

ROLE OF *N*-ACYLETHANOLAMINES IN PLANT DEFENSE RESPONSES:
MODULATION BY PATHOGENS AND COMMERCIAL ANTIMICROBIAL
STRESSORS

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N-acyl ethanolamines (NAEs) are a class of lipids recently recognized as signaling molecules which are controlled, in part, by their degradation by fatty acid amide hydrolase (FAAH). On the basis of previous studies indicating increased NAE levels in a tobacco cell suspension-xylanase elicitor exposure system and the availability of *FAAH* mutants, overexpressor and knockout (OE and KO) genotypes in *Arabidopsis thaliana*, further roles of NAEs in *A. thaliana* plant defense was investigated. The commonly occurring urban antimicrobial contaminant triclosan (TCS) has been shown to suppress lipid signaling associated with plant defense responses. Thus, a second objective of this study was to determine if TCS exposure specifically interferes with NAE levels.

No changes in steady state NAE profiles in *A. thaliana*-*Pseudomonas syringae* pv. *syringae* and *A. thaliana*-flagellin (bacterial peptide, flg22) challenge systems were seen despite evidence that defense responses were activated in these systems. There was a significant drop in enoyl-ACP reductase (ENR) enzyme activity, which catalyzes the last step in the fatty acid biosynthesis pathway in plants, on exposure of the seedlings to TCS at 10 ppm for 24 h and decreased reactive oxygen species (ROS) production due to flg22 in long term exposure of 0.1 ppm and short term exposure of 5 ppm. However, these responses were not accompanied by significant changes in steady state NAE profiles.

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS	iii
LIST OF FIGURES.....	vii
ABBREVIATIONS.....	x
1. GENERAL INTRODUCTION	1
2. BACKGROUND REVIEW	6
2.1 Introduction	6
2.2 Mechanism of Plant Response to Pathogens	7
2.2.1 Constitutive Barriers	7
2.2.1.1 Physical	7
2.2.1.2 Chemical.....	8
2.2.2 Inducible Responses.....	8
2.2.2.1 PAMP-Triggered Immune Responses (PTI)	9
2.2.2.2 Effector-Triggered Immune Responses (ETI).....	11
2.3 NAEs in Plants	13
2.3.1 Discovery in Plants and Context in Mammalian Work	13
2.3.2 Molecular Species and Abundance	14
2.3.3 NAE Metabolism.....	16
2.3.3.1 Synthesis.....	16
2.3.3.2 Degradation	16
2.3.4 Role in Cytoprotection: Physical and Chemical Stress	18
2.3.5 Role in Signaling.....	18
2.3.5.1 Growth.....	18
2.3.5.2 Defense.....	19
2.3.5.3 Possible Interactions with Other Signaling Pathways/Hormones	19
2.3.6 The Case for an NAE Signaling Role in Plant/Pathogen Interactions	20
2.3.6.1 Study of Tobacco Cell Suspension Exposure to Xylanase	20
2.4 Chemical Contaminants in Plants	21
2.4.1 Traditional Studies of “Legacy” Pollutants on Germination and Growth; Biomonitoring.....	21
2.4.2 Contaminant Interference with Plant Immunity.....	22
2.5 Commercial Antimicrobials in the Environment	23
2.5.1 Historical Use, Distribution and Bioaccumulation	23

2.5.1.1	TCS.....	24
2.5.1.2	MTCS.....	24
2.5.2	Mode of Action	24
2.5.2.1	Previous Work from Our Laboratory Demonstrating Effects of Antimicrobial Exposure on Plant Lipid Biosynthesis.....	25
2.6	A Direct Link between TCS Activity and Inhibition of Plant Defense Response Suggests Interference in Lipid Signaling as the Mechanism.....	25
2.6.1	TCS Inhibition of Plant Defense Responses	25
2.6.2	Hypothesized Connection to Lipid Messenger Signaling	27
2.7	Background Summary.....	28
2.7.1	Much is Known about Plant Defense Responses.....	28
2.7.2	Little is Known about the Role of NAEs in Plant Defense Responses.....	28
2.7.3	Nothing is Known about Chemical Contaminant Interference with Plant Immunity (i.e. Plant Immunotoxicity).....	28
2.8	Dissertation Objectives and Hypotheses	29
3.	MATERIALS AND METHODS.....	32
3.1	Growth of Adult Arabidopsis Plants	32
3.2	Well Plate Plant Growth Protocol	33
3.3	Enoyl-ACP Reductase Enzyme Activity Protocol (Serrano et al., 2007)	33
3.4	ROS Assay Protocol Modified from Serrano et al. (2007).	34
3.5	NAE Analysis Protocol	36
3.6	TCS and MTCS Analysis Protocol	40
4.	ADULT PLANT EXPOSURE TO PATHOGENIC BACTERIA.....	42
4.1	Introduction.....	42
4.2	Exposure Set-up for Adult Plant Experiments	44
4.3	Quantification of NAEs levels in Adult Plant Tissue	45
4.4	Results and Discussion.....	45
4.4.1	Results	45
4.4.2	Discussion	50
5.	SEEDLINGS EXPOSURE TO BACTERIAL ELICITOR	52
5.1	Introduction	52
5.2	Exposure Set-up for 5-day Seedlings and Methods for Analyses.....	53
5.2.1	NAE Analysis.....	54
5.2.2	ROS Analysis	54
5.3	Results and Discussion.....	54
5.3.1	NAE Analysis Results.....	54
5.3.2	NAE Analysis Discussion	59
5.3.3	ROS Analysis Results	60
5.3.4	ROS Analysis Discussion	62

6.	SEEDLINGS EXPOSURE TO BACTERIAL ELICITOR AND TCS	63
6.1	Introduction	63
6.2	Exposure Set Up for 5-day Seedlings and Methods of Analyses.....	64
6.3	Results and Discussion.....	65
6.3.1	Effects of TCS Exposure on ENR Enzyme Activity: Proteins Extract Exposures	65
6.3.2	Effects of TCS Exposure on ENR Enzyme Activity: 5-day Seedling Exposures	67
6.3.3	Effects of TCS Exposure and Combined Exposure to TCS and flg22 on NAE Profiles and TCS, M-TCS Accumulation	69
6.3.4	Discussion	74
7.	ZAT12 SEEDLINGS EXPOSURE TO BACTERIAL ELICITOR AND TCS.....	75
7.1	Introduction	75
7.2	Exposure and Analyses	76
7.3	Results and Discussion.....	77
7.3.1	NAE Profiles in Zat12 and WT 5-Day Seedlings	77
7.3.2	ROS Measurement in 5-Day Zat12 Seedlings	77
7.3.3	Changes in ROS Measurement on Short-Term Treatment of Seedlings to TCS Prior to Flg22 Exposure	79
7.3.4	Changes in ROS Measurement and Accumulation of TCS on Long-Term Treatment of Seedlings to TCS Prior to Flg22 Exposure.....	80
7.3.4.1	TCS Accumulation.....	80
7.3.4.2	ROS Analysis Discussion	81
7.3.5	Discussion	83
8.	CONCLUSIONS.....	84
8.1	Adult Plant Exposure to Pathogenic Bacteria: NAE Analyses	85
8.2	Seedlings Exposure to Bacterial Elicitor: NAE and ROS analyses	86
8.3	Seedlings Exposure to Bacterial Elicitor and TCS: NAE, TCS, ENR Activity Analyses.....	87
8.4	Zat12 Seedlings Exposure to Bacterial Elicitor and TCS: NAE, ROS and TCS Analyses	88
8.5	Summary and Future Work.....	89
	REFERENCES.....	91

LIST OF FIGURES

Fig. 2.1. Molecular species of N-acyl ethanolamines found in plants (Chapman, 2000) Reproduced with permission from Elsevier.	15
Fig. 2.2. Formation and degradation of NAEs (Chapman, 2000). Reproduced with permission from Elsevier.	17
Fig. 4.1. Steps involved in the vacuum infiltration process for adult plants (Katagiri et al., 2002).	44
Fig. 4.2. NAE levels on fresh mass basis from WT plants at 0 h and buffer/pss treated samples at 6 and 24 h.	46
Fig. 4.3. NAE levels on fresh mass basis from OE7 plants at 0 h and buffer/pss treated samples at 6 and 24 h.	47
Fig. 4.4. NAE levels on fresh mass basis in WT plants at 0 h and buffer/pss treated samples at 0.5 and 1.5 h.	48
Fig. 4.5. NAE levels on fresh mass basis from OE7 plants at 0 h and buffer/pss treated samples at 0.5 and 1.5 h.	48
Fig. 4.6. NAE levels on fresh mass basis from WT, OE7 plants at buffer/pss treated samples at 1.5 h.	49
Fig. 5.1. NAE levels in WT 5-day seedling samples, exposed to flg22 peptide elicitor for 90 minutes.	55
Fig. 5.2. NAE levels in WT 5-day seedling samples, exposed to flg22 peptide elicitor for 24 h.	56
Fig. 5.3. NAE levels in WT 5-day seedling samples, exposed to flg22 peptide elicitor for 24 h.	56
Fig. 5.4. NAE levels in WT 5-day seedling samples, exposed to flg22 peptide elicitor for 24 h.	57
Fig. 5.5. NAE levels in KO 5-day seedling samples, exposed to flg22 peptide elicitor for 24 h.	57
Fig. 5.6. NAE levels in OE2 5-day seedling samples, exposed to flg22 peptide elicitor for 24 h.	58

Fig. 5.7. <i>A. thaliana</i> WT 10-day seedlings exposed to flg22 at 1 μ M or DMSO solvent at 0.07% concentration.....	60
Fig. 5.8. <i>A. thaliana</i> WT 5-day seedlings exposed to flg22 at 1 μ M or DMSO solvent at 0.07% concentration.....	61
Fig. 5.9. The percentage change in flg22 (1 μ M) treated cumulative luminescence units relative to solvent control samples from different experiments.....	61
Fig. 5.10. Relative cumulative luminescence readings of solvent control and flg22 (1 μ M) treated samples.	62
Fig. 6.1. Protein extract from <i>A. thaliana</i> 4 week old plants exposed to Solvent methanol and various concentrations of TCS and methyl TCS (MTCS): 0.1, 1.0 and 10.0 ppm for 2 h.....	66
Fig. 6.2. Protein extract from <i>A. thaliana</i> 4 week old plants exposed to Solvent methanol and various concentrations of TCS: 0.01, 0.05, 0.1, 1.0 and 10.0 ppm for 2 h.....	66
Fig. 6.3. <i>A. thaliana</i> 5-day seedlings exposed to no treatment (control) and methanol (solvent) and various concentrations of TCS: 0.01, 0.1 and 1.0 ppm for 12 h	68
Fig. 6.4. <i>A. thaliana</i> 5-day seedlings exposed to no treatment (control) and methanol (solvent) and various concentrations of TCS: 0.1, 1.0, 1.0 and 10.0 ppm for 2 h.	68
Fig. 6.5. <i>A. thaliana</i> WT, KO and OE2 5-day seedlings exposed to no treatment (control) and methanol (solvent) TCS concentration of 10.0 ppm for 24 h.....	69
Fig. 6.6. NAE levels on fresh mass basis from WT plants treated with either a) solvent (0.1% ethanol+0.07% DMSO), b) flg22 (1 μ M) or c) flg22 (1 μ M) + TCS (10 ppm) for 24 h.....	71
Fig. 6.7. NAE levels on fresh mass basis from KO plants untreated (control) or treated with either a) solvent (0.1% ethanol+0.07% DMSO), b) flg22 (1 μ M) or c) flg22 (1 μ M) + TCS (10 ppm) for 24 h.....	71
Fig. 6.8. NAE levels on fresh mass basis from OE2 plants untreated (control) or treated with either a) solvent (0.1% ethanol+0.07% DMSO), b) flg22 (1 μ M) or c) flg22 (1 μ M)+ TCS (10 ppm) for 24 h.	72
Fig. 6.9. TCS accumulation on fresh mass basis in WT 5-day seedlings treated with either a) solvent (0.1% ethanol+0.07% DMSO), b) TCS 10 ppm c) flg22 (1 μ M) or d) flg22 (1 μ M) + TCS (10 ppm) for 24 h.....	72
Fig. 6.10. TCS accumulation on fresh mass basis in KO and OE2 5-day seedlings treated with either a) solvent (0.1% ethanol+0.07% DMSO) or b) TCS 10 ppm for 24 h.	73

Fig. 6.11. MTCS accumulation on fresh mass basis in KO and OE2 5-day seedlings treated with either a) solvent (0.1% ethanol+0.07% DMSO) or b) TCS 10 ppm for 24 h.	73
Fig. 7.1. Comparison of NAE levels in 5-day WT and Zat12: Luciferase seedlings on a fresh mass basis.	77
Fig. 7.2 a, b. Luminescence readings from 5-day Zat12 seedlings exposed to a) solvent 0.1% DMSO and b) flg22 1µM.	78
Fig. 7.3. Percentage change in relative luminescence unit readings from 5-day Zat12 seedlings exposed to a) solvent 0.1% DMSO and b) flg22 1 µM.	79
Fig. 7.4. Luminescence readings from 5-day Zat12 seedlings incubated with solvent (0.1% ethanol), 5 or 10 ppm TCS for 45 minutes.	80
Fig. 7.5. TCS accumulation on long term exposure of Zat12 5-day seedlings to TCS concentrations of 0.01, 0.1 and 1.0ppm.	81
Fig. 7.6. Relative luminescence readings from 5-day Zat12 seedlings exposed to solvent (0.07% DMSO (s) or 1uM flg22 (f). The seedlings were incubated with a) solvent 0.1% ethanol (Solvent) or b) 0.01 ppm TCS (0.01) or c) 0.1 ppm TCS (0.1) or d) 1.0 ppm TCS (1) from day 0.	82
Fig. 7.7. Relative luminescence readings from 5-day Zat12 seedlings exposed to solvent 1µM flg22. The seedlings were incubated with a) solvent 0.1% ethanol (s) or b) 0.01ppm TCS (0.01) or c) 0.1 ppm TCS (0.1) or d) 1.0 ppm TCS (1) from day 0.	82

ABBREVIATIONS

ABA	– Absciscic acid
ACP	– Acyl carrier protein
AOS	– Allene oxide synthase
<i>At</i>	– <i>Arabidopsis thaliana</i>
CB	– Cannabinoid
DMSO	– Dimethyl sulfoxide
ENR	– Enoyl ACP reductase
ETI	– Effector triggered immunity
ETYA	– Eicosatetraynoic acid
FAAH	– Fatty acid amide hydrolase
Flg22	– Flagellin
HPRG	– Hydroxyproline-rich glycoproteins
HR	– Hypersensitivity response
KO	– Knockout
LOX	– Lipoxygenases
MAPKs	– Mitogen activated protein kinases
MTCS	– Methyl triclosan
NAEs	– N-acylethanolamines
NAPE	– N-acylphosphatidyl ethanolamine
NATs	– N-acyl taurines
NB-LRR	– Nucleotide-binding (NB) leucine-rich repeat (LRR)
NDGA	– Nordihydroguaiaretic acid
OE	– Overexpressor
PAMP	– Pathogen associated molecular pattern
PCBs	– Polychlorinated biphenyls
PLD	– Phospholipase D
PPCPs	– Pharmaceutical and personal care products
PRPs	– Pathogenesis related proteins
PTI	– PAMP triggered immunity
RLKs	– Receptor like kinases
RLPs	– Receptor like proteins
RLU	– Relative luminescence units
ROS	– Reactive oxygen species
SA	– Salicylic acid
SAR	– Systemic acquired resistance
TCS	– Triclosan
WT	– Wild type

CHAPTER 1

GENERAL INTRODUCTION

Plants engage in defense responses in order to survive changes in the environment and attacks by herbivores and pathogens. Plants are constantly evolving and, in the rapidly changing environment of today, it is important to understand their survival responses to the physical, chemical and biological stressors they face as a part of a comprehensive effort for conservation of their critically important roles in agriculture and biodiversity.

Much work has been done in the investigation of plant pathogen defense responses. Plants respond immediately with short-term signals and also engage in long-term transcriptional changes to overcome such attacks in the future, developing systemic acquired resistance (SAR). Pathogen associated molecular pattern (PAMP) triggered immunity (PTI) or effector triggered immunity (ETI) are among these inducible responses. Overall there is a high level of complexity involved in the signaling and execution of plant defense responses which have been described in detail in several excellent reviews (Heath, 2000; Chisholm et al., 2006; Conrath, 2006; Garcia-Brugger et al., 2006; Ingle et al., 2006; Jones and Dangl, 2006; Bent and Mackey, 2007; Koornneef and Pieterse, 2008; Raffaele et al., 2009; Asselbergh et al., 2008).

N-acylethanolamines (NAEs) are an important class of lipid signaling molecules involved in the “endocannabinoid system” and are receiving a great deal of attention in vertebrates because of their potential therapeutic applications (Cravatt et al., 1995; Jonsson et al., 2006; Duncan et al., 2009). They are known to have important roles in their interaction with endocannabinoid receptors in the central nervous system (principally the CB1 receptor) and in peripheral tissues including immunoactive cells (principally the CB2 receptor) as well as serving as cytoprotective molecules (Mackie, 2006). NAEs also occur in plants and affect important processes such as germination and growth (Chapman et al., 1999; Blancaflor et al., 2003; Chapman, 2004). Exposure to exogenous NAEs causes inhibition of germination and growth in *Arabidopsis thaliana* (Blancaflor et al., 2003; Motes et al., 2005). With the development of transgenic lines which over-express or do not possess the principal enzyme responsible for the catabolism of NAEs, fatty acid amide hydrolase (FAAH), further studies were conducted to examine the role of NAEs in various plant processes (Wang et al., 2006; Teaster et al., 2007). The potential role of NAEs in plant defense responses was first suggested when it was shown that some NAEs dramatically increase in concentration on exposure of the tobacco cell cultures to fungal elicitors (Tripathy et al., 1999). Subsequently, it was shown that adult *A. thaliana* FAAH overexpressor plants are more susceptible to pathogen attack, developing necrosis much faster than wild type (Kang et al., 2008). However, in this study there was a decrease in the levels of NAEs in pathogen-treated samples in comparison with controls as opposed to the increase previously reported for tobacco

(Tripathy et al., 1999). A more detailed understanding of the possible role of NAEs in plant defense responses in seedling and adult plants is needed.

Plants must cope not only with pathogen stressors, with which they share millions of years of coevolution, but must also cope with an onslaught of very recently occurring chemical contaminants. Our modern urbanized societies have resulted in an almost instantaneous increased presence of chemical stressors, many of which now have a global distribution. One of the emerging classes of contaminants is the class known collectively as “pharmaceutical and personal care products” (PPCPs). Many of these products contain chemicals specifically designed to have potent biological activity. Of particular concern are those compounds designed as biocides which include the widely distributed antimicrobial compounds used in hundreds of household products.

The antimicrobial Triclosan (TCS) is a biocide which is widely distributed in the environment (Kolpin et al., 2002; Balmer et al., 2004; Bester, 2005). It tends to bioaccumulate in plant and animal systems (Balmer et al., 2004; Coogan, 2007; Coogan et al., 2007; Coogan and La Point, 2008) and studies have shown that it inhibits lipid biosynthesis in prokaryotes and plants (Heath et al., 1999; Serrano et al., 2007). Recently an examination of a library of over 120 small bioactive compounds for their ability to suppress defense responses to pathogen challenge in *A. thaliana*, revealed TCS to be the most suppressive compound in the library (Serrano et al., 2007). Interruption in lipid signaling was the hypothesized mechanism of defense inhibition. However, changes in defense responses and defense signaling pathways in plants exposed to chronic low levels or acute high levels of these antimicrobials have not been studied.

Although a great deal is known about the molecular biology of plant defense responses, the potential role of NAEs in these responses is only beginning to be appreciated. Similarly, the widespread environmental distribution of PPCPs has been well documented, but the risk that these compounds may represent for non-target organisms is still highly speculative and based primarily on studies of the effects on aquatic animals. Essentially nothing is known about the interaction of environmental chemical stressors and the health of defense response in plants. I have chosen the model system of *A. thaliana* in order to explore the role of NAEs in the defense response to pathogen challenge and how this response is influenced by exposure to the antimicrobial TCS.

The overarching objectives of my dissertation study were as follows:

Objective 1: To determine the role of NAEs in defense responses of adult *A. thaliana* and 5-day seedlings when pathogen challenged by a) quantification of the concentration profiles of short chain NAEs with and without challenge in wild-type and FAAH-altered genotypes and b) quantification of reactive oxygen species (ROS) production as an indicator of strength of response to the challenge.

Objective 2: To investigate, changes in NAE and ROS production observed in Objective 1 due to exposure to the antimicrobial compound TCS and quantify its inhibition of lipid biosynthesis as indicated by decreased Enoyl-ACP reductase enzyme activity, changes in NAE concentration profiles and modulation of ROS production.

These objectives were addressed in four studies that are briefly introduced at the end of the Chapter 2 background review and presented in detail in Chapters 4-7. Chapter

3 contains methods common to multiple experiments described in Chapters 4-7 while Chapter 8 provides the comprehensive conclusions of this work and ideas for further research.

CHAPTER 2

BACKGROUND REVIEW

2.1 Introduction

Plants are the basis of primary productivity on earth and play the important ecological role of providing food for all consumer organisms, including humans. Growing crops efficiently for food and bio-fuels has become extremely important with the growing world population and increasing energy demands. The man-made decrease in diversity of species across lands under management has increased the probability of severe destruction in short periods of time. Crop viability and productivity are influenced by a plethora of conditions. Physical stresses due to location (such as heat/cold, flooding/drought) along with chemical stressors such as environmental contaminants and biological stressors such as microbial pathogens and herbivores can all greatly alter productivity (Orcutt and Nilsen, 2000). The use of genetically modified seeds in crop production to increase yields may, in some cases, make them more susceptible to disease, herbivory and changes in the environment. A thorough understanding of all the factors involved in defense responses is important in order to develop better quality of seeds for better yields, productivity and sustainability.

