580

TaLpx1 - Clone	CACCGCTAGCATGACGTCCGACTTCC TGGGCTAC	GCTTAAGTTAGCATCGAACAGTTGC TGACACG	1986
TaLpx2 - Clone	CACCGCTAGCATGTTCGGCGTCGGCG GCATCGT	GCTTAAGCAGATGGAATCCTGTTGG GGATGCCCCTGCCCG	2591
TaLpx3 - Clone	CTAGCTAGCATGCTGCTGCACGGGCT GGTGGACCGGCTGACGG	CCGCTCGAGCAGATGGAGACGCTGT TGGGGATGCCCATGGCGGTGAGG	2637
LpxB1.1	CACCCCATCTACTACCCGGACGAC	GATCCGATCCAGATGATGGT	172
LpxA2	CACCGATGGAGCCCTTCGTCATC	GCTCGGTGAAGTTCCAGTTC	218
LpxA3	CACCGATCGAGCCGTTCGTGATT	TCGGTGAGCTTCCAGTTCTT	216
WhLOX1	CACCATGCGCTTGAGATGTCTTCC	ATGGCAGGGTACTCGTTCAC	207
WhLOX2	CACCGCGACACCATGAACATCAAC	GGGTAGTCCTCGATCAGCAG	229
WhLOX3	CACCCTTCACCATGGAGATCAACG	TGGCGTAGGGGTAGTCCTCT	233

4.5.6 SA Measurement

Quantification of total SA in *Arabidopsis* leaves was done using a modified biosensor strain of *Acinetobacter* sp., ADP1. Leaves were extracted in fresh LB medium (2.5 ml per gm of leaf) and sonicated on ice for 5 min and centrifuged. The supernatant was used for SA estimation using the biosensor strain as described previously (Huang et al., 2005; Huang et al., 2006).

4.5.7 Chemical Treatment

Salicylic acid (SA) treatment was performed by first preparing a stock solution of 10 mM sodium salicylate (Sigma Aldrich, St. Louis, MO) in water and diluted to a final concentration of 200 µM. Pre-treatment of *Arabidopsis* with SA was done by irrigating the potted plants followed by spray of 200 µM solution till run off one day before *Fg* inoculation. Plants irrigated and sprayed with water provided the controls. A stock solution of Methyl JA (MeJA) (Sigma Aldrich, St. Louis, MO) was prepared in 0.1% ethanol and diluted to a working stock of 200 µM in 0.1% ethanol. *Arabidopsis* plants were placed in an airtight plexiglass chamber and exposed for 24 h to MeJA vapors prior to *Fg* infection. Control plants were treated with 0.1% ethanol and water in a similar manner. Cotton plugs soaked with 1 ml of MeJA solution (200 µM) were placed in a petri plate at the centre of the plexiglass chamber (48 liter volume).

4.5.8 Isolation and Cloning of Putative Wheat Lipoxygenases

Wheat expressed sequence tags (ESTs) identified as putative lipoxygenases belonging to Ta*Lpx-1*, Ta*Lpx-2* and Ta*Lpx-3* loci (Table 4.3) were used as queries to search the wheat genome database, DFCI Wheat Gene Index (Dana-Farber Cancer Institute, http://compbio.dfci.harvard.edu/). This resulted in the identification of three tentative contigs

representing *Lpx-1*, *Lpx-2* and *Lpx-3* loci. Primers designed to amplify putative full length cDNAs from developing wheat tissue are listed in Table 4.4. Total RNA samples were extracted from roots of germinating seedlings of the wheat cultivar, Bobwhite. The cDNA amplified using primers for *Lpx-1* locus was named as *TaLpx1* and cDNA amplified using primers for *Lpx-3* was named as *TaLpx3*. Further, *TaLpx1* was cloned into a bacterial expression vector for further characterization (Table 4.4).

