9-LIPOXYGENASE OXYLIPIN PATHWAY IN PLANT RESPONSE TO BIOTIC STRESS

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Dissertation Prepared for the Degree of

DOCTOR OF PHILOSOPHY

UNIVERSITY OF NORTH TEXAS

May 2012

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UMI Number: 3533629

UMI 3533629

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Nalam, Vamsi J. 9-Lipoxygenase oxylipin pathway in plant response to biotic stress. Doctor of Philosophy (Molecular Biology), May 2012, 162 pp., 7 tables, 29 figures, chapter references.

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.

ACKNOWLEDGEMENTS

I would like to express my gratitude to my major professor, Dr. Jyoti Shah for his constant support and guidance. I appreciate his courage in accepting me as a PhD student even though my experience in the field of molecular biology and plant defenses was minimal. I also extend my thanks to my committee members, Dr. Brian Ayre, Dr. Kent D. Chapman, Dr. Rebecca Dickstein and Dr. Camelia Maier (Texas Woman's University). I would also like to thank the University of North Texas for providing me with financial support during the course of my study and for the facilities to carry out my research. I would like to thank all the past and present members of the Shah lab – Ratnesh Chaturvedi, Ragiba Makandar, Kataryzna-Lorenc Kukula, Kartikeya Krothapalli, Sujon Sarowar, Hossain Mondal, Joe Louis, Zulkarnain Chowdhry and last but not the least Vijay Singh – for being wonderful colleagues, assistance with lab techniques and also for their good humor that helped my during the most challenging times. Special thanks to Eyad 'kartman' Kattan and Guy Klossner for their help with everything from making soil to washing pots and dishes. I would also like to thank Jantana Keeretaweep and Dr. Kent Chapman for the assistance with oxylipin profiling. The members of the Chapman lab, Chris James, Patrick Horn, and Bikash Adhikari for making the fourth floor of the Life Science Building a happy place to work in.

I am especially grateful to my parents, sister and her family, who have been a pillar of support in my life. Finally, I am greatly indebted to the love of my life, Punya Nachappa, who has borne my innumerable shenanigans with a patience that never seems to end.

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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).

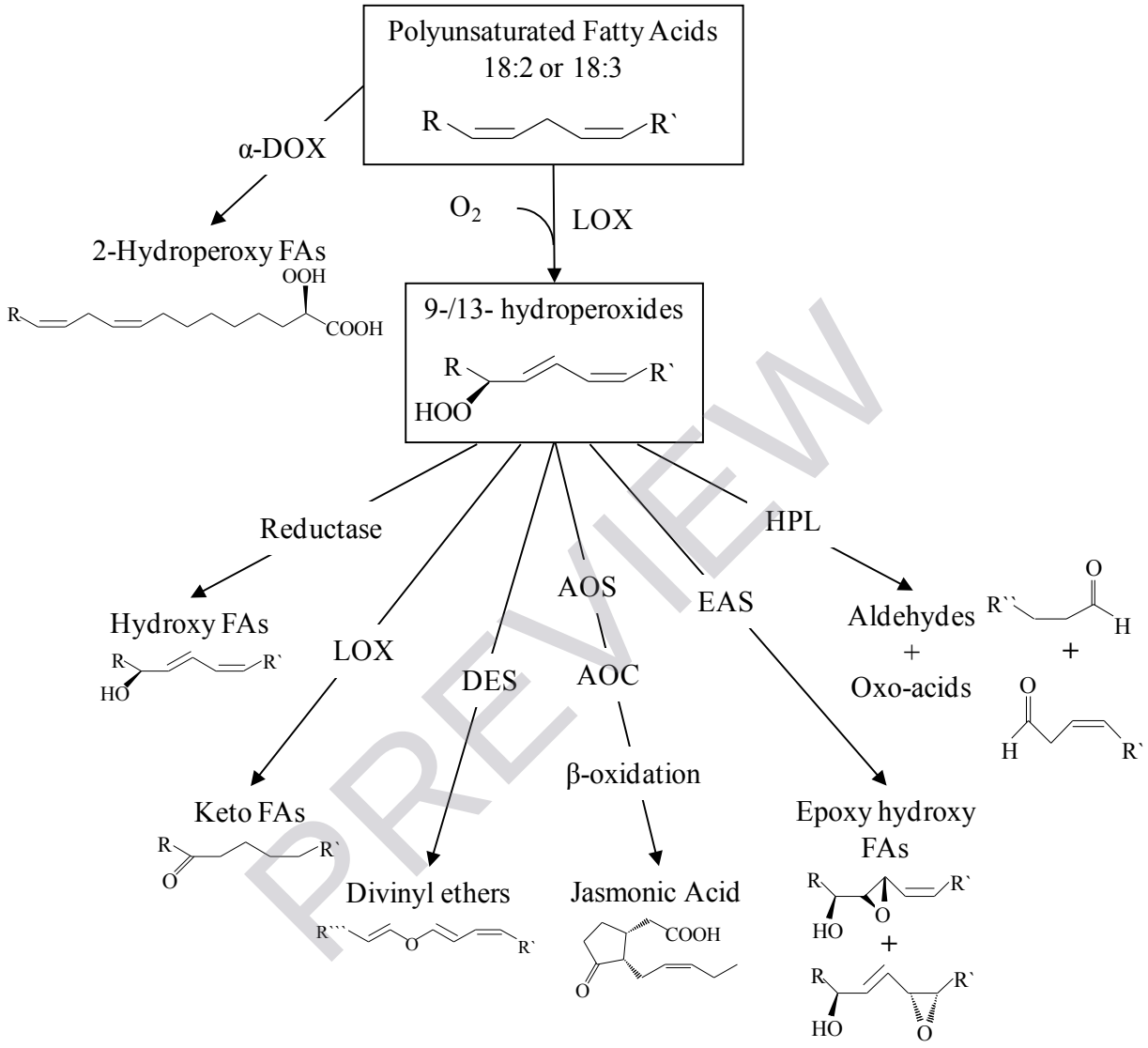

Figure 1. 1 Major pathways of oxylipin biosynthesis in plants from linoleic (18:2) or linolenic acid (18:3). LOX, Lipoxygenase; α-DOX, α-dioxygenase; DES, divinyl ether synthase; AOS, Allene oxide synthase; AOC, Allene oxide cyclase EPS, Epoxy alcohol synthase; HPL, Hydroperoxide lyase; FAs, Fatty acids.

1.2.1 Lipoxygenases

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