Plants are not mobile and cannot move away from stressors in any form, physical, chemical or biological. Hence plants have evolved mechanisms of response to all these

stressors. Considering that there are many different kinds of stressors, we observe plant defense responses at multiple levels, including immediate responses such as hypersensitive cell death as well as long term evolutionary genetic transformations. Examples of plant stressors that have played a role in the evolution of plant defense responses include microbial pathogens, insects, herbivores and physical stressors such as temperature, water availability, light, salt and nutrient availability. In this study, I have focused on pathogen stress as modeled by exposure to the bacterial peptide flg22. A summary of plant responses to pathogen stressors follows.

2.2 Mechanism of Plant Response to Pathogens

Defense mechanisms which are pre-existing or constitutive can be structural barriers or preformed antimicrobial compounds, such as cyanogenic glucosides, terpenoids, phenolics and hydroxamic acids. Activation of inducible defenses is based on recognition of an invasion. Induced structural barriers can include cell wall lignification, papillae formation, vascular occlusions, increased hydroxyproline-rich glycoproteins (HPRGs), and hypersensitive response (HR). Induced biochemical barriers include production of chemicals such as phytoalexins and pathogenesis related proteins (PRPs) (Turner, 1995).

2.2.1 Constitutive Barriers

2.2.1.1 Physical

Plant cells have walls made up of a matrix of non-cellulosic polysaccharides, cellulose and glycoproteins. The cell walls also contain lignin which is a hydrophobic phenolic polymer. The plant leaves and shoots are covered by a waxy layer called cuticle.

Pathogens and other microorganisms often attack this first line of defense by digesting some of the cell wall components. Cellulose is degraded by cellulases. Pectate matrices are degraded by the addition of water by glycoside hydrolases and by β -elimination of glycosidic bonds by polysaccharide lyases (Steven et al., 2000).

2.2.1.2 Chemical

Constitutive chemical defenses in plants include the presence of secondary metabolites. These chemicals can act in defense against microbes, insects and herbivores. They can also prevent the germination and growth of adjoining plants. Cyanogenic glycosides, terpenoids, hydroxamic acids and phenols are some examples of these compounds and newer compounds are still being discovered (Turner, 1995).

2.2.2 Inducible Responses

On the failure of the physical and chemical barriers, inducible resistance sets in and can range from a quick death of the infected area by hypersensitive response cell death to the expression of pathogenesis related proteins, initiation of signaling cascades that can trigger local defense responses such as PTI (PAMP triggered immunity) and ETI (effector triggered immunity) responses and systemic defense responses such as increase in systemic acquired resistance (SAR) and expression of defense genes (Chisholm et al., 2006; Jones and Dangl, 2006).

Some pathogenic virulence factors can attack the plant by degrading the cell wall. Some virulence factors such as coronatine, a nonhost specific phytotoxin produced by many of the pathovars of *Pseudomonas syringae*, increase the synthesis of compounds which limit the increase of salicylic acid (SA) that is a required for local defenses and for

the activation of SAR (Uppalapati et al., 2007). In such cases the plant may become diseased.

At the time of wounding and exposure to pathogen attack there is production of polyunsaturated fatty acids such as linolenic acid (18:3), linoleic acid (18:2), and hexadecatrienoic acid (16:2), which are released from plastidic membranes by phospholipases. These acids are catabolized by other enzymes to produce a class of lipids known as oxylipins, such as jasmonic acid, which are involved in signal transduction of such stresses (Kaloshian and Walling, 2005).

2.2.2.1 PAMP-Triggered Immune Responses (PTI)

Once a pathogen or its elicitor (virulent or avirulent) invade into the extracellular space of the plant through open pores such as stomata, and begins to cross the cell's walls, a host of defense responses are activated. These responses are based on the identification of pathogen associated molecular patterns (PAMPs) (Chisholm et al., 2006). The responses which are triggered to stop the pathogen from becoming established in the host are termed PAMP triggered immunity responses (PTI). Liposaccharides, flagellin of gram negative bacteria, bacterial elongation factor (EF-Tu), chitin and ergosterol of fungi are some examples of PAMPs capable of eliciting these responses.

PTI is part of the non-host resistance in plants and is activated in the presence of potential pathogens. If the PAMP binds to an appropriate receptor, it can lead to further activation of signaling events that can further increase the expression of the plant's basal responses in the form of increased physical barriers or induction of chemical responses (Ingle et al., 2006).

The conserved microbial features recognized by the plant cell-surface receptors induce MAP kinase signaling, transcription of pathogen-resistance protein genes, production of ROS, and also deposition of callose to reinforce the cell wall at sites of infection (Steven et al., 2000; Garcia-Brugger et al., 2006). The conserved plant motifs that recognize these patterns are mostly receptor-like kinases (RLKs) which have serine/threonine rich regions, receptor-like proteins (RLPs) and extracellular binding proteins that may form part of multicomponent recognition complexes (Sanabria et al., 2008). RLKs are structurally related to the polypeptide growth factor receptors of animals which consist of a large extracytoplasmic domain, a single membrane spanning segment and a cytoplasmic domain of the protein kinase gene family. Based on structural similarities in the extracellular domains, the RLKs are divided into three categories: the S-domain class, the leucine-rich repeat class (similar to mammalian Toll-like receptors, TLRs) and a class that has epidermal growth factor-like repeats. Both monocot and dicot plant species have been shown to exhibit these putative receptors (Morillo and Tax, 2006). The diversity of structural and functional properties of the ever growing number of plant RLKs represent a new area of investigation into cellular signaling in plants designed to identify the mechanisms by which plant cells perceive and respond to extracellular signals (Walker, 1994). If a plant is successful in triggering defense responses, it indicates the RLKs are successful in transferring the signals to mitogen activated protein kinases (MAPKs, MAPKKs).

Mitogen-activated protein (MAP) kinases are serine/threonine-specific protein kinases that respond to extracellular stimuli (mitogens) and regulate various cellular

activities, such as gene expression, mitosis, differentiation, and cell survival/apoptosis in both plants and animals. Some of the intracellular receptors acting in the signaling cascade can also belong to the nucleotide-binding (NB) leucine-rich repeat (LRR) class of receptors (Hirt, 1997).

PAMP signaling frequently involves signaling through WRKY transcription factors. The WRKY proteins contain one or two highly conserved WRKY domains characterized by a heptapeptide WRKYGQK and a zinc-finger structure. To date, genes encoding WRKY proteins have been identified only from plants. To regulate gene expression, the WRKY domain binds to the W box (TGAC(C/T) region) in the promoter of the target gene to modulate transcription (Zhang and Wang, 2005).

Fungal and bacterial elicitors can trigger fluxes of H^+ , K^+ , Cl^- and Ca^{2+} within minutes. These fluxes can be mediated by protein kinase cascades. Signaling cascades involving polyunsaturated fatty acids such as oxylipins can also occur. ROS such as H_2O_2 are generated by these elicitors and can be mediated by several different signaling cascades (for example: cell wall peroxidases, gene expression). It can lead to changes such as a shift in flux of Ca^{2+} , activation of G proteins and induction of phospholipases.

2.2.2.2 Effector-Triggered Immune Responses (ETI)

Plants have also developed more specialized mechanisms to defend against pathogens and elicitors which are new to the plant. These are referred to as effector triggered immunity (ETI) responses (Jones and Dangl, 2006). Effectors are the chemical substances released into the plant host by the pathogen. When the PAMPs are not recognized by the plant or the virulence of the pathogen does not abate, then the plant

generates a second line of defense. Effectors that enable pathogens (*avr* proteins) to overcome PTI are recognized by specific disease resistance (R) genes. Most R genes encode NB-LRR, nucleotide binding (NB) and leucine rich repeat (LRR) domains. NB-LRR proteins are broadly related to animal NOD/NLR proteins. Plant *R* gene families have LRR domains with a diversifying selection. They have evolved over time to respond to different species of pathogen or changes in pathogen virulence. *R* genes are polymorphic. The polymorphism is generated by (i) single base mutations or (ii) small insertion/deletions or (iii) intragenic recombination with equal/unequal exchange, to generate more/fewer *R* genes at the locus. A B-lectin extracellular domain has also been discovered recently as a functional R protein domain. The immediate steps following recognition and activation of R proteins remain unclear (Bent and Mackey, 2007).

Plant defense responses activated upon *avr/R* recognition are often accompanied by a hypersensitive response (HR), which is mediated by a number of elicitors and secondary messengers, including ROS and accumulation of endogenous salicylic acid (SA). Neighboring as well as distant host cells can increase cell wall lignification and produce phytoalexins and pathogenesis-related (PR) proteins. The systemic activation of these defense responses, for example development of SAR (indicated by accumulation of endogenous SA and HR), can lead to broad-spectrum resistance to many fungal, bacterial, and viral pathogens throughout the plant.

In contrast to incompatible plant-pathogen interactions resulting in HR and systemic acquired resistance (SAR), a compatible interaction resulting in disease can occur in the absence of a specific *avr/R* gene interaction. The pathogen is virulent, and

the host is susceptible. Many of the same host responses involved in *avr/R*-mediated resistance also occur in compatible interactions, but they are activated more slowly or at a lower magnitude (Ham et al., 1998). A variety of *A. thaliana* defense-related genes have been identified whose products appear to function specifically downstream of *avr/R*-recognition (Glazebrook, 2001).

The guard hypothesis for plant defense response indicates that the plant R proteins are sensitive to changes/modifications in certain cellular targets of bacterial effectors. Upon sensing these changes there is activation of defense responses. In *A. thaliana*, for example, RIN4 protein activity is monitored by RPM1 (R protein) and when RIN4 levels are low, defense responses are activated when AvrB and AvrRpm1 are perceived. RSP2 (R protein) also activates defense responses when AvrRpt2 is sensed along with changes in RIN4 levels, thus indicating that guarding a virulence target is an effective way of a plant defending itself with a limited number of R-proteins (Marathe and Dinesh-Kumar, 2003).

2.3 NAEs in Plants

2.3.1 Discovery in Plants and Context in Mammalian Work

N-Acylethanolamines (NAEs) were first identified in soy and peanut extracts in the 1950s. NAEs were also ubiquitously observed in animal plasma, brain and other tissues at low levels. It was found that levels increased upon injury. Specifically, initial studies had identified a long chain polyunsaturated ethanolamine, known as anandamide, to be an endogenous ligand for the cannabinoid CB1 receptor (which acts in the central

nervous system) and CB2 receptors (which act in the peripheral nervous system and the spleen) in the animal and human systems (Schmid et al., 1990; Cravatt et al., 1995).

Numerous studies in animal systems have indicated that endocannabinoids play an important role in modulating behavioral responses to acute, inflammatory, and neuropathic pain stimuli. There are broadly two groups of lipid signaling molecules, the *N*-acylethanolamines and the monoacylglycerols (Cravatt and Lichtman, 2004) that are currently considered to be primary ligands in the endocannabinoid system.

Studies in plant systems for the role of NAEs have been fairly recent, starting about a decade ago (Chapman, 2004). Initial identification and quantification of several NAE species was done in both monocot and dicot plants. Total NAE content of about 0.5 to 2.0 µg/g fresh mass of tissue was found in desiccated seeds (Chapman et al., 1999).

2.3.2 Molecular Species and Abundance

NAEs are made up of an ethanolamine moiety attached to the carboxylate end of a fatty acid. The species found most prominently in plant tissue range from 12 to 18 C even numbered chain lengths. Anandamide is not endogenous in plant tissue. The carbon chains can be saturated, mono or polyunsaturated. Saturated NAEs are most stable.

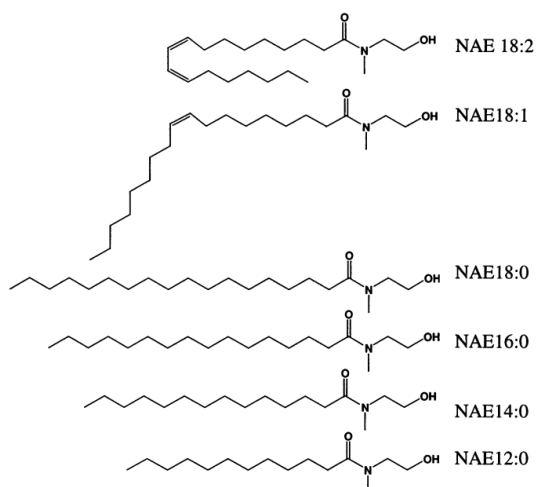


Fig. 2.1. Molecular species of N-acylethanolamines found in plants (Chapman, 2000) Reproduced with permission from Elsevier.

Extensive studies on levels in desiccated seeds indicated that the 16:0 and 18:2 species are most abundant followed by lower amounts of 12:0 and 14:0. From studies on legume plant seeds, the total NAE concentrations varied over a wide range, up to 3 orders of magnitude on a fresh weight basis and 2 orders of magnitude on an extracted lipid weight basis (Venables et al., 2005). The legume plant *Bauhinia congesta* (orchid tree) had the lowest value of 0.3 $\mu\text{g/g}$ and *Medicago truncatula* cv. Jemalong had the highest value of 44.6 $\mu\text{g/g}$.

The amounts of NAEs in seeds decrease drastically at the time of imbibition and germination. Studies on wild type (WT) *A. thaliana* seeds and seedlings indicate that the total levels were approximately 2 $\mu\text{g/g}$ fresh mass and 0.2 $\mu\text{g/g}$ fresh mass, respectively (Wang et al., 2006). The amounts of particular NAE molecular species are not exactly in proportion to the corresponding free fatty acids but rather more in proportion with the corresponding precursor compounds, the *N*-acylphosphatidyl ethanolamines (NAPEs).

2.3.3 NAE Metabolism

2.3.3.1 Synthesis

NAEs are the product of hydrolysis of *N*-acylphosphatidyl ethanolamines (NAPE) by phospholipase D (PLD). NAPE is a minor, endogenous constituent of plant seeds (Chapman and Moore, 1993; Sandoval et al., 1995). One of the pathways by which it is formed is the *N*-acylation of phosphatidyl ethanolamine in plant cells in the presence of NAPE synthase. This reaction was observed to occur in plant cells which were exposed to pathogen elicitors for several hours (Chapman et al., 1995). Therefore it was proposed that NAPE metabolism plays a role in the signaling cascade started due to pathogen perception.

2.3.3.2 Degradation

NAEs are hydrolyzed by the enzyme fatty acid amide hydrolase, (FAAH), an integral membrane protein. The products are the corresponding fatty acid and ethanolamine. FAAH inactivates a large and diverse class of endogenous signaling lipids consisting of NAEs and *N*-acyl taurines (NATs) (McKinney and Cravatt, 2006). FAAH also hydrolyzes fatty acid primary amides such as oleamide (Cravatt et al., 1995).

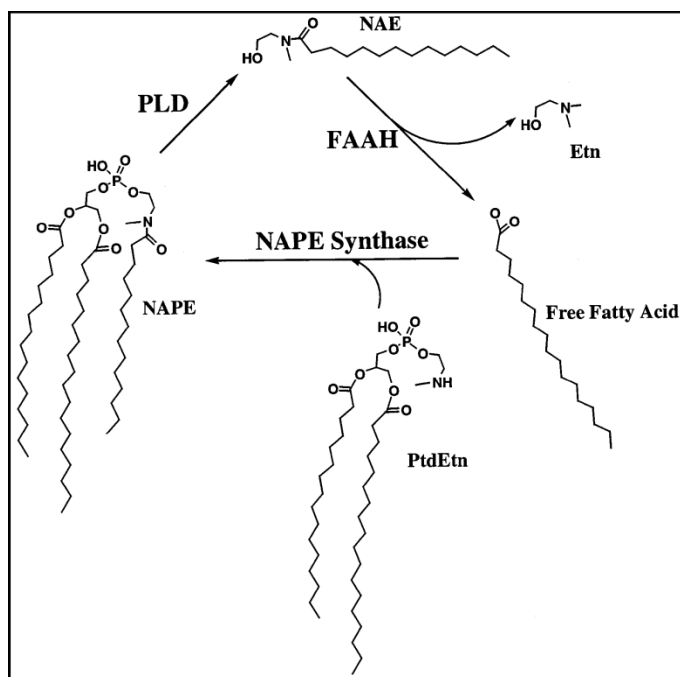


Fig. 2.2. Formation and degradation of NAEs (Chapman, 2000). Reproduced with permission from Elsevier.

The plant fatty acid amide hydrolase was identified in *A. thaliana* by collaboration between groups of researchers at University of North Texas and at Noble Foundation. It was determined that five amino-acid residues important for catalysis by rat FAAH were absolutely conserved within the FAAH sequences of different plant species such as *A. thaliana*, *Medicago truncatula* and *Oryza sativa* (Shrestha et al., 2006). Though the overall sequence of plant and mammalian FAAH homologues are different, this conserved domain indicated similar structure and activity relationships.

NAEs are also metabolized by the oxylipin pathway, which uses lipoxygenases (LOX enzymes) to convert the NAEs into NAE-oxylipins. Two widely used LOX inhibitors, 5,8,11,14-eicosatetraynoic acid (ETYA) and nordihydroguaiaretic acid

(NDGA) reduced NAE-oxylin formation in a concentration-dependent manner, confirming the LOX pathway (Shrestha et al., 2002).

A study of the metabolites of NAEs and sub-cellular distribution of the enzymes involved in degradation indicated that LOX and allene oxide synthase (AOS) were distributed both in membrane and cytosol-enriched fractions, whereas FAAH (also referred to as AHase) was localized almost exclusively to microsomes (Shrestha et al., 2002).

2.3.4 Role in Cytoprotection: Physical and Chemical Stress

Interrelations of phospholipase D (PLD) and NAEs in the context of plant exposure to stress have been investigated. The mechanical wounding of leaves in WT *A. thaliana* and those deficient in PLD indicated that PLD and its isoforms are involved in wound-induced metabolism of polyunsaturated fatty acids (Zien et al., 2001). Other studies have indicated that PLD is involved in responses to cold, drought, wounding, and pathogens (Wang, 2005; Bargmann and Munnik, 2006).

The up-regulation of NAE precursor NAPE in cell cultures exposed to pathogens, the release of NAEs in animal systems on wounding and other stressors also indicates that there may be multiple sites/roles for NAEs in cytoprotection from physical and chemical stressors (Tripathy et al., 1999; Chapman, 2004; Cravatt and Lichtman, 2004).

2.3.5 Role in Signaling

2.3.5.1 Growth

The overall levels of endogenous NAEs fall drastically during the imbibition and germination of *A. thaliana* seeds (2 µg/g fresh mass) to seedlings (~0.2 µg/g fresh mass).

In mature plants, the levels are even lower ($\sim 0.05 \mu\text{g/g}$ fresh mass) (Kilaru et al., 2007). During seed germination and seedling establishment, *AtFAAH* expression and NAE hydrolase activity increase along with concomitant depletion of endogenous NAEs (Wang et al., 2006). Studies on seedling germination and growth when exposed to exogenous levels of NAE 12:0 indicate that this causes change in levels of PLD and indirectly causes a remodeling of the microtubule and actin cytoskeleton. The plant morphology indicates a drastic inhibition of growth in exposed seedlings (Blancaflor et al., 2003; Motes et al., 2005).

2.3.5.2 Defense

NAPE formation in plant cells following treatment with pathogen elicitors indicated the possibility that NAPE metabolism was involved in signaling pathogen perception (Chapman et al., 1995). Research with tobacco cell culture and leaves has indicated an increase in NAE levels post inoculation with an elicitor (Tripathy et al., 1999). NAEs are metabolized in two pathways, a) by the fatty acid amide hydrolase (FAAH) enzyme to give rise to free fatty acids (Wang et al., 2006) and b) by the lipoxygenase pathway with unsaturated NAEs 18:2 and 18:3 to produce NAE-oxylipins (Stelt et al., 2000). The authors speculate that by acting as competitive inhibitors to FAAH, NAE-oxylipins may play a role in maintaining NAE levels in vivo and also have other physiological roles in defense yet to be determined.

2.3.5.3 Possible Interactions with Other Signaling Pathways/Hormones

NAE and abscisic acid (ABA) levels are depleted during seed germination, and both metabolites inhibit the growth of *A. thaliana* seedlings. Similar to the effect of

exogenous application of NAE 12:0 on growth of seedlings, the application of exogenous ABA reduces growth. On exposure to both ABA and NAE 12:0, there was a more dramatic reduction in germination and growth than either compound alone (Wang et al., 2006). Further studies by transcript profiling and gene expression on NAE-treated seedlings, FAAH overexpressor seedlings and ABA insensitive mutants indicated that changes in the ABA pathway had adverse affects on normal functioning of NAE signaling. NAE metabolism interacts with ABA in the negative regulation of seedling development and normal seedling establishment depends on the reduction of the endogenous levels of both metabolites (Teaster et al., 2007). FAAH overexpressors are resistant to NAEs and show larger vegetative growth compared to Col-0 WT plants, suggesting a possible interaction with growth hormones such as auxin and cytokinin (Wang et al., 2006).

2.3.6 The Case for an NAE Signaling Role in Plant/Pathogen Interactions

2.3.6.1 Study of Tobacco Cell Suspension Exposure to Xylanase

An earlier study of cell suspension exposure to a fungal elicitor, xylanase, indicated that NAPE biosynthesis increased 1 to 2 h after xylanase treatment and this was preceded by a rapid hydrolysis of NAPE (Chapman et al., 1995). A PLD-type activity was identified in tobacco membranes that hydrolyzed NAPE to NAE in vitro. Release and accumulation of NAE (Chapman et al., 1998) with a corresponding decrease in NAPE has prompted further interest in a possible role for NAE in elicitor-plant interactions. In tobacco cells, NAE 14:0 was the major NAE component which was responsive to xylanase exposure (Tripathy et al., 1999).