4.5.9 LOX Reaction and Identification of LOX products

The cDNAs of *AtLOX1* and *TaLpx-1* were cloned in pET28c vector (Novagen, Schwalbach, Germany) using primers listed in Table 4.2. Recombinant proteins were expressed in BL21 (DE3) cells (Invitrogen). Isopropyl β-D-1-thiogalactopyranoside (IPTG) at 1 mM concentration was used to induce protein expression for a 5 h period. The cells were then harvested and lysed by sonication in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM Imidazole, pH 8.0 adjusted with NaOH). The cell lysates obtained by centrifugation at 10,000 g from 10 min to remove insoluble proteins were incubated with 2 mg 18:2 (linoleic acid) or 18:3 (linolenic acid) (Cayman chemicals) at room temperature for 1 hour and reduced with $NaBH_4$. Lipids were extracted and enriched in chloroform an analyzed via GC-MS as previously described (Kilaru et al., 2011).

4.5.10 Generation of RNAi Wheat Transgenics

Wheat EST sequences for putative wheat LOXs (*Lpx* and *WhLOX*s, Table 4.3) were amplified from germinating seedlings. To generate RNAi constructs for suppression of wheat lipoxygenases, 200-300 bp putative EST sequences corresponding to *Lpx-1*, *Lpx-2* and *Lpx-3* loci

were amplified. The RT-PCR products so obtained were cloned in pANDA-Mini vectors for wheat transformation which allows the cloning of gene/PCR fragment of interest as inverted repeats that are separated by a GUS linker (Miki and Shimamoto, 2004). The RNAi-constructs and plasmid containing the *Ubi:bar* construct (pAHC20) (Christensen and Quail, 1996) were co-bombarded into immature wheat embryos. Regeneration of wheat embryos was performed as previously described (Makandar et al., 2012). The numbers of independent transgenic lines obtained are listed in Table 4.3.

4.5.11 Molecular Analyses Transgenic Wheat

The DNA and RNA from leaves and germinating seedlings of wheat plants were extracted. Genomic PCR was used to monitor the presence of RNAi constructs using the forward primers for the respective line and a GUS linker primer in the T_1 generation (Table 4.4). In the T_2 and T_3 generations, RT-PCR analysis using insert specific primers was used to determine the level of gene suppression.

4.5.12 Statistical Analysis

For all assays, analysis of means using the General Linear Model (GLM) was used to separate the means (Minitab v15).

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CHAPTER 5

SUMMARY

Oxylipins play diverse roles during plant development and response to biotic stresses. In plants, oxylipins such as jasmonic acid play important roles as signal molecules in developmental processes and in response to attack by pathogenic microorganisms and insect pests (Mosblech et al., 2009, 2010; Wu and Baldwin, 2010). The importance of other oxylipins such as those derived from 9-LOXs is not well understood. The focus of this dissertation research was therefore to study the role of the 9-LOX pathway in plant response to various biotic stressors. In *Arabidopsis*, 9-LOXs facilitate infestation by the green peach aphid and infection by the fungal pathogen, *Fg*. Additionally, 9-LOX activity is also required for the induction of SAR.

The co-evolution of plants and their interacting insect pests/microbes has resulted in the development of complex interactions that determine the outcome (Walling, 2009). Whereas plants have evolved a variety of defense and tolerance mechanisms against pathogen attack and insect herbivory (Howe and Jander, 2008), the invading organisms have evolved countering strategies to bypass and/or overcome the barriers employed by plants (Walling, 2009). In *Arabidopsis*, 9-LOX activity is induced in response to aphid infestation, *Fg* and bacterial infection suggesting 9-LOX synthesized oxylipin(s) play an important role in plant response to various biotic stressors. The requirement of 9-LOX activity for the induction of SAR indicates a role in systemic promotion of defenses, whereas the same biochemical activity facilitates infestation by aphids and infection by *Fg* which suggests that certain pests/pathogens utilize plant responses for their own benefit.