The addition of NAE 14:0 inhibited xylanase-induced alkalinization when added 10 min prior to or at the same time as xylanase. Later addition of the NAE 14:0 after elicitor treatment was marginally effective, if at all. To analyze whether this inhibitory action of NAE 14:0 was elicitor specific or a more general phenomenon, other elicitors (cryptogein, harpin and ergosterol) were tested. Similar effects were observed with these elicitors. The inhibition by NAE 14:0 was also concentration dependent.

Xylanase treatment also induced PAL gene expression and it was noted that application of exogenous NAE 14:0 also produced the same effect. The same effect was also noted in tobacco plant leaves which were 8-16 weeks old. In tobacco plant leaves exposed to xylanase and cryptogein elicitors it was also noted that the NAE levels jumped 10 to 50 fold after a 10 minute exposure.

The authors concluded that future work should be aimed at addressing the precise mechanisms of NAE action in whole plant tissue with regard to pathogenic exposure (Tripathy et al., 1999). Results of my work with adult *A. thaliana* exposure to the pathogen *Pseudomonas syringae* pv. *syringae* are discussed in Chapter 4. My results with 5-day old seedling exposures to the pathogen peptide flg22 are discussed in Chapters 5 and Chapter 6.

2.4 Chemical Contaminants in Plants

2.4.1 Traditional Studies of “Legacy” Pollutants on Germination and Growth; Biomonitoring

Legacy pollutants (also called criteria or priority pollutants) were manufactured in large quantities for commercial purposes and are persistent in the environment. Their

chemical use was varied, such as in herbicides or pesticides or as a key ingredients in other manufacturing processes. Some examples are polychlorinated biphenyls (PCBs) and DDT. Heavy metals such as mercury and lead also belong to the legacy contaminants. The emergence of improved techniques for detection and quantification of these contaminants at very low levels has sustained interest in their potential long-term effects (Daughton, 2001). Many studies have been conducted on the effects of these pollutants in animals (Yoder et al., 1999). The effects of pesticides and heavy metals have been studied on a few agriculturally important plant species along with a few model plants (Islam et al., 2007; Sharma and Dietz, 2009; Ramel et al., 2009). Studies on more complex matrices involving a number of legacy pollutants are difficult to replicate in the laboratory settings and difficult to analyze effectively in field studies. Toxicity endpoints traditionally used have largely been limited to germination, root elongation and adult growth in plants. To my knowledge, no studies of the effects of environmental contaminants on plant immunity have been conducted.

2.4.2 Contaminant Interference with Plant Immunity

Contaminants of different types have varied effects on the plant physiology. In nature, plants are exposed to a wide range of these substances. Pollutants can be organic, heavy metal or poisonous gases. New classes of contaminants that are emerging include the class to which the contaminants in this study belong, the PPCPs, and they are rapidly increasing in quantities in our global water and soil systems (Richardson et al., 2005; Xia et al., 2005). One common underlying theme with exposure to any pollutant is the notion that if the plant spends sufficient energy resources in order to adapt to a constant source

of contamination, resources for other important physiological processes such as defense are compromised. If the contaminant is present at acutely toxic levels, the plant may be unable to overcome the effects of the contamination and die. Plants not acutely affected may become susceptible to secondary effects associated with chronic exposure.

As indicated previously, the antimicrobial contaminants that are the subject of my study have been specifically identified as potent inhibitors of plant defense responses (Serrano et al., 2007). To my knowledge, the immunotoxicity *per se* of environmental contaminants has not been examined in plants.

2.5 Commercial Antimicrobials in the Environment

2.5.1 Historical Use, Distribution and Bioaccumulation

The discovery of antibiotics, starting with penicillin along with the widespread use of heavy metals in treating parasitic diseases changed the way man began to combat infectious diseases (Drews, 2000). In order to decrease the spread of a number of infectious diseases affecting populations of both animals and humans, there has been a marked increase in the production and application of antimicrobials in a variety of ways (Kunin, 1993). Antimicrobials or antiviral medicines are not only used in the time of disease, but are added into a number of other products such as toothpastes, soaps, detergents, other personal care products and even toys and towels as disease prevention strategy (Glaser, 2004). This widespread use of antimicrobials has, in turn, raised many questions regarding the increasing resistance of pathogens to antimicrobials thus decreasing their effectiveness. Another major cause of concern over the widespread use of antimicrobials is the impact seen in the environment in water and sediment. These

organochemicals tend to bioaccumulate in aquatic organisms, animals and plants (Coogan et al., 2007). They can cause acute toxicity and, in low doses, may be impacting the reproductive and immune systems in the organisms. Specific antimicrobial compounds of interest are described below.

2.5.1.1 TCS

TCS is a chlorinated phenoxyphenol with a chemical name of 2,4,4'-Trichloro-2'-hydroxydiphenyl ether. It is quite stable against hydrolysis but is easily metabolized to methyl-TCS. It has a log K_{ow} (octanol-water partition coefficient, a measure of lipophilicity of the molecule) of 4.8 and hence is relatively hydrophobic. It can cross cell membranes easily and once inside the cell, can inhibit lipid biosynthesis. TCS blocks the active site of an enzyme called Enoyl-acyl carrier-protein reductase (ENR), which is necessary in synthesis of fatty acids in both prokaryotes and plants (Heath et al., 1999; Surolia and Surolia, 2001; Glaser, 2004; Serrano et al., 2007).

2.5.1.2 MTCS

Methyl-triclosan (M-TCS) is a metabolite of TCS with an increased log K_{ow} of 5.4 (Balmer et al., 2004) and is therefore more bioaccumulative than the parent TCS. It is more stable than TCS in the environment. It can be used as a model compound for the study of the behavior of urban lipophilic compounds (Stevens et al., 2009). Little is known about the biological activity of this metabolite (Glaser, 2004).

2.5.2 Mode of Action

Enoyl-ACP reductase (also referred to as ENR or FabI) is an enzyme that belongs to the fatty acid synthase II family of enzymes in plants. It catalyzes the final step of fatty

acid elongation cycle, converting trans-enoyl-ACP to acyl-ACP (Pidugu et al., 2004). As discussed above, TCS inhibits the action of ENR by binding to it. Two lines of research regarding possible effects of this inhibition in plants have been initiated (1) examination of fatty acid synthesis by quantification of labeled acetate uptake and (2) direct analysis of enoyl-ACP reductase (ENR) enzyme activity.

2.5.2.1 Previous Work from Our Laboratory Demonstrating Effects of Antimicrobial Exposure on Plant Lipid Biosynthesis.

The effect of short term exposure to TCS on plant lipid biosynthesis has been studied in two ways in our laboratory. The first, was an examination of the effect of TCS exposure on rate of incorporation of radio-labeled acetate into lipid stores in the aquatic alga *Sp. cladophora* (Coogan, 2007). This study indicated a dramatic decrease in lipid biosynthesis associated with this short term exposure to relatively high concentrations of TCS. The second, part of the current study, was an examination of the effect of TCS and M-TCS exposure on the ENR activity in *A. thaliana*.

2.6 A Direct Link between TCS Activity and Inhibition of Plant Defense Response Suggests Interference in Lipid Signaling as the Mechanism

2.6.1 TCS Inhibition of Plant Defense Responses

Serrano's group (Serrano and Guzman, 2004; Serrano et al., 2007) exposed 7-day *A. thaliana* seedlings to 120 small molecules with known biological activities. Four of the chemicals, TCS, oxytriazine, fluazinam and cantharidin decreased plant defense responses to pathogen elicitors. TCS was identified in this work as a potent inhibitor of all tested elicitor-triggered immune responses (elicitor-activated *ATL2* gene expression,

FLS2 receptor endocytosis, and flg22-triggered oxidative burst and MOD1 enoyl-ACP reductase inhibition).

The GUS reporter system (GUS is β -glucuronidase) is useful in plant molecular biology to monitor gene expression (Jefferson et al., 1987). Monitoring glucuronidase activity provided evidence that, on exposure to pathogen elicitors, *ATL2* mRNA accumulation occurs rapidly and transiently, indicating the expression of *ATL2* genes which could target proteins involved in SA or JA signaling pathways (Serrano and Guzman, 2004). In the TCS study (Serrano et al., 2007) it was reported that constitutive *ATL2* gene expression occurred mainly in the leaf primordia and vasculature on treatment with the elicitor cellulysin and GUS activity increased four-fold. On treatment of the plants with TCS for two hours prior to elicitor treatment, this four-fold increase in GUS activity was reduced by 70%.

A 22 amino acid sequence (flg22) of the conserved N-terminal part of flagellin is known to activate plant defense mechanisms. Flagellin perception in *A. thaliana* functions via the receptor-like-kinase, FLS2 (flagellin-sensitive-2) (Chinchilla et al., 2006). More than 900 genes are affected upon flg22 treatment. Other reactions are protein phosphorylation, mitogen-activated protein (MAP) kinase signaling, transcriptional gene activation and triggering of *FLS2* internalization by endocytosis (Robatzek et al., 2006). *FLS2* endocytosis results in production of ROS (Gomez-Gomez et al., 1999). Serrano et al. (Serrano et al., 2007) found that on *FLS2* endocytosis was impaired by 90% on a 30 minute exposure to 10 ppm TCS.

2.6.2 Hypothesized Connection to Lipid Messenger Signaling

Studies of herbicidal activity on long term exposure of plants to TCS were done in the 1980s and 90s (Bhargava and Leonard, 1996). This herbicidal activity was proposed to be due to physiological shutdown of plant processes because of the sustained inhibition of fatty acid biosynthesis.

The *MOD1* gene in *A. thaliana* encodes an enoyl-ACP reductase, which is a subunit of the fatty acid synthase complex that catalyzes de novo synthesis of fatty acids. *Mod1* mutants show decreased fatty acid biosynthesis and amount of total lipids. There are pleiotropic effects on plant growth and premature cell death (Zhonglin et al., 2000). A 30 minute exposure of the 7-day seedlings to 10 ppm TCS produced inhibition of MOD1 enoyl-ACP reductase (ENR) activity by 70% (Serrano et al., 2007). This reduced activity in lipid biosynthesis could have a negative effect on signaling lipids.

Along with this drastic drop in ENR activity, other rapid responses occurring from 30-120 minutes exposures, such as, *ATL2* gene expression inhibition, FLS2 inhibition and ROS bursts led the authors to speculate that TCS effects were mediated through interruption of lipid signaling cascades (Serrano et al., 2007). These results, when viewed in the context of the previous studies indicating a role for NAEs in plant defense signaling, led us to ask the question whether TCS inhibition of plant defense responses might be due to interference in capacity for effective NAE signaling.

2.7 Background Summary

2.7.1 Much is Known about Plant Defense Responses

Plants in the environment are constantly battling attacks in the form of herbivores, bacterial and fungal attacks and physical and chemical stressors. Plants form the backbone of the world ecosystem. Hence understanding plant defense responses has received a great deal of attention which has resulted in a relatively detailed understanding of the molecular events relating to plant-pathogen interactions.

2.7.2 Little is Known about the Role of NAEs in Plant Defense Responses

NAEs are an important class of lipid signaling molecules being studied widely in mammalian systems for therapeutic use. They have demonstrated roles in central nervous system and immune function signaling. These molecules have also been identified in plants and are known to affect plant processes such as germination and growth. Initial studies have shown that their levels dramatically increase on interaction with fungal elicitors. However, relatively little is known about the details of how these compounds might directly or indirectly influence plant defense responses.

2.7.3 Nothing is Known about Chemical Contaminant Interference with Plant

Immunity (i.e. Plant Immunotoxicity)

The water and sediments of many of our water bodies, natural and man-made, are increasingly becoming polluted by many different xenobiotics. Emerging classes of contaminants are pharmaceutical and personal care products. These products are of special concern because they are specifically designed to have biological activity. Antimicrobials may be important in this context because:

1. They are widely distributed in the environment.
2. They tend to bioaccumulate in plant and animal systems.
3. They may interrupt lipid messenger signaling important in plant defense responses.

2.8 Dissertation Objectives and Hypotheses

As stated in the Chapter 1, the overarching objectives of my dissertation are

Objective 1: To determine the effect on NAEs, in adult *A. thaliana* and 5-day seedlings when pathogen challenged by a) quantification of the concentration profiles of short chain NAEs with and without challenge in wild-type and FAAH-altered genotypes and b) quantification of ROS production as an indicator of strength of response to the challenge.

Objective 2: To investigate, changes in NAE and ROS production observed in Objective 1 due to exposure to the antimicrobial compound TCS and quantify its inhibition of lipid biosynthesis as indicated by decreased Enoyl-ACP reductase enzyme activity, changes in NAE concentration profiles and modulation of ROS production.

The experimental design and null hypotheses proposed are summarized below:

1. Adult Plant Exposure to Pathogenic Bacteria

Evaluation of WT and *FAAH*-altered genotype plants for effects of pathogen challenge on the following metrics: changes in NAE levels, changes in gene expression and salicylic acid (SA) levels. Experiments involving changes in gene expression and SA levels were performed in Dr. Mysore's lab at the Nobel Foundation, Ardmore, OK. Plants grown at the Nobel foundation were subsequently analyzed for NAE concentration profiles as part of my study. The null hypothesis was that there is no significant difference in NAE

levels in plants subjected to buffer or pathogen challenge by *P. syringae* and control plants within a given genotype (H_{01}).

2. Seedlings Exposure to Bacterial Elicitor

Exposure of WT and FAAH altered genotype 5-day seedlings to the bacterial elicitor flg22 to determine effects on the following metrics: generation of ROS and alteration in NAE concentration profiles. The null hypotheses were that there is no significant change in NAE concentration profile (H_{02a}) and there is no significant change in ROS production in seedlings subjected to elicitor when compared to solvent control treatment (H_{02b}).

3. Seedlings Exposure to Bacterial Elicitor and TCS

Exposure of WT and FAAH altered genotype 5-day seedlings treated with TCS to determine effects on the following metrics: changes in Enoyl-ACP reductase enzyme activity and in NAE concentration profiles/levels. These results were combined with those from Experiment Two to form null hypotheses: there is no significant change in NAE concentration profiles in seedlings exposed to TCS, elicitor or TCS + elicitor when compared to solvent control treatments (H_{03a}), there is no significant difference in enzyme activity or NAE concentration profiles among genotypes (H_{03b}) and there is no significant change in ENR activity on exposure to the TCS metabolite MTCS when compared to solvent controls (H_{03c}).

4. *Zat12* Seedling Exposure to Bacterial Elicitor and TCS

Exposure of 5-day *Zat12 A. thaliana* seedlings (these seedlings have a luciferase reporter gene fused to *Zat12* promoter) to flg22, paraquat and TCS to determine effects on ROS

generation. The null hypothesis tested is that there will be no significant changes in ROS production due to long term exposure to TCS (H_{04}).

CHAPTER 3

MATERIALS AND METHODS

The materials and methods described in this chapter are those which were used in more than one experiment. Plant tissue used in the analyses were either adult plants grown in autoclaved soil under short day conditions for 4-5 weeks or seedlings grown in well plates (12, 24 or 96-wells) or volumetric flasks (125 ml or 250 ml) grown in Murashige-Skoog half media with 1% sucrose solution.

3.1 Growth of Adult Arabidopsis Plants

Materials: sterilized seeds (col-0: WT, *FAAH* OE and KO genotypes), autoclaved soil, arabidopsis fertilizer, growth equipment, DI water.

Arabidopsis plants of different genotypes were initially surface sterilized and then planted in autoclaved soil. Seeds were surface sterilized by placing them in a 2 ml microcentrifuge tube and adding 1 ml of 70% ethanol solution and mixing the contents for 3 minutes. This was followed by removal of alcohol and addition of 20% bleach solution for 3 minutes. The seeds were then rinsed with water 3 times and planted in soil or refrigerated at 4°C for 3 days in order to stratify the seeds. Soil and equipment for growth was obtained from Lehle Seeds Company (Round Rock, TX). Soil was placed in aluminum trays and autoclaved in a slow exhaust cycle for 20 minutes. After cooling, the soil was ready to be used in the individual pots of the growth trays. After sufficient

moistening of the soil and addition of fertilizer, one to two seeds were added to each pot and the tray placed in the growth chamber.

3.2 Well Plate Plant Growth Protocol

Materials: sterilized well plates from Corningware™ (Corning, NY), MS media, PNT vitamins mixture, seeds.

Surface-sterilized seeds were stratified for four days at 4°C. Seedlings were dropped into sterile 12, 24 or 96-well Corningware™ sterile micro titer plates containing approximately 10-12, 5-6 or 1-2 seeds per well, respectively, in Murashige and Skoog (MS) liquid ½ medium containing 1% sucrose. Briefly the ½ medium consisted of MS macro and micro nutrient mixtures (10%), MES buffer (250 mg/l), myo-inositol (50 mg/l); nicotinic acid (0.25 mg/l), pyridoxine*HCl (0.25 mg/l) and thiamine *HCl (0.05 mg/l). All solutions were obtained from Fisher Scientific (Springfield, NJ). The plates were incubated at 22 - 24°C with continuous shaking at 100 rpm in a growth chamber under 16/8 light/dark conditions with light intensity of 250 µmoles/m²/s. At the appropriate time for analysis (5, 10, and 7- 14 days), the plants were removed from the growth chamber and used for analysis.

3.3 Enoyl-ACP Reductase Enzyme Activity Protocol (Serrano et al., 2007)

Materials: 10 mM sodium phosphate buffers pH 9.0 (with DTT) and 6.2; Bradford assay reagent, β-NADH sodium salt and crotonyl-CoA powder were obtained from Sigma Chemical Co. (St. Louis, MO). Glass tissue homogenizers used for extraction were from Kontes (Vineland, NJ).

Total protein was extracted from ground seedlings (up to ~1g) using ~ 600 μ l – 1 ml of sodium phosphate buffer (10 mM) with 1 mM dithioerythritol (DTT) at a pH of 9. Protein concentration was determined by the Bradford assay. 50 μ g of protein was dissolved in 500 μ l of the reaction buffer (10 mM sodium phosphate at pH 6.2 and 140 μ M NADH). Approximately 400 μ l of 10 mM sodium phosphate buffer at pH 6.2 was added to yield a final reaction volume of 1 ml. Enoyl-ACP reductase enzyme activity was monitored by the decrease of absorbance at 340 nm due to oxidation of NADH at room temperature (22°C). 120 μ M crotonoyl-CoA was used as substrate. The reaction was initiated after addition of substrate and monitored for at least 15 minutes with absorbance readings taken once every minute. Reaction control was also used in order to verify the specificity of NADH to substrate.

3.4 ROS Assay Protocol Modified from Serrano et al. (2007)

Materials: Luminol powder (A8511), horse radish peroxidase (HRP) powder (P6782), luciferin (L6882, a lyophilized sodium salt) and paraquat (856177) were obtained from Sigma (St. Louis, MO) and elicitor (flg22) from Chi Scientific (Maynard, MA). Synergy 2 plate reader from Biotek Instruments (Winooski, VT) and well plates (CLS 3912) from Corningware™ (Corning, NY) were used in analyses.

H₂O₂-dependent luminescence of luminol was measured (Glazener et al., 1991). Hydrogen peroxide in the presence of horseradish peroxidase and hydroxide salt (used to dissolve luminol) decomposes to form oxygen. Luminol (5 amino- 2,3-dihydro-1,4-phthalazine dione) is oxidized to form a highly reactive dianion which reacts with peroxide to form a trianion, in an excited state. When the trianion returns to ground state,

there is release of light at a wavelength of 425 nm. This emission of light is termed chemiluminescence and can be quantified proportionally to the amount of hydrogen peroxide in the reaction system.

Experiments were performed by using whole seedlings, individual leaves and leaves cut in equal parts. The samples were initially soaked in DI water for at least four hours or overnight.

Stock solution of luminol was prepared by dissolving 17 mg in 1 ml of 200 mM KOH solution. Stock HRP was prepared by dissolving 10 mg in 1 ml of DI water. Subsequent dilutions of stocks were performed using DI water and final concentrations in experimental wells were 1 mM for luminol and 1 μ g for HRP. Average well volume was 150 μ l in Corning™ white-96 well plates.

Solvent (0.1% DMSO) alone or solvent with 100 μ M flg22 was added to achieve a final concentration of 1 μ M. The sample was then placed in Synergy2 plate reader. Readings of luminescence were noted every 5 to 10 seconds for 20 to 60 minutes. This procedure was modified as described below for use with *Zat12* plants.

The *Zat12* gene is a representative of genes that respond to multiple environmental stress conditions. It was identified initially as a light stress-response gene. Transgenic plants expressing the reporter gene luciferase under the promoter of *Zat12* gene were grown to study the transcriptional activation of *Zat12* in abiotic stress conditions. Gain and loss-of-function plants were used to further characterize the results and it was shown that *Zat12* gene could control activation of response to light and oxidative stress (Davletova et al., 2005). *Zat12* transgenic plant seeds were kindly

provided by Dr. Ron Mittler, Univ. of Nevada at Reno. The 5-day seedling samples were grown as described in section 2.3. Individual intact seedlings were subsequently placed in de-ionized (MQ water, obtained from Milli-Q Purification System, Millipore, Bedford, MA) water for at least four hours or overnight. The samples were then placed in fresh MQ water and treated with solvent (0.1% DMSO) or elicitor (flg22, 1 μ M) and luciferin (1 mM) for 20 minutes and subsequently luminescence readings were recorded every 30 s using a Synergy 2 plate reader for 30 minutes to one hour.

In experiments that included exposure to TCS, the samples were exposed to the compound TCS (at various concentrations) for 45 to 120 minutes along with control samples exposed to solvent (<0.1%EtOH) alone. Subsequently, luciferin and the elicitor were added to the sample and sample readings were recorded on the Synergy 2 instrument every 5-10 s for 30 minutes to one hour.

3.5 NAE Analysis Protocol

Materials: Isopropyl alcohol, chloroform and hexane were obtained from Fisher Scientific (Springfield, NJ), NAE internal standards were synthesized using deuterated ethanolamine from New England Nuclear (Boston, MA) and acyl chlorides from Nu-Check Prep (Elysian, MN) or ordered from Cayman Chemicals (Ann Arbor, MI), Bead beater and glass beads and vials were from Biospec (Bartlesville, OK). PVDF 22 μ M filters, autosampler vials, derivatizing agent *N,O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) were from Alltech (Deerfield, IL). 25 ml capacity glass tubes were from Sigma, St. Louis, MO.

Method used for extracting and analyzing NAEs from plant leaves:

Step 1: Extraction of samples:

Approximately 1 gram (or as required) of undamaged leaves was carefully removed from the plants and added to a bead-beater vial containing one-third volume of 2.5 mm glass beads. Immediately, 2 ml of 70°C isopropyl alcohol (IPA) was added and the vial was placed in a water bath at 70°C for 30 minutes. The samples were homogenized by placing the vials in a bead-beater, for approximately 3 minutes and immediately placed in water-bath at 70°C to stop any enzyme activity.

Internal standards, which were deuterated (mixture of d4 12:0, 18:0 and 20:4 or mixture of d4 16:0, 20:1 NAEs) were added at 250 ng or 100 ng to the vial. After a thorough mixing by vortex, Fisher Scientific (Springfield, NJ), they were transferred to a 25 ml glass tube. The vial was rinsed with 1 ml of CHCl₃ and contents were transferred to the same glass tube and covered with PTFE-lined cap. The sample in tube was thoroughly mixed using a vortex and placed in the refrigerator overnight at 4°C. The samples were inspected to ensure that the solution was monophasic. If solution was biphasic, small amounts of IPA were added and solution mixed until it became monophasic.

Step 2: Back-extraction of samples:

After overnight extraction the samples were vortexed and placed in a centrifuge for 5-6 minutes at 1500 rpm. The top layer of clear extract was decanted into a clean MeOH-rinsed tube. The sample tube was subsequently rinsed with 2 ml of 2:1 IPA: CHCl₃ 3x times, adding each rinse to previous extract.

The IPA from the extraction solvent was removed by back-extraction with 1M KCl solution. Briefly, additional 1.5 – 2 ml of CHCl_3 was added to the extraction solution, then 3-4 ml of 1M KCl solution was added and the solution was thoroughly mixed by vortex. It was then centrifuged for 4-5 minutes at ~1000 rpm. The top aqueous layer was removed. The procedure of adding KCl solution was repeated until there was little change in the volume of extract (about 3x times). On the last rinse cycle, the entire aqueous layer was carefully removed and the tube containing the CHCl_3 extract was placed in a RapidVap™ nitrogen evaporator (Labconco, Kansas City, MO). When the volume of solvent reduced to less than 1 ml, it was filtered through a 0.22 μM PVDF single use syringe filter into a pre-weighed sample vial. The sample was evaporated to dryness to get the total lipid mass. It was then reconstituted in CHCl_3 to 100 μl . If an emulsion formed, 1 ml increments of 2:1 IPA: chloroform were made followed by vortex, and centrifuged until the emulsion disappeared.

Step 3: Clean-up by HPLC

Solvents used: Hexane: IPA.

A 100 μl of sample was injected into an Agilent 1100 HPLC system (Palo Alto, CA) with autosampler and automatic sample collection features. The column used was an Alltech (Deerfield, IL) semi-preparative silica column (10 X 250 mm, 10 μM particle size) at a flow rate of 4.5 ml/min. Mobile phases A and B were hexanes and isopropyl alcohol respectively. The gradient was 100% A until 5 minutes with an immediate increase to 10% B held until 16 minutes followed by a linear increase to 50% B at 20 minutes which was held for 20 minutes at which point starting conditions were

reestablished. Total run time was 60 minutes with an NAE collection window from 12.5-15.5 minutes.

Step 4: Prep for GC/MS

The sample collected from the HPLC clean-up run was evaporated under N₂ gas to ~1 ml and then transferred to an auto-sampler vial and evaporated just to dryness. Bis(trimethylsilyl)trifluoroacetamide (BSTFA, 50 µl), a trimethylsilyl derivatizing agent, was added in order to decrease polarity and increase volatility of the sample in the GC/MS. Derivatization was conducted in an oven for 30 min @ 55°C and then evaporated just to dryness under N₂ gas. Derivatized NAEs were reconstituted with 50 µl of chloroform and transferred into a limited-volume insert (200 µl volume) and placed in a GC/MS auto sampler for analysis.

Step 5: GC/MS analysis

Sample analysis was conducted using a gas chromatograph, Agilent 6890N (Palo Alto, CA) coupled with a 5973 mass selective detector MS operated in selected ion mode, 70 eV. Injector temperature was 260°C and the column (Alltech, Deerfield, IL; EC-5 30 m, 0.25 mm i.d., 0.25 µm film) was programmed to increase from 40 to 220°C at 50 °C/min and then to 285 at 5°C/min. The column was then allowed to bake out at 300°C. Carrier gas used was helium at a constant inlet pressure of 8.0 psi; inlet temperature was at 260°C with a 2 µl, splitless injection. The MS was operated in the single ion monitoring mode (SIM) from 7 to 25 minutes during which time all compounds of interest eluted from the column.

The standard curve was prepared by injection of 2.0 ng of each internal standard with target NAEs ranging from 0.04 to 20 ng. Final NAE concentrations were calculated on the basis of fresh seed weight and total extracted lipid weight.

3.6 TCS and MTCS Analysis Protocol

Method used for extracting and analyzing contaminants from plant leaves.

Step 1: Extraction of lipids

Total lipid extraction steps as described in the method 2.5 (step 1 and step 2), for NAE analysis were used, along with the addition of 50 ng of the appropriate internal standards for contaminant analyses also. Once lipid mass was noted, sample was reconstituted in methylene chloride and analyzed for TCS, MTCS content on GC/MS.

If NAE analysis was not required of the samples (e.g. blank sample) the sample's fresh mass was noted and it was added to a bead-beater vial with one-third volume of 2.5 ml glass beads and 1 or 2 ml of 1:1 ethyl acetate (EA): Hexane mixture along with appropriate amounts of internal standard (50 ng of ^{13}C TCS, ^{13}C MTCS). The samples were homogenized, and the mixture was filtered. The vial and filter were rinsed 3x with the extraction solvent and subsequently the sample was evaporated to dryness to get lipid mass (if any) and reconstituted to an appropriate final volume for GC/MS.

Step 2: GC/MS analysis

Sample analysis was conducted on an Agilent (Palo Alto, CA) 6890 GC coupled with a 5973 mass selective detector MS, 70-eV. GC conditions were helium carrier gas at 480 hPa, inlet temperature at 260°C (2 μl , pulsed pressure at 170 KPa for 0.5 min, splitless injection), and column (Alltech, Deerfield, IL, USA; EC-5 30 m, 0.25 mm i.d.,

0.25 μm film) with temperature initially at 40°C with a 1-min hold followed by a 50°C/min ramp to 140°C with a 5-min hold and increased to 190 °C for 24 minutes. The sample run was then allowed to bake out at 300°C, with a total run time of 55 minutes.

An eight point standard curve was established with analyte concentrations from 5 to 1000 pg/ μl and internal standard concentration at 500 pg/ μl . The MS was operated in the single ion monitoring mode (SIM) with target and three confirmatory masses monitored (50 ms dwell time) for each compound.

CHAPTER 4

ADULT PLANT EXPOSURE TO PATHOGENIC BACTERIA

Response of *A. thaliana* exposure to the non-host pathogen *Pseudomonas syringae* pv. *syringae*: Changes in NAE levels (Work in collaboration with Nobel foundation, (Kang et al., 2008))

4.1 Introduction

According to (Heath, 2000), non-host resistance is the most common type of resistance to a pathogen offered by a plant species. The author suggests that the non-host resistance is caused primarily by the activation of defense genes, which in turn could be due to the activation of one or more signaling pathways involving MAP kinases and protein phosphatases, rather than the inducement of programmed cell death or hypersensitive response.

Pathogens attack plants via their secretion (entry into plant) of virulent proteins in several secretion pathways I through VI. Type III secretion involves the transport of the bacterial effector proteins across the membranes of both the pathogen and the host cells. Once effector proteins are inside the host cell or apoplastic space they can be recognized by 'R' proteins, which can lead to gene-for-gene resistance. Changes in ion fluxes and generation of ROS are two of the results of recognition. There is also generation of nitric oxide (NO) which could help in creating a zone where there is hypersensitive response (HR) in order to contain the infection. Usually HR is also accompanied by the

production of salicylic acid (SA), which is one of the key endogenous signaling compounds that activate systemic acquired resistance in plants. Several strains of the pathovar *Pseudomonas syringae*, pathovars *P.s. tomato*, *P.s. maculicola*, *P.s. pisi*, *P.s. glycinea*, *P.s. phaseolicola* and *P.s. atropurpurea* infect the *A. thaliana* plant (Katagiri et al., 2002). A study of the *P. syringae* pv. *glycinea* (Psg) and *P. syringae* pv. *phaseolicola* (Psp) infection on *A. thaliana* plants, which are non-hosts to these strains, indicated accumulation of salicylic acid (SA) and pathogenesis-related gene expression at inoculation sites (Mishina and Zeier, 2007). The induction of these defenses is largely dependent on bacterial type III secretion.

The identification of the FAAH analogue gene in *A. thaliana* led to the development of mutant lines of *A. thaliana faah* knockouts which had elevated levels of endogenous NAEs while FAAH overexpressors had reduced NAEs. Some of the characterizations of these mutant seedlings were that the seedlings of *faah* knockouts (KO) became hypersensitive to the growth inhibitory effects of exogenous NAE, whereas overexpressors seedlings were more tolerant to NAE (Wang et al., 2006). *AtFAAH* overexpressors (OE2, OE7, and OE11) also exhibited enhanced seedling growth suggesting that the timely depletion of NAEs might be involved in seed germination and early seedling establishment (Wang et al., 2006). The availability of plants with altered FAAH expression is being used for more detailed studies of NAE metabolism and how it impacts plant development and responses to the environment.

4.2 Exposure Set-up for Adult Plant Experiments

Adult plants were grown in mesh covered soil pots under conditions suitable for growth of high density plant material (short-day growth conditions) in order to increase amount of plant material available for analysis. Subsequently the samples were exposed to pathogens by the use of spray solution or by the use of vacuum infiltration for two minutes.

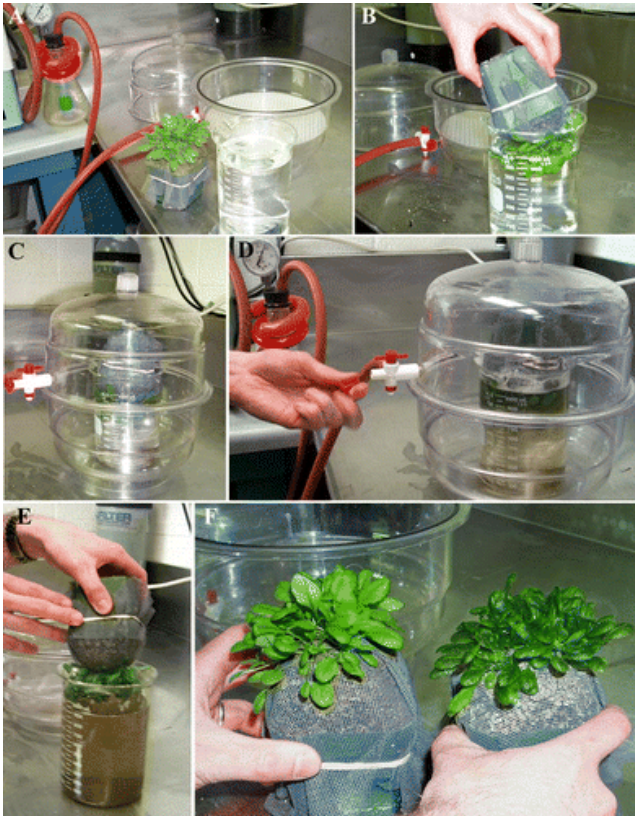


Fig. 4.1. Steps involved in the vacuum infiltration process for adult plants (Katagiri et al., 2002). The mesh covered pot is inverted into the beaker containing the bacterial suspension and vacuum infiltration of the plants is done for two minutes while in the sealed bell jar (A-C). Vacuum pressure is released by unplugging the valve stopcock and the pot of plants is removed from the bacterial suspension (D-E). Comparison of uninoculated (left) and vacuum-infiltrated plants (right) shows the inoculated leaves to appear water-soaked (F). Reproduced with permission from American Society of Plant Biologists.

Vacuum filtration usage decreases the amount of variation in the infection of leaves of the plants. Fig. 4.1 (from (Katagiri et al., 2002)) indicates the steps involved in the vacuum infiltration process. The bacterial concentration used was 5×10^6 CFU/ml in a buffer containing 0.01% of Silwet L-77 (OSI, Danbury, CT).

4.3 Quantification of NAEs levels in Adult Plant Tissue

Each sample for a given treatment was a mix of fully expanded mature leaves of about 1 g from each of the control (0 h) and treated plants (6 and 24 h; 0.5 and 1.5 h). The treated plants were vacuum infiltrated with buffer control or *P. s. syringae* (non-host pathogen) at 5×10^6 CFU/ml.

NAE extraction, enrichment and identification/quantification by gas chromatography/mass spectrometry (GC/MS) were performed as described in the section 3.5. Final NAE concentrations were calculated on both fresh weight and lipid mass basis.

4.4 Results and Discussion

4.4.1 Results

Results indicated that there was no significant difference in total or individual control, buffer and pathogen treated samples for WT (Fig. 4.2) or *AtFAAH* overexpressing, OE7 plants (Fig. 4.3) when measured at either 6 or 24 h post-inoculation.

Vacuum infiltration of the leaves resulted in a transient increase in water weight of leaf tissue. Thus, my initial experiments were conducted at 6 and 24 h post-inoculation to allow reestablishment of normal water content. The NAE levels in adult leaf tissue (Fig. 4.2-4.3) were much lower than the corresponding seeds and 8-day seedlings (Kilaru et al., 2007). Total NAE levels in WT seeds averaged about 1800 ng/g fresh mass basis.

NAE levels in 8-day seedlings dropped to a fifth of the levels in seeds. The average levels in WT adult leaf tissue varied between 50-100 ng/g on a fresh mass basis. The dominant molecular species was 18:2 in seeds, seedlings and adult tissues.

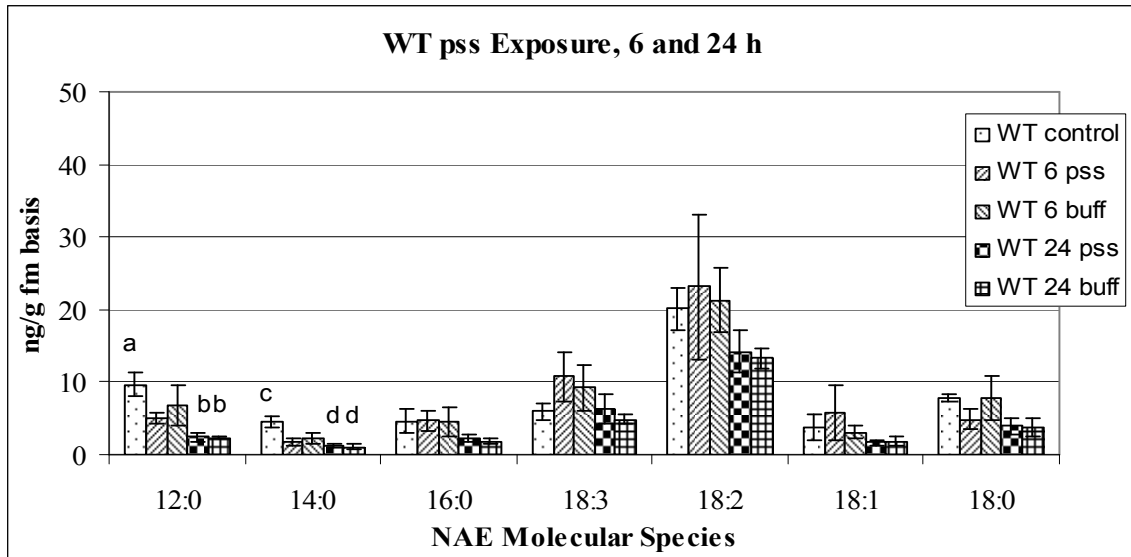


Fig. 4.2. NAE levels on fresh mass basis from WT plants at 0 h and buffer/pss treated samples at 6 and 24 h. Different lowercase letters identify treatment means that are significantly different (Dunn's multiple comparison, $p < 0.01$) for a NAE type. In NAE types where there are no significant differences, they are not labeled. Errors bars indicate one standard deviation. $n = 3, 4, 5, 5, 5$ respectively in columns left (L) to right (R).

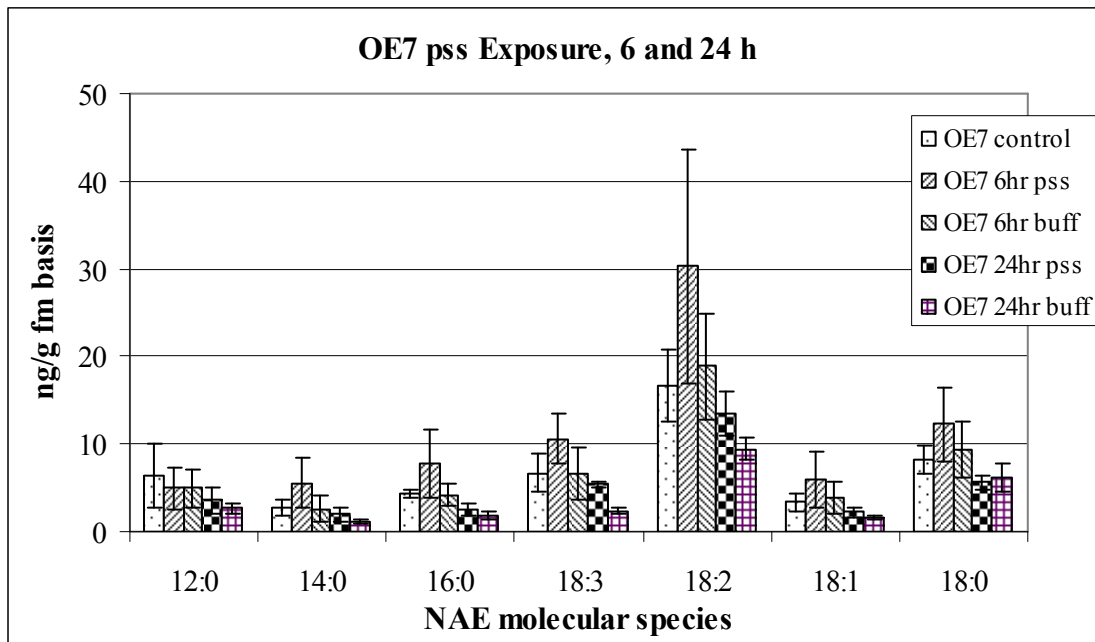


Fig. 4.3. NAE levels on fresh mass basis from OE7 plants at 0 h and buffer/pss treated samples at 6 and 24 h. There are no statistically significant differences in values based on treatment. Errors bars indicate one standard deviation. $n = 5, 5, 4, 4, 5$ respectively in columns L to R.

Since xylanase-elicited tobacco plants had elevated NAE levels in only 30 minutes post infection (Tripathy et al., 1999), I decided to examine earlier time points despite the complicating factor of increased variability in concentration associated with the transient uptake of water during infiltration. Two time points of analysis of 0.5 and 1.5 h were selected. An initial experiment with WT and OE7 plants was conducted at both time points. This was subsequently repeated with WT, OE7 and OE11 at control and 1.5 h. As seen in earlier experiments, overall NAE levels were lower in OE plants than WT. Figures 4.4 and 4.5 present the values of individual NAE levels at control, 0.5 and 1.5 h post-inoculation for WT and OE7 respectively. Neither data set indicated any consistent change in NAE profile associated with pathogen exposure.

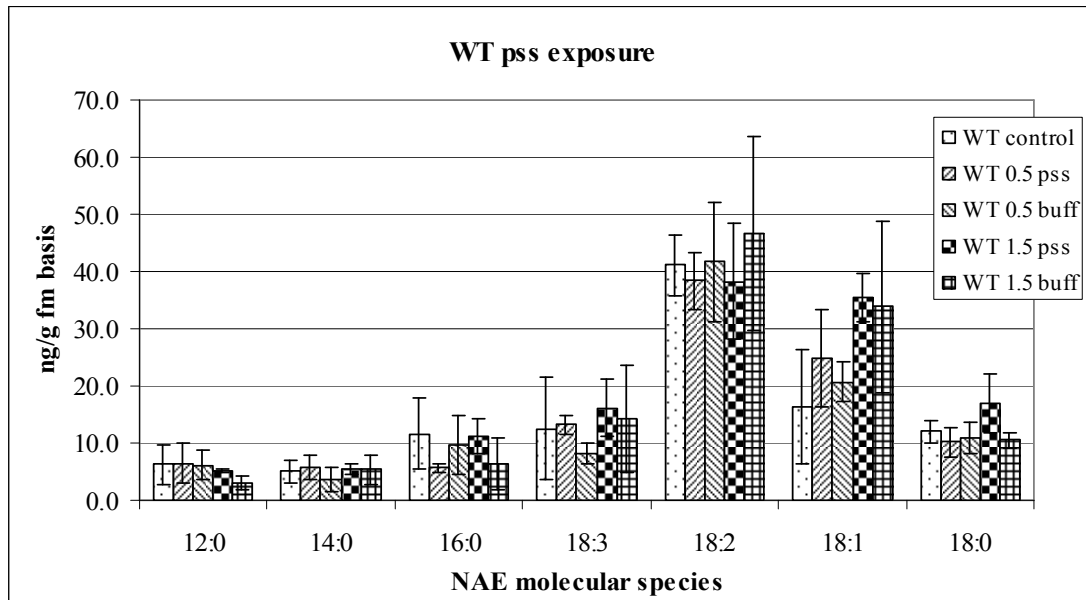


Fig. 4.4. NAE levels on fresh mass basis in WT plants at 0 h and buffer/pss treated samples at 0.5 and 1.5 h. There are no statistically significant differences in values based on treatment. Errors bars indicate one standard deviation. $n = 3, 3, 4, 4, 4$ respectively L to R.