The findings presented in this dissertation suggest that 9-LOX-oxylipins contribute to host susceptibility to aphids by enabling phloem and xylem sap consumption in addition to

promoting GPA fecundity. However, since 9-LOXs also influence plant defenses (Gao et al., 2008; López et al., 2011; Vicente et al., 2011), it is plausible that the impact of LOX5-synthesized oxylipins on facilitating GPA infestation is mediated by an antagonistic relationship with *Arabidopsis* defenses against GPA. Indeed, in *Arabidopsis* 9-LOX activity facilitates local infection by *Fg* by the suppression of SA-mediated defenses. Although, SA- and JA- mediated defenses were unaffected in the leaves of GPA-infested *lox5* mutants, at this point the influence of 9-LOX-oxylipins on plant defense signaling pathways in response to GPA-infestation cannot be ruled out. These findings suggest that GPA and *Fg* have evolved to either manipulate and/or evade a plant defense response mediated by 9-LOX activity to enable their successful colonization of the host plant. LOX5-derived oxylipins also impact insect population on an aphid diet, suggesting that LOX5-derived lipids may directly impact insect physiology. Similarly, these oxylipins could also impact *Fg*. The availability of genomic tools, for example RNAseq should permit characterizing the impact of *lox5* mutation and LOX5-derived oxylipins on gene expression in GPA and *Fg*, and hence the impact of these mutations and oxylipins on insect and fungal physiology.

The induction of *LOX5* activity in the roots of GPA-infested plants highlights an important role for the below ground organ in response to infestation by a foliar pest. The role of roots in plant response to insect pests and pathogens has often been underestimated. Roots are active participants in the "shoot-root-shoot loop" which involves the transmission of signals from the attacked tissue to below ground organs, their metabolic reconfiguration and a subsequent translocation of secondary metabolites involved in defense upwards to the shoot (Erb et al., 2009a). Although examples exist of root derived metabolites that enable plants to tolerate above ground herbivory (Erb et al., 2009b; Erb et al., 2009a), the findings presented here indicate that

aphids have adapted to utilize a root-derived metabolite to facilitate infestation of above ground parts. Further, the induction of *LOX5* activity in roots of GPA-infested plants implies the translocation of a plant or GPA-derived signal(s) to the roots. The identification of the source of the signal(s) will be essential to determine if the increase in *LOX5* activity in roots is a plant- or aphid-induced response.

The increased accumulation of SA and the hyper-activation of SA-mediated defenses in response to *Fg* infection in the 9-LOX mutants indicates that 9-LOX activity suppresses the activation of SA-mediated defenses. The breakdown of resistance in 9-LOX mutants by the exogenous application of MJ suggests that *Fg* exploits the antagonistic cross-talk between the two signal molecules (Kunkel and Brooks, 2002) to enable proliferation on the host. The requirement of SA for basal resistance to *Fg* in *Arabidopsis* and the dichotomous role of JA mediated signaling by contributing to susceptibility during early stages of infection and resistance during the later stages of infection by *Fg* provides additional support to this hypothesis (Makandar et al., 2010; Makandar et al., 2012). Further experimentation is needed to determine if the potential cross-talk is the result of *Fg* effectors or a direct result of 9-LOX activity.

The requirement of SA for basal resistance to bacterial pathogens and induction of systemic defenses during incompatible interactions is well established (Durrant and Dong, 2004). However, although basal resistance to bacterial pathogens is unaffected, the activation of systemic acquired resistance is compromised in the 9-LOX mutants. The inability of the 9-LOX mutants to generate a SAR signal(s) and the presence of 9-LOX derived oxylipins in petiole exudates from GPA-infested plants raises the possibility that bacterial infection also induces the accumulation of these compounds in the vascular sap. Alternatively, the possibility exists that 9-LOX activity is required for the synthesis of a mobile signal molecule(s). Further, the inability of

the 9-LOX mutants to perceive a SAR signal(s) that is present in Avr-Pet-ex from wild-type plants suggests that 9-LOX activity is also required in distal leaves. Whether 9-LOX activity is required for the perception of the SAR signal(s) or for the activation of downstream responses after signal(s) perception requires further experimentation.

In summary, the induction of 9-LOXs in response to an insect pest, a fungal pathogen and during bacterial infection suggests an important role for 9-LOX derived oxylipins in plant response to these varied biotic stresses. Certain pests and pathogens however have seemed to acquired adaptations to evade/modulate 9-LOX mediated responses to enable their proliferation and growth on the host.

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