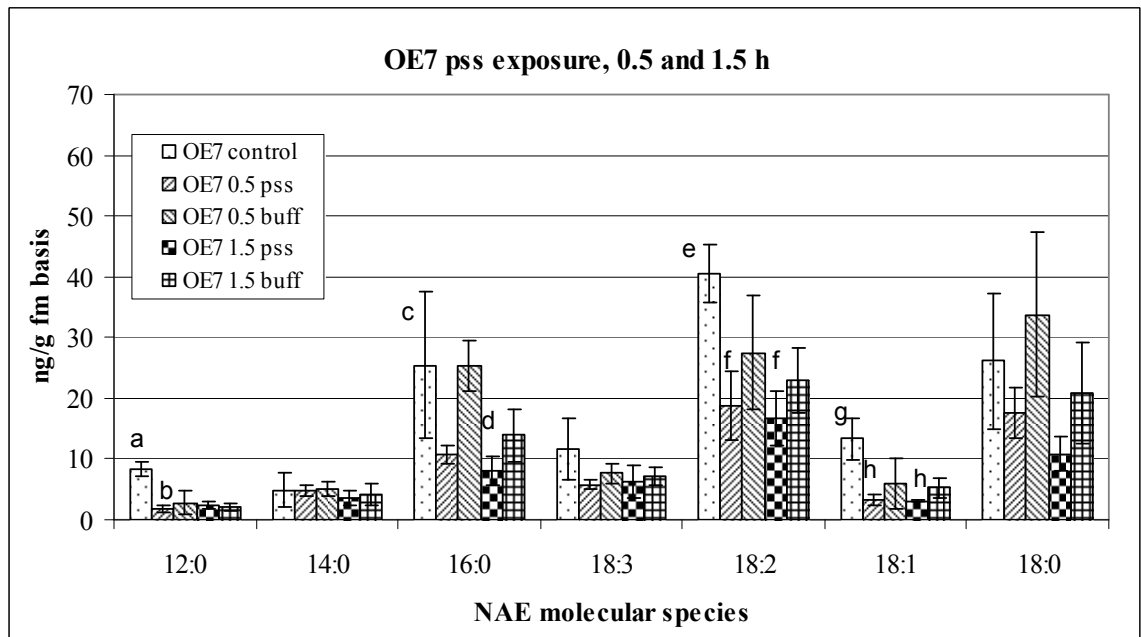


Fig. 4.5. NAE levels on fresh mass basis from OE7 plants at 0 h and buffer/pss treated samples at 0.5 and 1.5 h. Different lowercase letters identify treatment means that are significantly different from control (Dunn's Multiple Comparison, $p < 0.05$). In NAE types where there are no significant differences, they are not labeled. Errors bars indicate one standard deviation. $n = 4, 4, 4, 4, 4$ respectively L to R.

A second set of experiments with WT and OE7 plants was performed at a time point of 1.5 h post-inoculation in order to confirm absence of a pathogen effect (Fig. 4.6). Total NAE concentrations observed in this experiment were lower than the previous experiment. The total NAE levels of WT buffer and pss treated samples were 22 and 21 ng/g and 15 and 19 ng/g, respectively, in OE7 samples. Once again, overall NAE levels in OE7 samples were lower than those of WT samples as seen in seeds and 8-day seedlings. However, there was no significant difference in comparison of each individual NAE between the genotypes or in comparison of control and pathogen-treated plants.

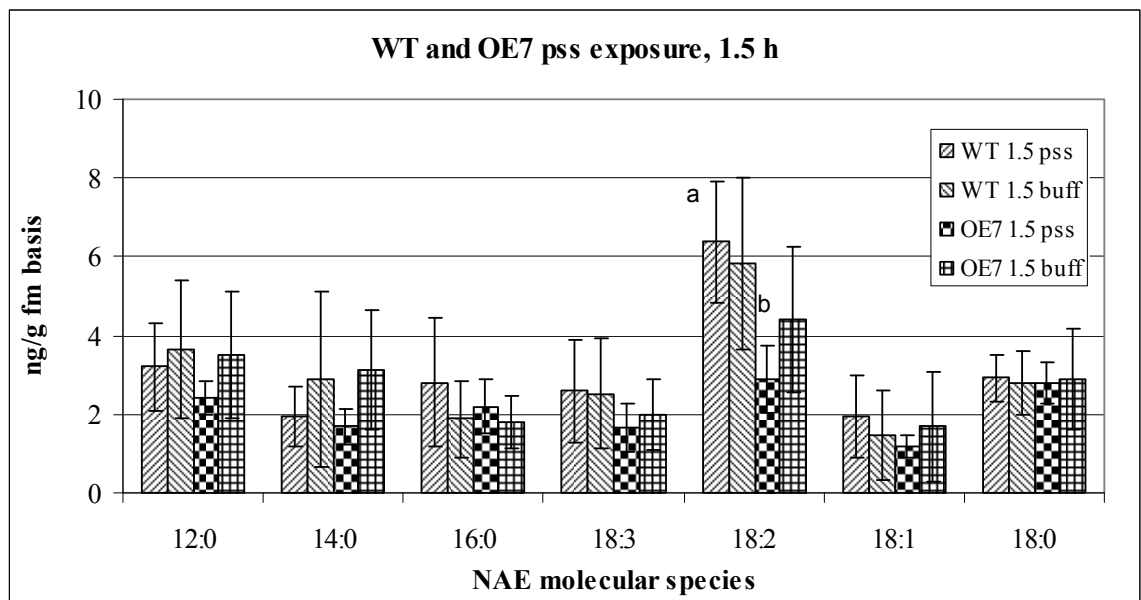


Fig. 4.6. NAE levels on fresh mass basis from WT, OE7 plants at buffer/pss treated samples at 1.5 h. Different lowercase letters identify treatment means that are significantly different from control (Dunn's Multiple Comparison, $p < 0.05$). In NAE types where there are no significant differences, they are not labeled. Errors bars indicate one standard deviation. $n = 5, 5, 4, 5$ respectively L to R.

Over the course of these experiments, the results indicated that variation in NAE levels could be influenced by the age of the leaves selected as well as the amount of

water present in the samples. Thus further investigations (Experiments 2, 3 and 4) of effects were conducted with 5-day seedlings which would have higher and more consistent NAE concentrations.

The null hypothesis tested in experiment one (H_{01}) was that there will be no significant difference in NAE levels in plants subjected to no treatment, buffer and bacterial solution treatments in a given genotype. My results were consistent with the null hypothesis H_{01} .

4.4.2 Discussion

The changes in NAE profiles in *A. thaliana* plants were not as dramatic as seen in the work with tobacco cell culture and leaf samples exposed to fungal elicitors. In the tobacco study, increase in NAE 14:0 was found to induce the expression of the defense gene PAL, similar to several pathogen associated signaling molecules such as salicylic acid and systemin (Tripathy et al., 1999).

The current study focused on changes in NAE profiles in *A. thaliana* based on *Pseudomonas syringae* infection. There were lower levels of plant phytohormones (JA, ABA and free and conjugated SA) in FAAH overexpressor plants and these plants were more susceptible to infection (Kang et al., 2008), indicating a depression in PAMP associated signaling. Recent related study of *AtFAAH* overexpressor plants, in which there was loss of hydrolytic activity caused by site-directed mutagenesis, there was no growth enhancement and no NAE tolerance, which is seen in typical *AtFAAH* overexpressor plants. These seedlings were hypersensitive to ABA and hyper susceptible to non-host pathogens to a degree similar to the overexpression of the native *AtFAAH*.

The authors conclude that *AtFAAH* influences plant growth and interacts with ABA signaling and plant defense through distinctly different mechanisms (Kim et al., 2009).

The major resistance of *A. thaliana* plants to different strains of the bacteria is thought to be due to gene-for-gene resistance. However, there can be antagonistic interaction in the two defense system responses associated with gene-for-gene or PAMP (Kim et al., 2005). Studies of xylanase-tobacco elicitor systems indicated that there are short-term changes in ion fluxes at the plasma membrane and in long-term generation of ethylene, production of phytoalexin defense compounds (Chapman, 2004). The role of NAEs in relation to these responses is unknown. Therefore, since I did not see substantial change in the NAE profiles associated with *Pseudomonas syringae* infection, further investigations (Experiments 2 and 3, in Chapters 5 and 6 respectively) were performed with the well characterized bacterial PAMP elicitor flg22 using 5-day seedlings, which have higher levels of NAEs.

CHAPTER 5

SEEDLINGS EXPOSURE TO BACTERIAL ELICITOR

Response of 5-day *A. thaliana* seedlings exposed to bacterial elicitor molecule flg22:

Changes in NAE levels and production of ROS.

5.1 Introduction

The best characterized small molecule which elicits pathogen associated molecular patterns (PAMPs) based responses from plants is the bacterial flagellin molecule flg22, a 22 amino acid peptide. The flg22 peptide is an elicitor for whole *A. thaliana* plants, resulting in responses such as callose deposition, oxidative burst, ethylene production, and induction of defence-related genes such as PR1 and PR5 (Gomez-Gomez et al., 1999). Flagellin is recognized by FLS2, a transmembrane leucine-rich repeat (LRR) receptor kinase. FLS2 is found in roots, stems and flowers, leaf epidermal cells and stomatal guard cells (Bauer, 2003). I exposed 5-day seedlings grown in sterile conditions to flg22 to determine if there were changes in NAE concentration profiles associated with exposure.

ROS are generated during the normal metabolic processes in plants. They play a role in plant growth and defense responses. ROS in growth contributes to the development of cells. NADPH is oxidized by NADPH oxidases (NOXs) to produce the superoxide radical (Gapper and Dolan, 2006). Some of these NOX proteins play a role in root development via involvement in ABA-signaling pathway (Kwak et al., 2003). The

NOX proteins are now called RBOHs (respiratory burst oxidase homologs) (Yoshioka et al., 2008). ROS signaling plays a variety of roles in plant development. For example, rate of growth in a tissue can be stimulated by increased ROS production. Ca^{2+} concentration gradients created by ROS accumulation can induce root hair growth and dissolution of xyloglucan polymers due to $\cdot\text{OH}$ radical and H_2O_2 production promotes secondary cell wall formation (Laloi et al., 2004; Gapper and Dolan, 2006). There are more than 150 genes involved in the regulation of ROS levels in plants (Mittler et al., 2004).

In plant defense, upon perception of a pathogen attack due to recognition of PAMPs or other elicitor molecules, there is activation of NADPH oxidases, peroxidases and nitric oxide production which in turn lead to production of ROS, H_2O_2 and superoxide radical. The production of ROS can set into action signaling pathways that can increase the production of SA which will induce SAR, hypersensitive response which will cause cell death of infected cells, cell wall strengthening and defense gene expression (Torres et al., 2006). MAP kinases play an important role in ROS signaling pathways (Hancock et al., 2006). Thus, the production of ROS on exposure to an elicitor indicates the activation of various signaling cascades.

I exposed 5-day *A. thaliana* seedlings grown in sterile conditions to flg22 to characterize expected levels of H_2O_2 production associated with exposure.

5.2 Exposure Set-up for 5-day Seedlings and Methods for Analyses

The seeds were stratified for 3 days at 4°C and subsequently placed in 12-well plates containing 1 ml of $\frac{1}{2}$ MS media with 1% sucrose in each well. The plants were placed in a growth chamber with 16/8 day/night cycle at 22°C on a rotary shaker at 100-

120 rpm. Two time points were assessed: 90 minutes and 24 h. For the samples for 24 h exposure, flg22 or solvent (0.07% DMSO) only solutions were added at day 4 and samples collected 24 hours later. The concentrated flg22 stock was at 1 mM in 70% DMSO.

5.2.1 NAE Analysis

The seedling samples were carefully blotted dry and weighed (up to nearest 0.1 mg) for fresh mass. Subsequent steps in analysis followed those described in section 3.5.

5.2.2 ROS Analysis

Individual intact seedlings were placed in de-ionized (MQ) water for at least four hours. The samples were then subsequently placed in fresh MQ water (to remove any oxidases present in solution) and treated with stock solutions to achieve 1 mM luminol and 1 μ M of HRP concentrations. Then solvent alone or solvent with 100 μ M flg22 was added to yield a final concentration of 1 μ M. The sample was then placed in Synergy2 plate reader from Bio-Tek Instruments (Winooski, VT). Readings of luminescence were noted every 5 to 10 seconds for 20 to 60 minutes.

5.3 Results and Discussion

5.3.1 NAE Analysis Results

An initial analysis of WT plants was conducted in order to determine an appropriate exposure to the bacterial peptide flg22 to measure changes in NAE concentration profiles. The exposures were for 90 minutes (100 nM and 1 μ M) and 24 h (1 μ M). At 90 minutes there was no significant difference between the solvent and flg22-exposed samples (Fig. 5.1). In the 90 minutes exposure (Fig. 5.1) the relative contribution

of 16:0 was higher than in the typical NAE profile in these samples but no significant changes in NAE concentrations were associated with the flg22 exposure. The exposure for 24 h at a concentration of 1 μ M showed slightly increased levels of NAE 18:2 (Fig. 5.2). I decided that a long exposure of 24 h was probably a better endpoint for measuring the changes, if any, in the steady state levels of NAEs. Two more sets of experiments with WT plants at 24 h exposure and 1 μ M concentration were performed to assess whether the elevated NAE 18:2 concentration was repeatable. The overall levels of total NAEs varied between \sim 100 and \sim 150 ng/g, as seen in Figs. 5.2 to 5.4. However, there was no consistent pattern of NAE change associated with flg22 treatment.

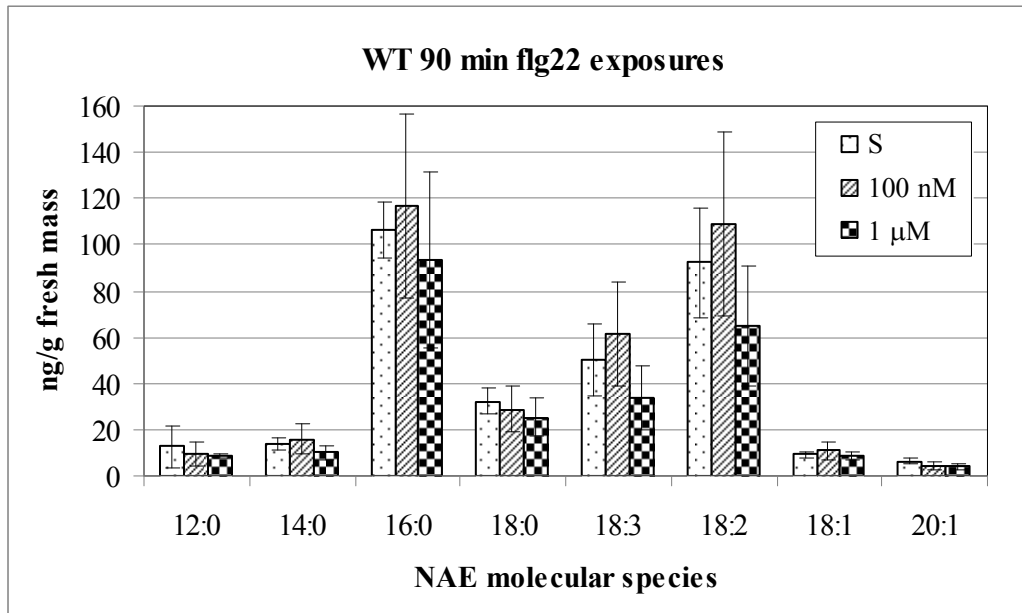


Fig. 5.1. NAE levels in WT 5-day seedling samples, exposed to flg22 peptide elicitor for 90 minutes. Treatments included solvent, concentrations at 100 nM and 1 μ M. In NAE types where there are no significant differences, they are not labeled. Error bars indicate one standard deviation. $n = 4, 4, 3$.

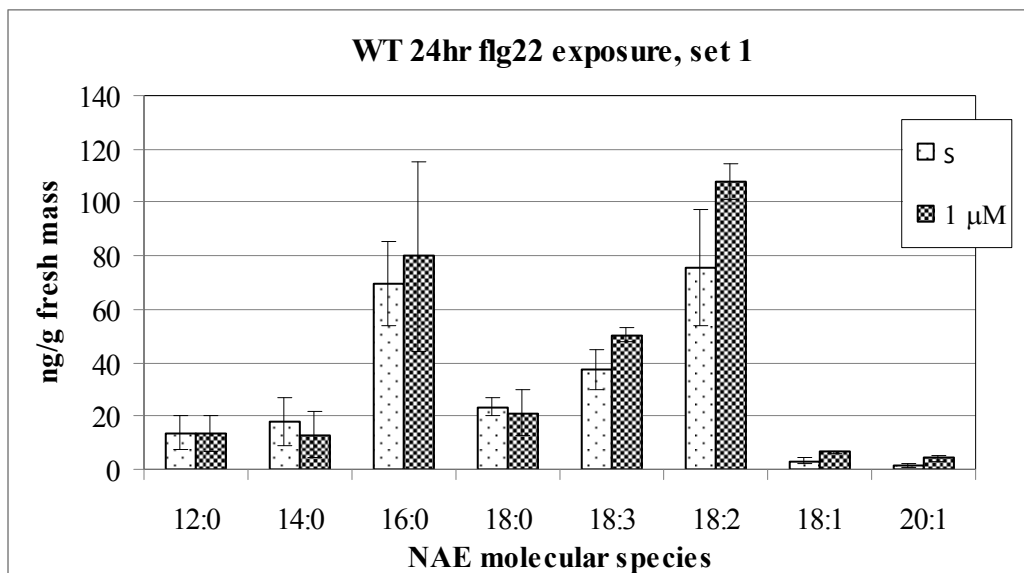


Fig. 5.2. NAE levels in WT 5-day seedling samples, exposed to flg22 peptide elicitor for 24 h. Treatments included solvent (0.1% DMSO) and concentration of flg22 at 1 μ M. Results are of set 1 of 3. Different lowercase letters identify treatment means that are significantly different from control. In NAE types where there are no significant differences, they are not labeled. Error bars indicate one standard deviation. $n = 4$.

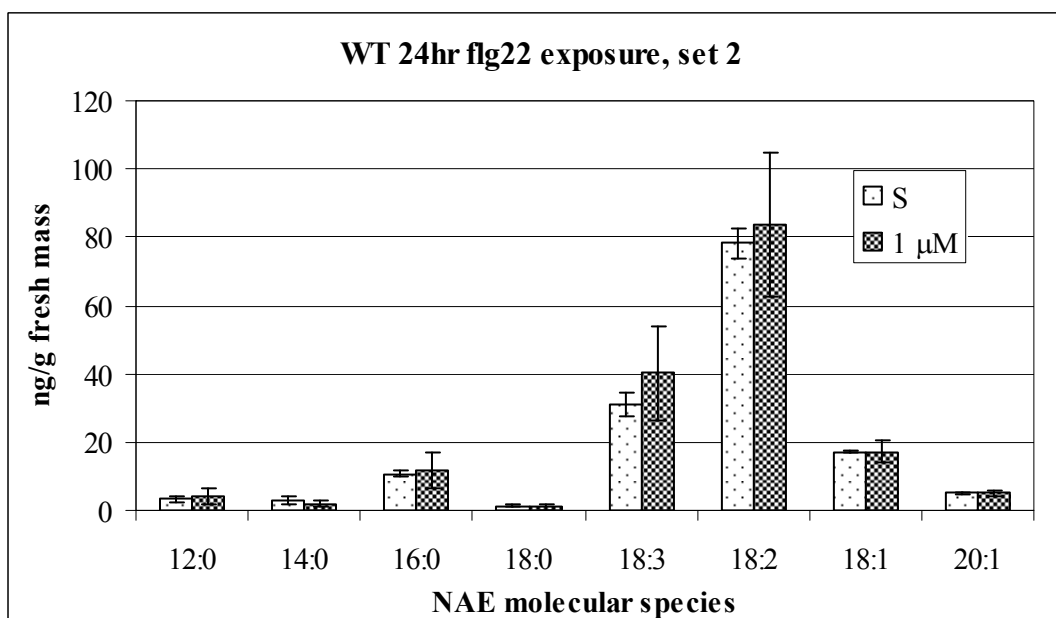


Fig. 5.3. NAE levels in WT 5-day seedling samples, exposed to flg22 peptide elicitor for 24 h. Treatments included solvent (0.1% DMSO) and concentration of flg22 at 1 μ M. Results are of set 2 of 3. There are no statistically significant differences in values based on treatment. Error bars indicate one standard deviation. $n = 3$.

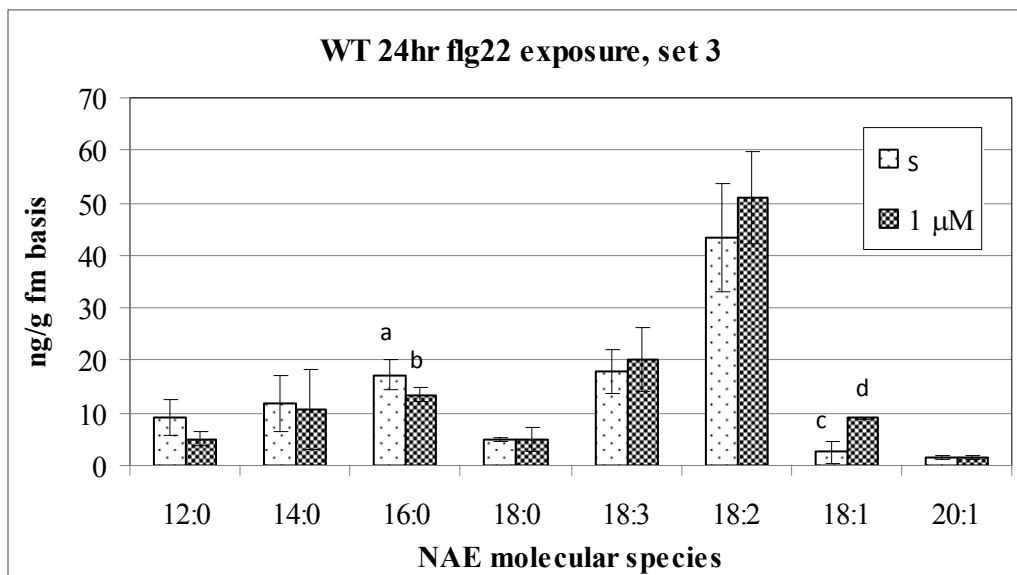


Fig. 5.4. NAE levels in WT 5-day seedling samples, exposed to flg22 peptide elicitor for 24 h. Treatments included solvent (0.1% DMSO) and concentration of flg22 at 1 μ M. Results are of set 3 of 3. Different lowercase letters identify treatment means that are significantly different from control. In NAE types where there are no significant differences, they are not labeled. Error bars indicate one standard deviation. $n = 3$.

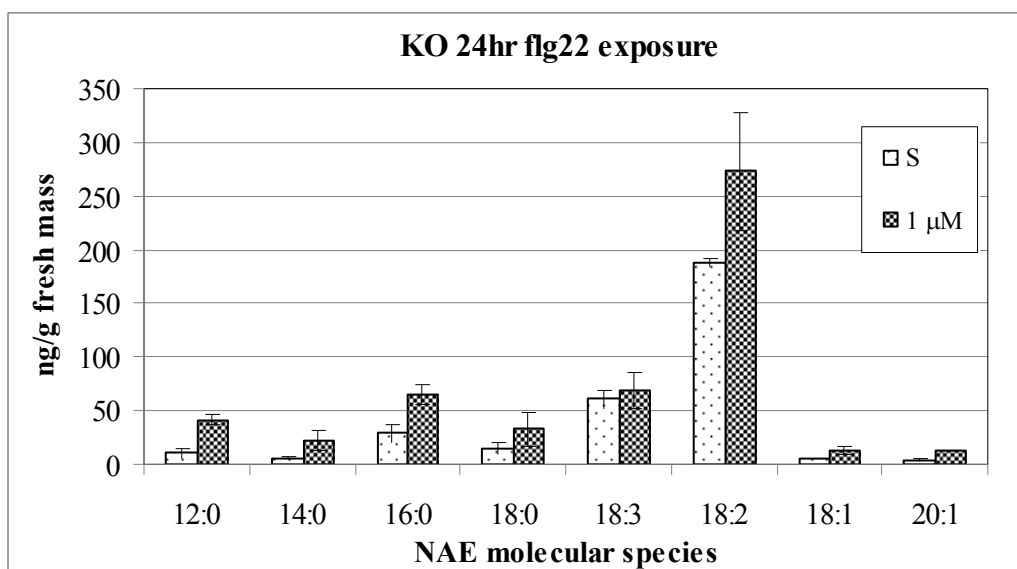


Fig. 5.5. NAE levels in KO 5-day seedling samples, exposed to flg22 peptide elicitor for 24 h. Treatments included solvent (0.1% DMSO) and concentration of flg22 at 1 μ M. Due to a small $n = 2$, statistical calculations were not performed. Error bars indicate the range.

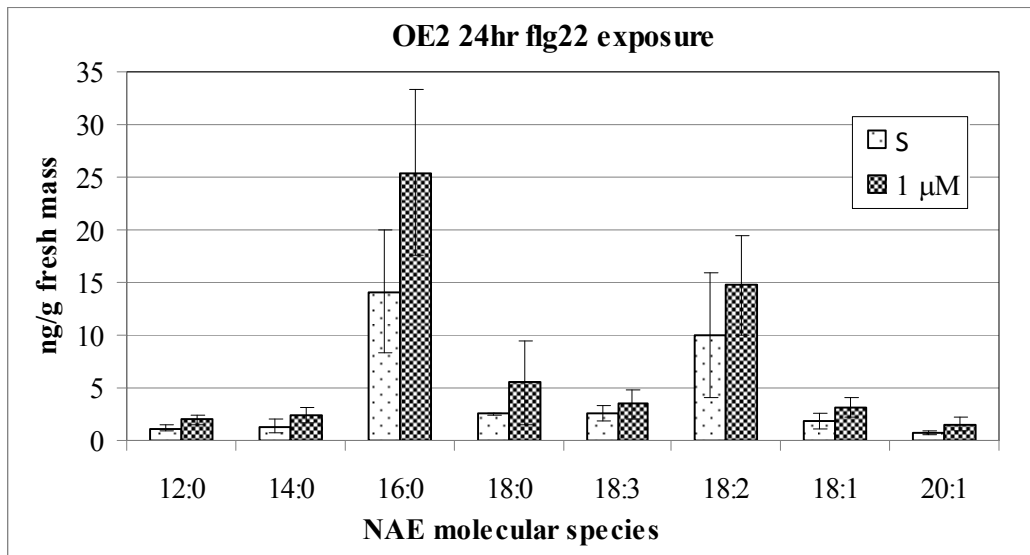


Fig. 5.6. NAE levels in OE2 5-day seedling samples, exposed to flg22 peptide elicitor for 24 h. Treatments included solvent (0.1% DMSO) and concentration of flg22 at 1 μ M. There are no statistically significant differences in values based on treatment. Error bars indicate one standard deviation. $n = 3$.

Experiments with different FAAH genotypes, KO and OE2 were also performed in order to determine if the altered genotypes influenced NAE response to flg22 challenge (Figs. 5.5 and 5.6). Once again, as was seen in the WT plants, NAE profiles for solvent and flg22 exposed plants were similar. However, these data for the OE2 samples, like those of Fig. 5.1 and 5.2 for WT, had an unexplained higher relative contribution of NAE 16:0.

The null hypothesis to be tested H_{02a} was that there will be no significant changes in NAE levels in seedlings subjected to elicitor and solvent treatment. My results for 90 minute and 24 hr exposure of WT, OE and KO plants were consistent with the null hypothesis.

5.3.2 NAE Analysis Discussion

As seen in results from study involving adult tissue and *Pss*, in this study there was once again no significant change in NAE profiles on exposure of 5-day seedlings to the bacterial elicitor flg22, known to elicit PAMP mediated defense responses in *A. thaliana*. Thus the expected increase in NAE levels on exposure to elicitor seen in earlier tobacco cell culture and leaf exposures to the fungal elicitor xylanase (seen within 10 minutes) was not demonstrable in my work with *A. thaliana* exposure to flg22 (measured at 90 minutes and 24 h) or *Pseudomonas syringae* pv. *syringae* (Chapter 4). These time points can be considered as steady state time points NAE analysis. Thus an earlier time-point, 10 minutes post exposure should have been considered for analysis.

There was a decrease in PAMP associated signaling responses such as ROS and flg22 induced endocytosis in plants exposed to TCS (Serrano et al., 2007). There was also decrease in lipid synthesis due to decrease in enzyme activity involved in the FASII pathway. The authors suggested that the TCS induced suppression of the ROS response to pathogen might be related to interference with synthesis of lipid signaling. I hypothesized that this might include interference with the demonstrated role of NAE signaling in the tobacco response to xylanase challenge. Since I failed to see changes in *A. thaliana* exposed to flg22, I conducted additional experiments to verify the induction of an ROS response to flg22 in my system (the following section of this chapter) and the TCS inhibition of this ROS response and ENR activity while monitoring NAE profiles and TCS accumulation (Chapters 6 and 7).

5.3.3 ROS Analysis Results

Initial studies involving exposure of 10-day *A. thaliana* seedlings to flg22 resulted in rapid release of ROS in the form of H_2O_2 . There was a peak in luminescence for samples exposed to flg22 between 8-11 minutes. Typical luminescence plots for control and flg22-exposed samples are presented in Fig. 5.7 for 10-day leaf pieces and Fig. 5.8 for 5-day seedlings.

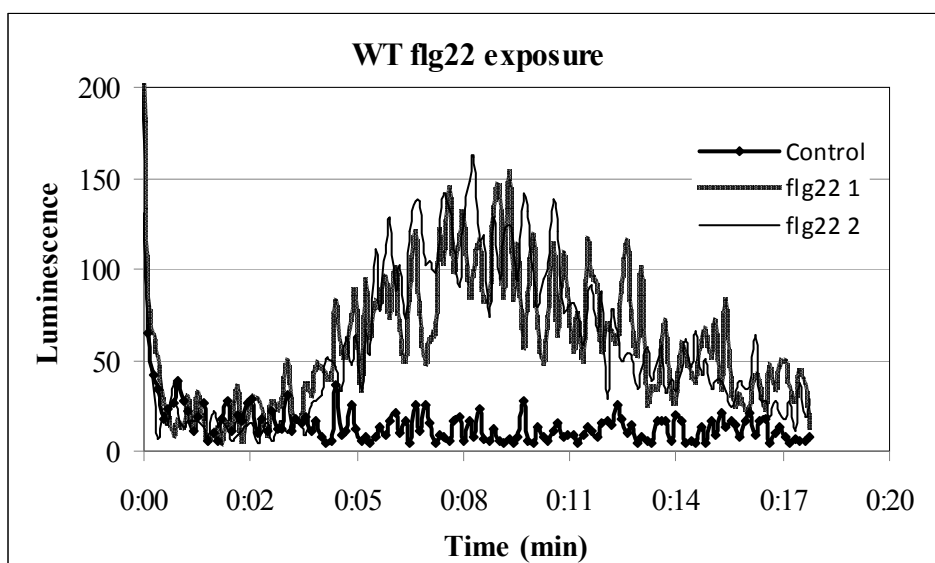


Fig. 5.7. *A. thaliana* WT 10-day seedlings exposed to flg22 at 1 μ M or DMSO solvent at 0.07% concentration.

Over the course of several experiments based on 1 or 2 solvent-exposed and 3-4 flg22-exposed samples, a wide range of magnitude of response to flg22 was observed. The differences in solvent control and flg22 treated samples averaged from these experiments, as total area under the luminescence curve, are shown in Fig. 5.9. Although there was a consistent positive increase in H_2O_2 production in flg22 exposed plants, the range of response varied over orders of magnitude. Despite this consistent elevation in

H₂O₂ production with flg22 exposure, the variability in response prohibited any conclusions regarding genotypic differences in response (Fig. 5.10).

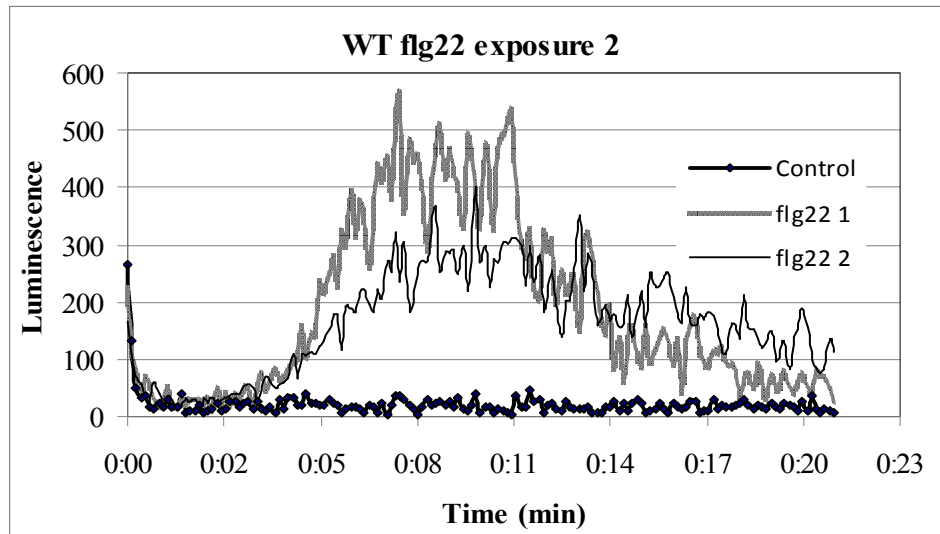


Fig. 5.8. *A. thaliana* WT 5-day seedlings exposed to flg22 at 1 μ M or DMSO solvent at 0.07% concentration.

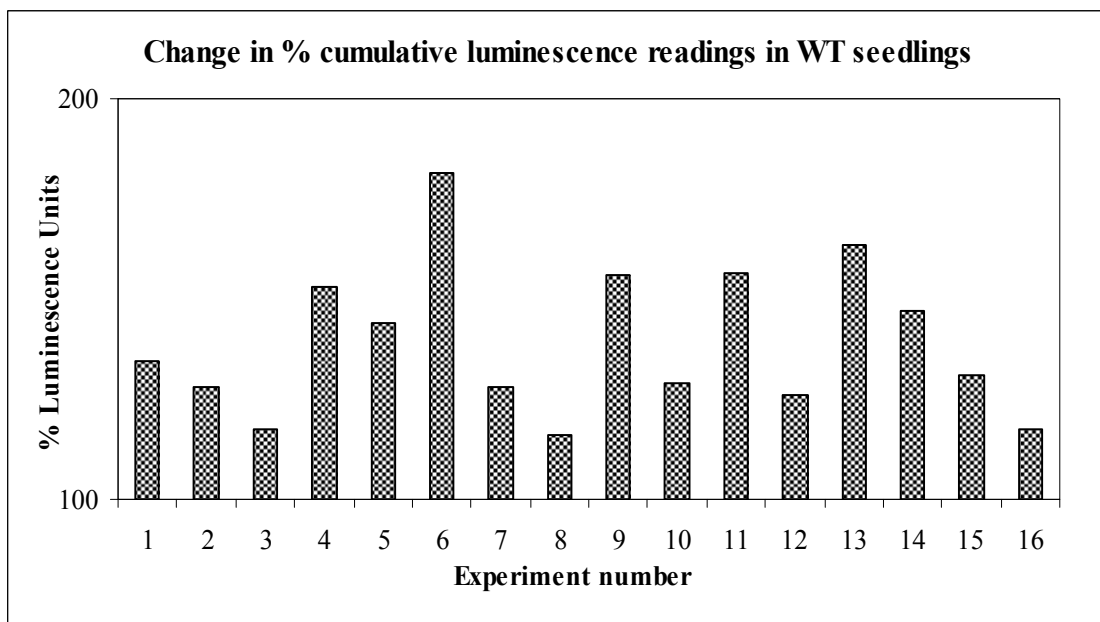


Fig. 5.9. The percentage change in flg22 (1 μ M) treated cumulative luminescence units relative to solvent control samples from different experiments. Actual readings ranged from 49335 to 2342553. On average the solvent control had 1 or 2 replicates and flg22 samples had 3 to 6 replicates.

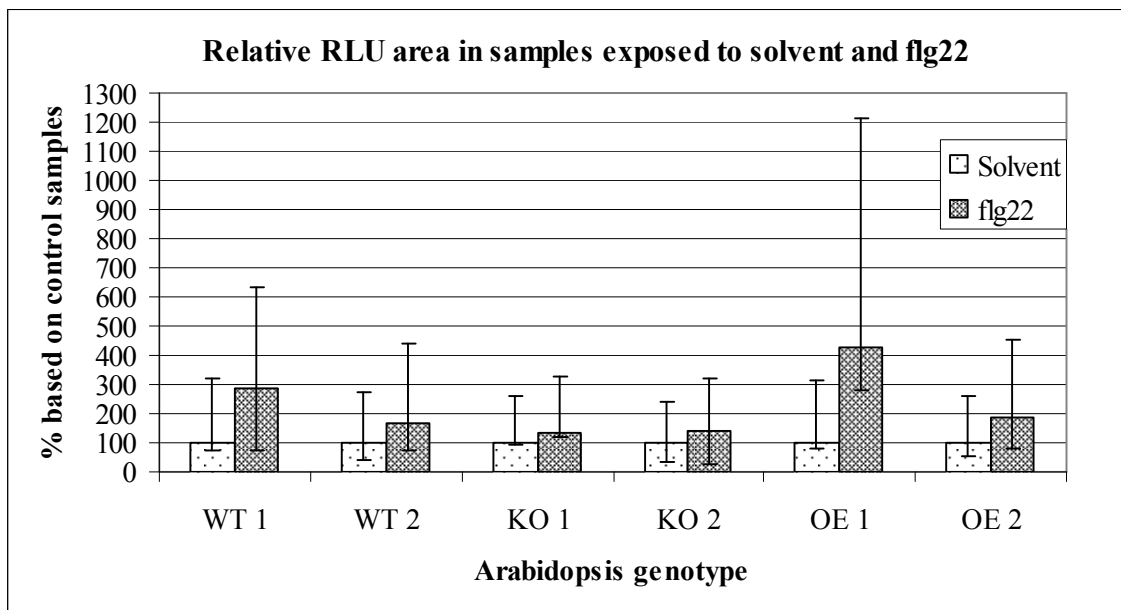


Fig. 5.10. Relative cumulative luminescence readings of solvent control and flg22 (1 μ M) treated samples. Mean area of luminescence units calculated on basis of control samples, having a value of 100. Error bars indicate the range based on control samples. $n = 3$ in each data set.

5.3.4 ROS Analysis Discussion

Due to the (i) variability in the amounts of actual luminescence reading obtained, (ii) lack of any substantial differences in ROS luminescence readings from different genotypes, (iii) difficulty in obtaining tangible results due to extreme sensitivity of the luminol and horseradish peroxidase system and (iv) variability due to orientation of leaves in the experimental well chamber, no clear conclusions could be drawn from the exposure of the WT, KO and OE2 samples, except that there was an increase in H_2O_2 production on exposure to flg22 in all three genotypes at a concentration of 1 μ M. Thus the results were not consistent with the null hypothesis H_{02b} . However, the quantitative inconsistency of the H_2O_2 response led me to focus on the use of Zat12 genetically modified *A. thaliana* seedlings for monitoring of ROS response (Chapter 7).

CHAPTER 6

SEEDLINGS EXPOSURE TO BACTERIAL ELICITOR AND TCS

Response of 5-day *A. thaliana* seedlings exposed to TCS and bacterial elicitor flg22:

Changes in NAE levels and Enoyl-ACP Reductase enzyme activity, and accumulation of TCS.

6.1 Introduction

Plant cells produce fatty acids in the plastids with the starting molecule acetyl-CoA. The investigation of the role of lipid signaling in plant defense responses has resulted in the discovery of many lipids involved in the process. Plants physical barrier, cutin, is a polyester of hydroxy and epoxy-hydroxy C16 and C18 fatty acids. Some pathogens recognize cutin and release elicitors. Plants identify microbial lipid molecules called lipopolysaccharides (LPSs) as pathogenic elicitors. Disruption in plant sphingolipid metabolism, lipid modification (myristoylation and/or palmitoylation) for localization of Avr proteins to the plasma membrane are some of the initial responses in plant defense. Fatty acids are substrates for the synthesis of oxidized lipids and regulation of the activity of enzymes involved in plant defense responses (Shah, 2005). The jasmonic acid pathway involves plant oxylipins, which are oxidized fatty acids. Unsaturated fatty acid levels control the expression of several R genes (Chandra-Shekara et al., 2007). Very long chain fatty acids are required for the biosynthesis of the plant cuticle and the generation of sphingolipids (Raffaele et al., 2009).

In plants, the synthesis of fatty acids is accomplished through the fatty acid synthesis (FASII) pathway which is shared with bacteria and is considered to be an excellent target for the development of new antibacterial agents. The last step in the FASII pathway involves the enzyme enoyl-ACP reductase (ENR) enzyme which catalyzes the hydrogenation of trans-unsaturated double bonds to form saturated ACP. TCS effects on ENR enzyme in the malarial bacteria *Plasmodium falciparum* have been studied extensively (Kapoor et al., 2004). In *A. thaliana*, it has been reported that the MODI gene encodes for the ENR enzyme. The mutant knock out plant *modI* has overall lower lipid content. Several phenotypic effects are seen in this mutant and include chlorotic and curly leaves, distorted siliques, and premature senescence of primary inflorescences, reduced fertility, and semi-dwarfism (Mou et al., 2000). As discussed earlier, studies with *A. thaliana* (Serrano et al., 2007) indicate that TCS is a potent inhibitor of ENR enzyme with a short term exposure of 10 ppm.

The present study followed the changes in ENR enzyme activity based on short-term (2 h) exposure of protein extract and long-term (12 h and 24 h) and short-term exposure (2 h) of 5-day seedlings to TCS. Also, NAE profiles were quantified in 5-day seedlings exposed to TCS.

6.2 Exposure Set Up for 5-day Seedlings and Methods of Analyses

An initial study was conducted with protein extracted from four-week old *A. thaliana* leaf samples in order to examine the effect of short term exposure of 2 h to various TCS and M-TCS concentrations on the ENR enzyme activity. Methanol was used as a solvent control.

Five day seedlings were assessed for changes in activity at 2, 12 and 24 h. Typical exposure set up for samples included the use of a sterile 12-well plate with 1 ml of media. The seedlings were grown in the plate or transferred to it from volumetric flasks. On day 4/5, treatments of solvent, TCS or flg22 were added using minimum volume additions. The plates were used for analysis after 2, 12 or 24 h.

A protocol based on that described in (Serrano et al., 2007), as described in section 3.3, was used to assess the changes in ENR enzyme activity.

6.3 Results and Discussion

6.3.1 Effects of TCS Exposure on ENR Enzyme Activity: Proteins Extract Exposures

Increasing TCS concentrations caused a steady decrease in *MOD1* enzyme activity. There was a significant drop in activity between solvent and both 1 and 10 ppm treatments, although comparison of 1 and 10 ppm treatments showed no significant differences. These results are shown in the Fig. 6.1, where the x-axis describes the treatment and y-axis denotes the activity of the various treatments when compared to the control mean set at 100%. In this short term exposure, the effect of MTCS at 0.1 ppm – 10 ppm on the rate was not significantly different from controls. MTCS was found to be ineffective in reducing ENR activity at concentrations as high as 50 ppm (results not shown).

On a repeat of the previous experiment, significant reductions in ENR activity were seen at lower TCS concentrations of 0.1 and 0.05 ppm, but not at 0.01 ppm. The results of the second experiment with protein extract at different TCS concentrations are seen in Fig. 6.2.

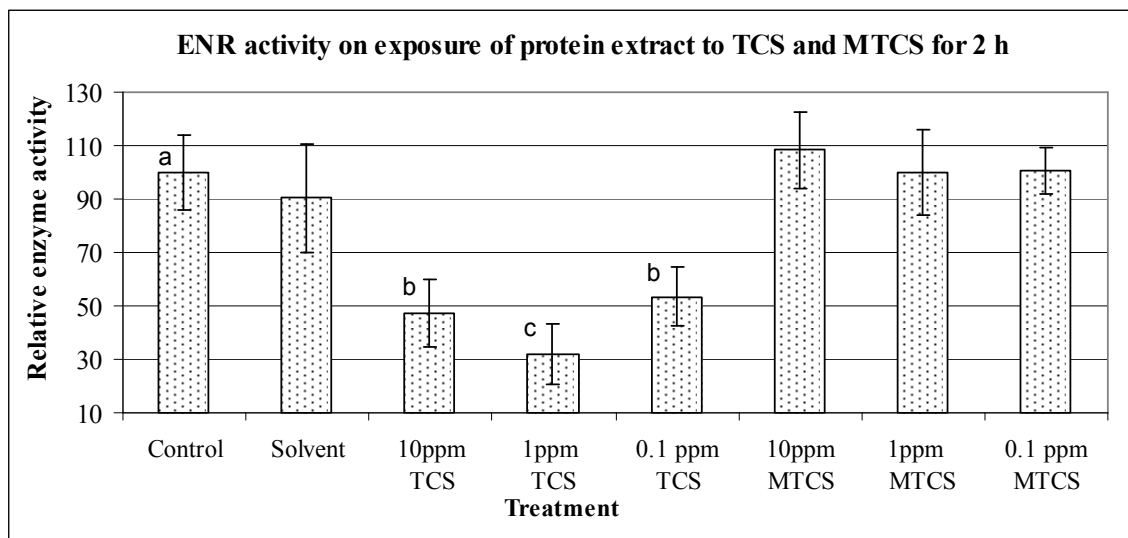


Fig. 6.1. Protein extract from *A. thaliana* 4 week old plants exposed to Solvent methanol and various concentrations of TCS and methyl TCS (MTCS): 0.1, 1.0 and 10.0 ppm for 2 h. Points indicate an average of 25 readings subsequent to adding substrate. Treatments significantly different from controls are indicated by different letters (Tukey–Kramer $p < 0.05$). Error bars indicate one standard deviation.

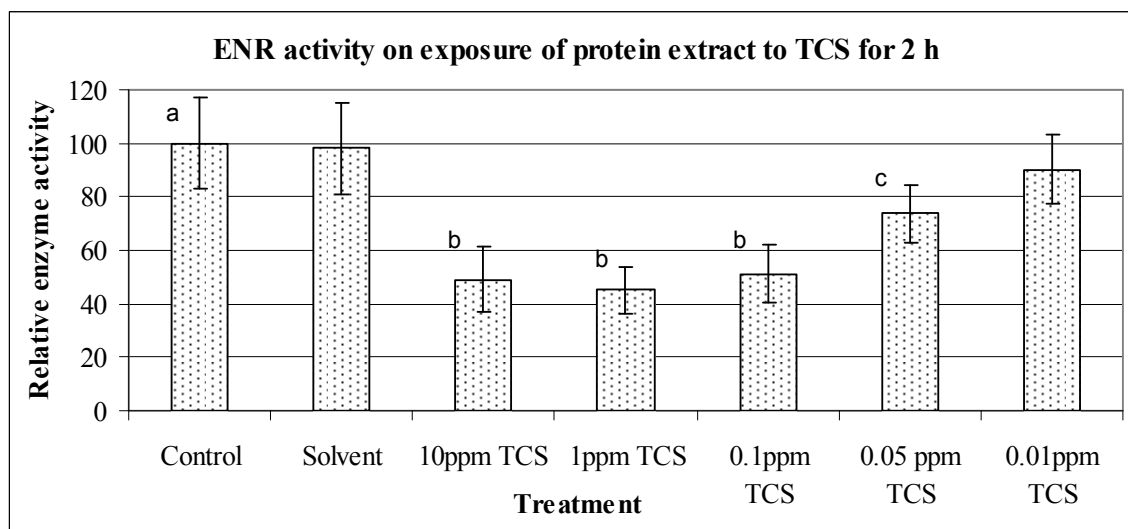


Fig. 6.2. Protein extract from *A. thaliana* 4 week old plants exposed to Solvent methanol and various concentrations of TCS: 0.01, 0.05, 0.1, 1.0 and 10.0 ppm for 2 h. Points indicate an average of 22 readings subsequent to adding substrate. Treatments significantly different from controls are indicated by different letters (Tukey–Kramer $p < 0.05$). Error bars indicate one standard deviation.

6.3.2 Effects of TCS Exposure on ENR Enzyme Activity: 5-day Seedling Exposures

Analysis of NAE profiles and ROS measurement studies in subsequent experiments were performed with 5-day seedlings as they quantified higher NAE levels (discussed in Chapter 4). Young 5-day seedlings are also not stressed due to growth in liquid media. Thus enzyme activity studies, discussed in this section were also performed with 5-day seedlings.

Analysis of ENR activity was initially performed with 5-day WT seedlings at two different time points of 2 and 12 h. In the 12 h exposure, the treatments included control, solvent, 0.01 ppm, 0.1 ppm and 1.0 ppm TCS. ENR activity decreased at 0.1 and 1.0 ppm treatments, however there was no statistically significant difference in ENR activity at 0.01 ppm TCS (Fig. 6.3). In the 2 h exposure, the treatments included control, solvent, 0.1 ppm, 1.0 ppm and 10.0 ppm TCS. With this short-term exposure, significant depression of ENR activity was seen only at the highest exposure of 10 ppm TCS (Fig. 6.4).

Based on the 2 h and 12 h exposure results, I decided that subsequent exposures would be conducted at 10 ppm with an exposure time of 24 h to ensure a consistent TCS-induced depression of ENR activity. Further experiments were repeated with at least two or three replicates in each treatment.

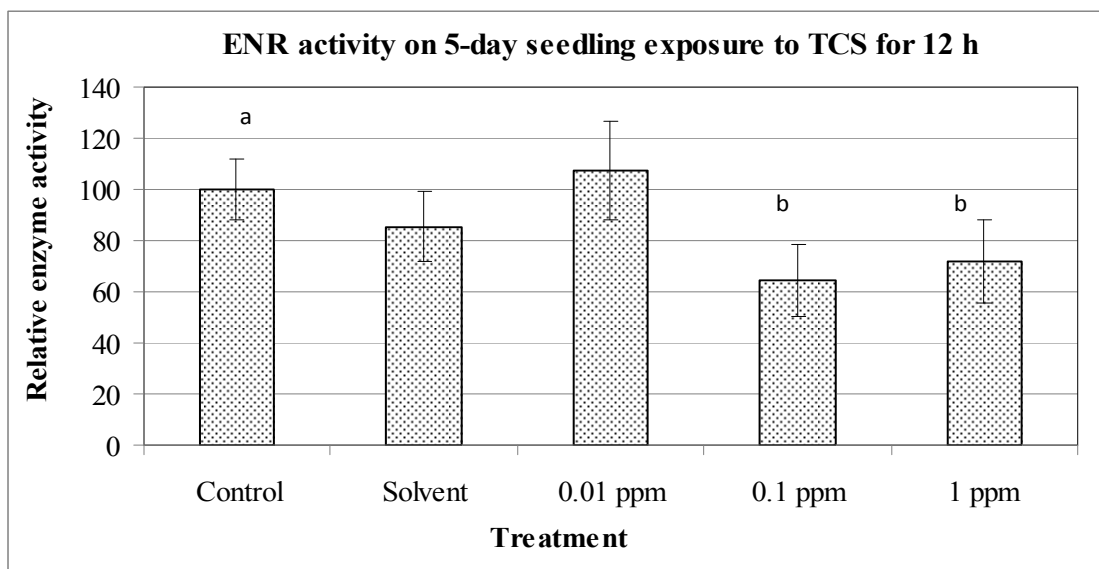


Fig. 6.3. *A. thaliana* 5-day seedlings exposed to no treatment (control) and methanol (solvent) and various concentrations of TCS: 0.01, 0.1 and 1.0 ppm for 12 h. Points indicate an average of at least 25 readings subsequent to adding substrate. Treatments significantly different from controls are indicated by different letters (Dunn's multiple comparison, $p < 0.05$). Error bars indicate one standard deviation.

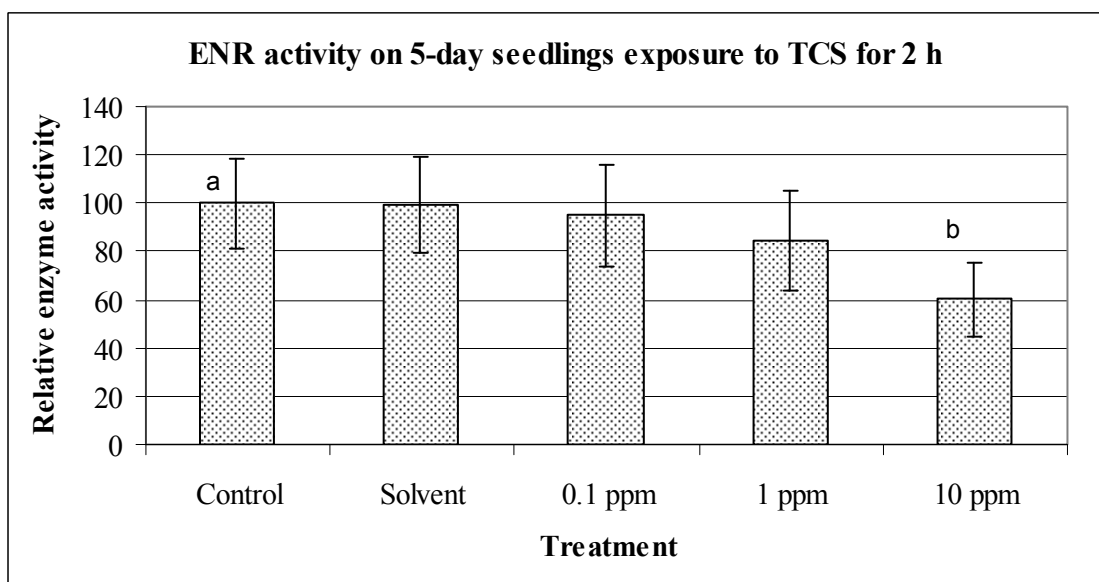


Fig. 6.4. *A. thaliana* 5-day seedlings exposed to no treatment (control) and methanol (solvent) and various concentrations of TCS: 0.1, 1.0, 1.0 and 10.0 ppm for 2 h. Points indicate an average of at least 25 readings subsequent to adding substrate. Treatments significantly different from controls are indicated by different letters (Dunn's multiple comparison, $p < 0.05$). Error bars indicate one standard deviation.

Next, the effect of the 24 h exposures of 5-day seedlings to 10 ppm TCS were compared among the WT and FAAH-altered genotypes (Fig. 6.5). ENR enzyme activity was not significantly affected by DMSO solvent addition but was significantly depressed to levels between 20 and 40% of that of controls in all three genotypes with no apparent differences among genotypes.

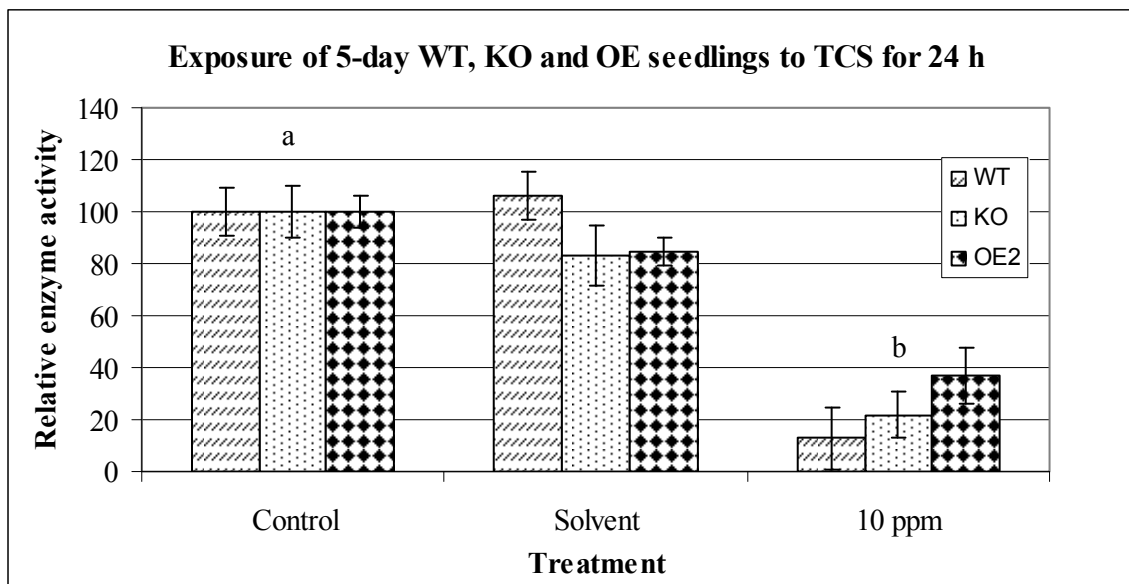


Fig. 6.5. *A. thaliana* WT, KO and OE2 5-day seedlings exposed to no treatment (control) and methanol (solvent) TCS concentration of 10.0 ppm for 24 h. Points indicate an average of 25 readings from three replicates subsequent to adding substrate. Similar lowercase letters identify treatment means that do not significantly differ (Tukey–Kramer $p < 0.05$). Error bars indicate one standard deviation.

6.3.3 Effects of TCS Exposure and Combined Exposure to TCS and flg22 on NAE

Profiles and TCS, M-TCS Accumulation

In a trial experiment, NAE profiles were analyzed for WT samples exposed to solvent (0.07% DMSO + 0.1% Ethanol), TCS (10 ppm), flg22 (1 μ M) and flg22 (1 μ M) + TCS (10 ppm). There were no apparent changes in NAE levels based among these

treatments (results not shown). The experiment was subsequently repeated with three replicates of control, solvent, flg22 and flg22+TCS and once again failed to demonstrate significant differences in NAE levels (Fig. 6.6). Similar experiments conducted with KO and OE2 5-day seedlings treated with control, solvent, flg22 and flg22+TCS also showed no significant treatment effect for any of the NAEs (Figs. 6.7 and 6.8). As expected, overall NAE concentrations were elevated in the KO experiment (Fig. 6.7) and depressed in OE2 experiment (Fig. 6.8) relative to the WT experiment (Fig. 6.6).

Evaluation of TCS accumulation was conducted on extracts prior to their clean-up and analysis for NAE levels. WT samples exposed to TCS at 10 ppm for 24 h had significant accumulation of TCS that was eliminated by the flg22 treatment (Fig. 6.9). Background concentrations of TCS in the control solvent samples were below practical quantitation limits. On addition of TCS alone, the TCS content in the seedlings increased by approximately 1000 fold. The surprising elimination of TCS accumulation in the flg22+TCS treated samples may have been due to the presence of flg22 or its solvent DMSO. Accumulation experiments conducted with KO and OE2 plants exposed to only TCS at 10 ppm resulted in tissue TCS concentrations similar those seen in the WT exposure to TCS (Fig. 6.10).

There was generation of the TCS metabolite MTCS in the 10 ppm exposed samples (Fig. 6.11). Variability of MTCS accumulation was greater than that seen for the percent TCS which precluded detection differences among genotypes.

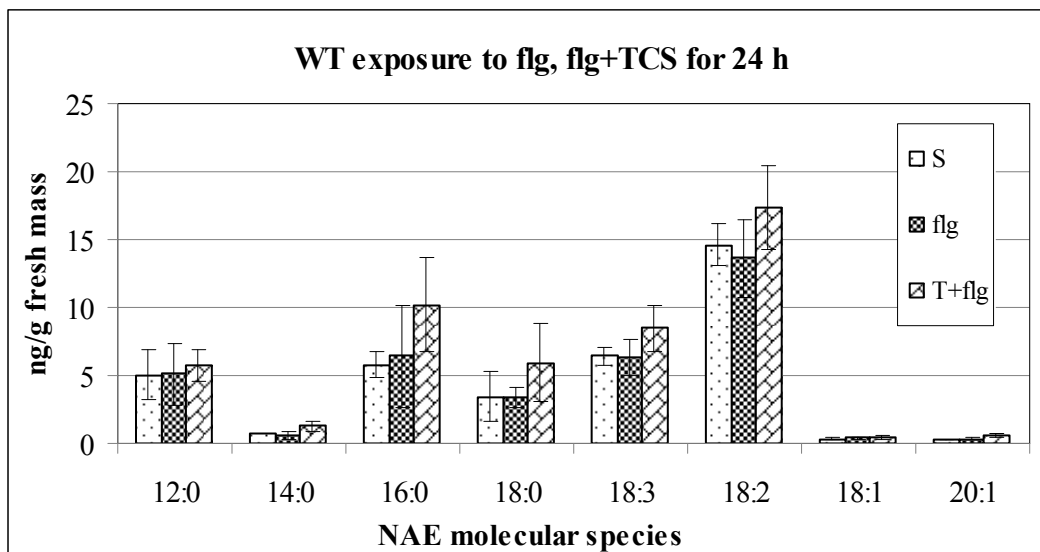


Fig. 6.6. NAE levels on fresh mass basis from WT plants treated with either a) solvent (0.1% ethanol+0.07% DMSO), b) flg22 (1 μ M) or c) flg22 (1 μ M) + TCS (10 ppm) for 24 h. In NAE types where there are no significant differences, they are not labeled. Error bars indicate one standard deviation. $n = 3$ for all treatments.

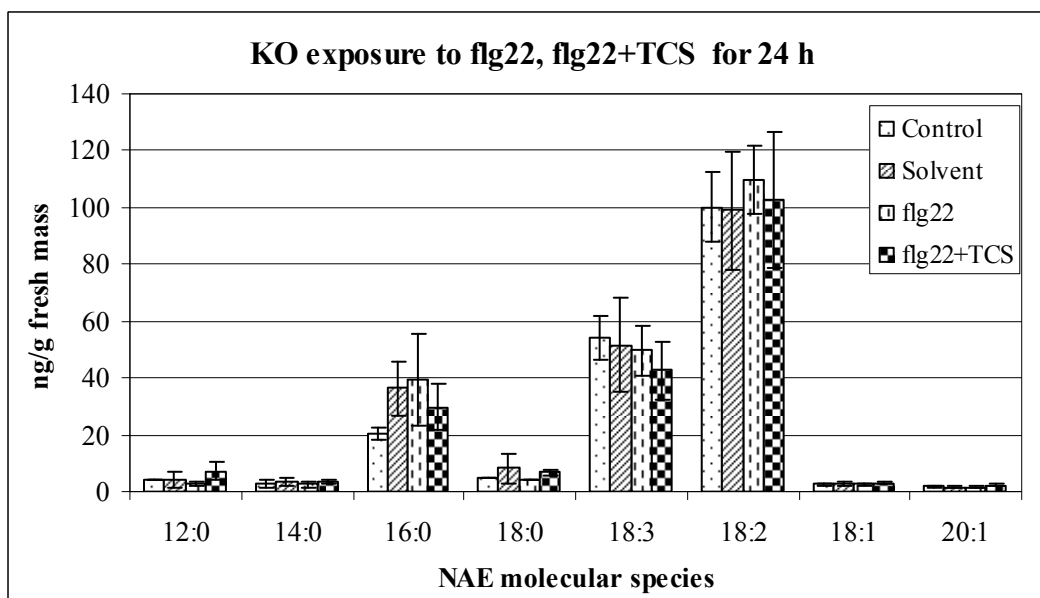


Fig. 6.7. NAE levels on fresh mass basis from KO plants untreated (control) or treated with either a) solvent (0.1% ethanol+0.07% DMSO), b) flg22 (1 μ M) or c) flg22 (1 μ M) + TCS (10 ppm) for 24 h. There are no statistically significant differences in values based on treatment. Error bars indicate one standard deviation. $n = 4$ for all treatments.

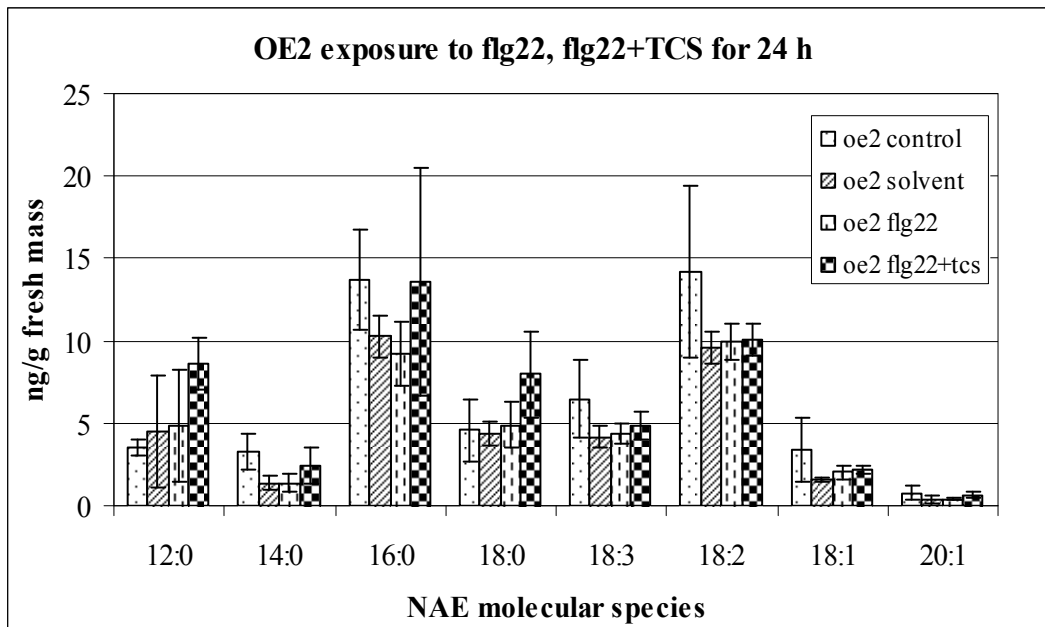


Fig. 6.8. NAE levels on fresh mass basis from OE2 plants untreated (control) or treated with either a) solvent (0.1% ethanol+0.07% DMSO), b) flg22 (1 μ M) or c) flg22 (1 μ M)+ TCS (10 ppm) for 24 h. There are no statistically significant differences in values based on treatment. Error bars indicate one standard deviation. $n = 3$ for control, 5 for other treatments.

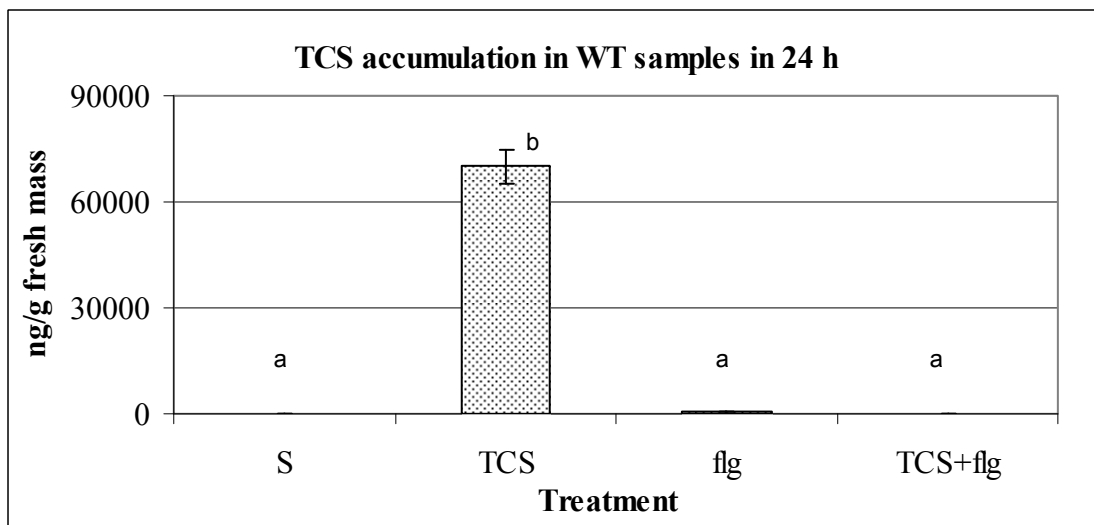


Fig. 6.9. TCS accumulation on fresh mass basis in WT 5-day seedlings treated with either a) solvent (0.1% ethanol+0.07% DMSO), b) TCS 10 ppm c) flg22 (1 μ M) or d) flg22 (1 μ M) + TCS (10 ppm) for 24 h. Similar lowercase letters identify treatment means that do not significantly differ (Tukey–Kramer $p < 0.05$). Error bars indicate one standard deviation. $n = 3$ for all treatments.

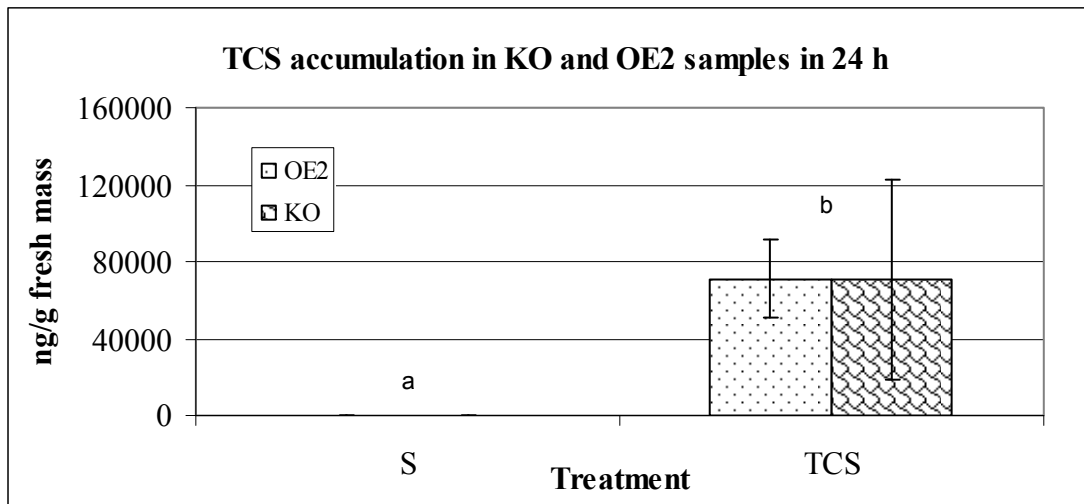


Fig. 6.10. TCS accumulation on fresh mass basis in KO and OE2 5-day seedlings treated with either a) solvent (0.1% ethanol+0.07% DMSO) or b) TCS 10 ppm for 24 h. Similar lowercase letters identify treatment means that do not significantly differ (Tukey–Kramer $p < 0.05$). Error bars indicate one standard deviation. $n = 3$ for all treatments.

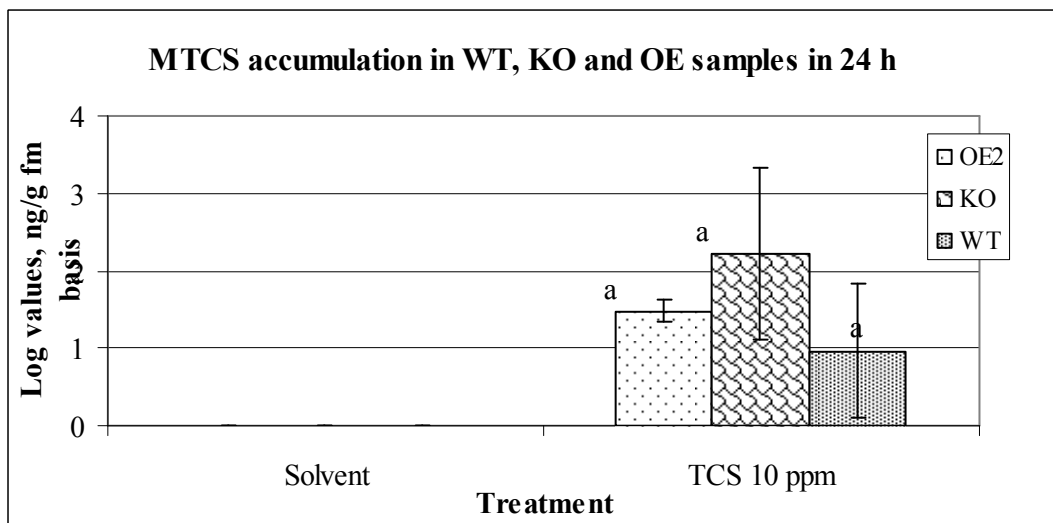


Fig. 6.11. MTCS accumulation on fresh mass basis in KO and OE2 5-day seedlings treated with either a) solvent (0.1% ethanol+0.07% DMSO) or b) TCS 10 ppm for 24 h. Similar lowercase letters identify treatment means that do not significantly differ (Tukey–Kramer $p < 0.05$). Error bars indicate one standard deviation. $n = 3$ for all treatments.

Based on the requirements of Objective 2 the null hypotheses to be tested H_{03a} was that there will be no significant change in NAE levels in seedlings exposed to TCS,

flg22, TCS + flg22 and solvent treatments. My results were consistent with this null hypothesis.

Null hypothesis H_{03b} was that TCS exposure would not alter ENR enzyme activity when compared to controls. My results indicate that this null hypothesis can be rejected. Exposure concentrations of 0.05–10 ppm, with exposure times of 2–24 h, significantly decreased ENR enzyme activity in protein extracts and whole 5-day seedlings.

Null hypothesis H_{03c} was that MTCS exposure would not alter ENR enzyme activity when compared to controls. My results are consistent with this hypothesis, with MTCS concentrations of up to 50 ppm resulting in no change in ENR enzyme activity.

6.3.4 Discussion

As seen in results from Experiment One and Two, there is no significant effect of TCS exposure on NAE levels with and without exposure to flg22. On long term exposure of seedlings to TCS at 1 ppm there was no phenotypic difference. However at 10 ppm the plants did not show much growth (results not shown).

TCS inhibited ENR activity at 10 ppm and there was significant bioaccumulation of TCS at 10 ppm TCS exposure. The bioconcentration factors (ratio of tissue concentration to exposure concentration) in Fig. 6.10 were 7.0 and 0.146 in TCS (10 ppm) and TCS (10 ppm) + flg22 exposed samples. In both KO and OE2 samples it was 7.1. MTCS accumulation relative to TCS levels were 0.01, 0.04 and 1.5 in WT, OE2 and KO seedlings respectively. The decrease TCS accumulation in samples treated to flg22+TCS could be due to a rapid cell death causing inhibition of TCS uptake or changes in availability of TCS, which need further investigation.

CHAPTER 7

ZAT12 SEEDLINGS EXPOSURE TO BACTERIAL ELICITOR AND TCS

Response of Zat12 *A. thaliana* 5-day seedlings exposed to bacterial elicitor flg22, and TCS: Comparison of WT and Zat12 NAE profiles, effects of TCS on ROS production and accumulation of TCS.

7.1 Introduction

Zat12 is a zinc-finger protein that is involved in the signaling of many biotic and abiotic stresses (Rizhsky et al., 2004). Zat12 expression is activated at the transcriptional level during different abiotic stresses, such as oxidative, osmotic, salinity, high light and heat stresses and in response to wound-induced systemic signals. The Zat12 promoter has been fused with a luciferase reporter gene in order to assess the activation of Zat12 transcription (Davletova et al., 2005). Low temperatures, high salinity, drought, application of superoxide inducing methyl viologen (commercial form: paraquat) and wounding all resulted in activation within half an hour as indicated by analysis of luciferase activity. Studies determining the characterization of Zat12 indicated it is required for *Apx1*, H₂O₂ responsive transcript, expression and plant protection during oxidative stress (Rizhsky et al., 2004). With the availability of a Zat12: Luciferase genotype seeds, courtesy of Dr. Mittler, I decided to explore the capability of flg22 to elicit response and the capability of TCS to inhibit response using the reporter system. In the assay, firefly luciferin from the firefly *Photinus pyralis* is used as a substrate for

luciferase enzyme (Davletova et al., 2005). Upon transcription and expression of the Zat12 which is fused with luciferase promoter there is production of oxyluciferin and energy in the form of light. The light emitted can be measured using a luminometer.

7.2 Exposure and Analyses

Intact seedlings were placed in MQ water for at least 4 hours. Subsequently the seedlings were carefully placed in Corning white bottomed 96- well plates. Luciferin was added for a final concentration of 1 mM. Solvent (DMSO, 4 μ l) or flg22 (1 μ M) was added and the samples were allowed to incubate for approximately 20 minutes. Subsequently the luminescence was measured using the Synergy2 plate reader.

Each sample consisted of an individual 5-day seedling resting in MQ water for at least four hours prior to transfer into 96-well white corning well-plate with fresh MQ water. The samples were placed in sets of 8; usually four samples were used as solvent controls and four samples had addition of flg22 at 1 μ M.

If there was addition of TCS to the analysis, then some of the samples were placed in fresh MQ water, and some with MQ water with the requisite TCS concentration. The solvent used for dissolving TCS was a 10% solution DMSO for a high concentration of 1000 ppm. DMSO concentrations in final exposure solutions ranged from 0.001 to 0.1%.

ROS measurement was performed as described in section 2.4. NAE analysis was performed as described in section 3.5

7.3 Results and Discussion

7.3.1 NAE Profiles in Zat12 and WT 5-Day Seedlings

A comparison of NAE levels in Zat12 and WT 5-day seedlings indicated that their general profiles were similar (Figure 7.1). However, Zat12 plants had increases in 14:0, 16:0, 18:1 and 18:0 and an absence of 20:1. This was interpreted as an indication that the modified Zat12 genotype does not greatly alter general ability for normal NAE metabolism.

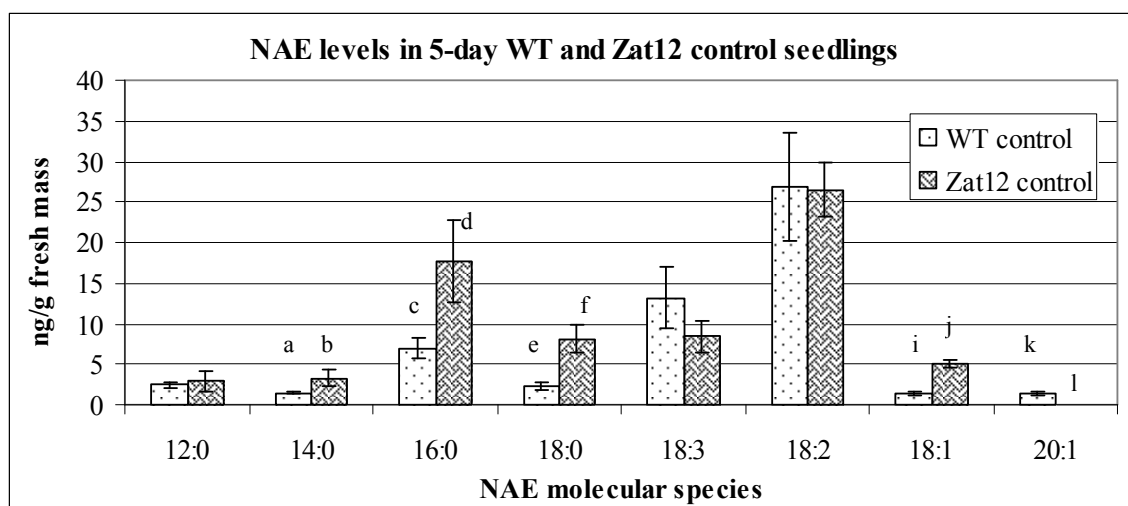


Fig. 7.1. Comparison of NAE levels in 5-day WT and Zat12: Luciferase seedlings on a fresh mass basis. Lowercase letters identify treatment means that are significantly different (Tukey–Kramer $p < 0.05$) for a NAE type. In NAE types where there are no significant differences, they are not labeled. $n = 8$.

7.3.2 ROS Measurement in 5-Day Zat12 Seedlings

The general pattern of response of Zat12 plants treated with flg22 (Fig. 7.2b) was a marked increase in luminescence while solvent-treated samples (Fig. 7.2a) showed a leveling or drop in luminescence. The average fresh mass of these seedlings varies from 0.5 to 1.0 mg which, among other variables can lead to the variation in the luminescence

readings. Figure 7.3 summarizes the percentage changes in RLU in solvent and flg22 (1 μ M) treated samples normalized to solvent control response of 100%. There is significant increase in luminescence at 15 minutes and it continues at 25 minutes subsequent to placing the samples in the plate reader.

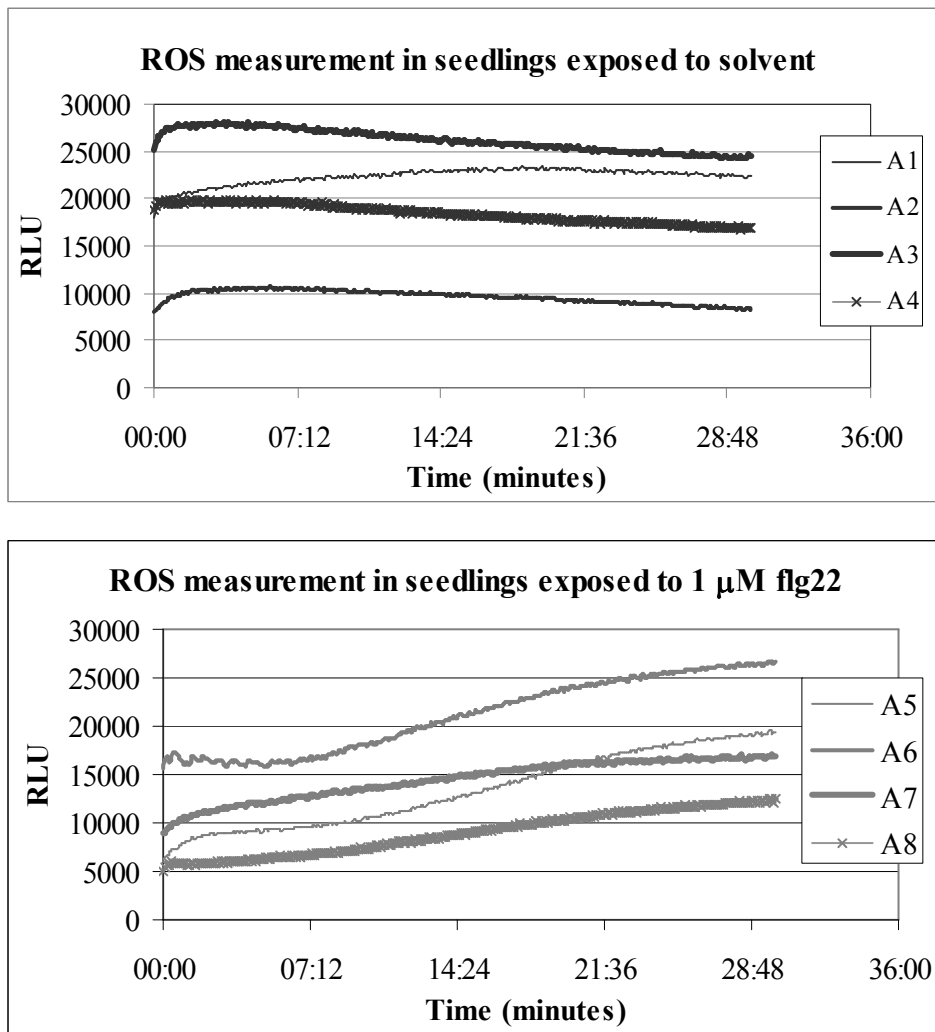


Fig. 7.2 a, b. Luminescence readings from 5-day Zat12 seedlings exposed to a) solvent 0.1% DMSO and b) flg22 1 μ M. Readings were taken after the addition of luciferin and waiting for 20 minutes. Flg22 exposed samples (A5-A8) show an increase in luminescence where as the solvent (control, A1-A4) samples show a leveling of the luminescence readings.

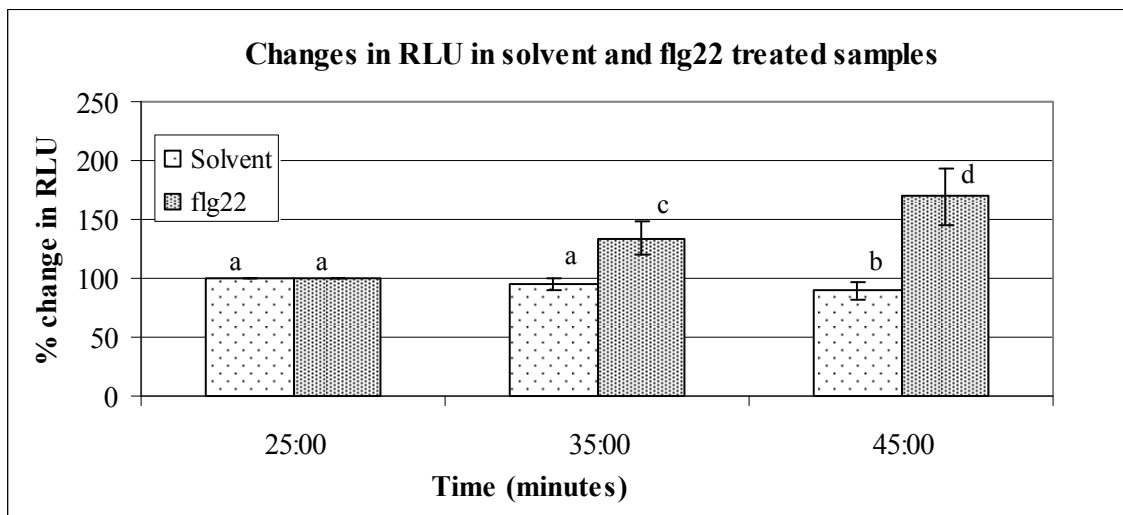


Fig. 7.3. Percentage change in relative luminescence unit readings from 5-day *Zat12* seedlings exposed to a) solvent 0.1% DMSO and b) flg22 1 μ M. Readings at time of 25 minutes were considered as 100% and changes at two other time points of 35 min and 45 min were assessed. Lowercase letters identify treatments/ time points where readings are significantly different (Tukey–Kramer $p < 0.05$) from readings at 5 minutes. Error bars indicate one standard deviation. $n = 8$.

7.3.3 Changes in ROS Measurement on Short-Term Treatment of Seedlings to TCS

Prior to Flg22 Exposure

On a short term exposure to TCS at a high concentration of 10 ppm for 45 minutes, there was a decrease in the overall amount of luminescence produced (Fig. 7.4), due to a possible suppression of *Zat12* expression. The increase in luminescence was also delayed in the TCS exposed samples. However, it is to be noted that the TCS exposed samples do start responding to the flg22 once they are not exposed to TCS for even a short length of time (e.g. increased response at 40 minutes in Fig. 7.4). This recovery was more noticeable in samples which were exposed to 5 ppm TCS than 10 ppm TCS.

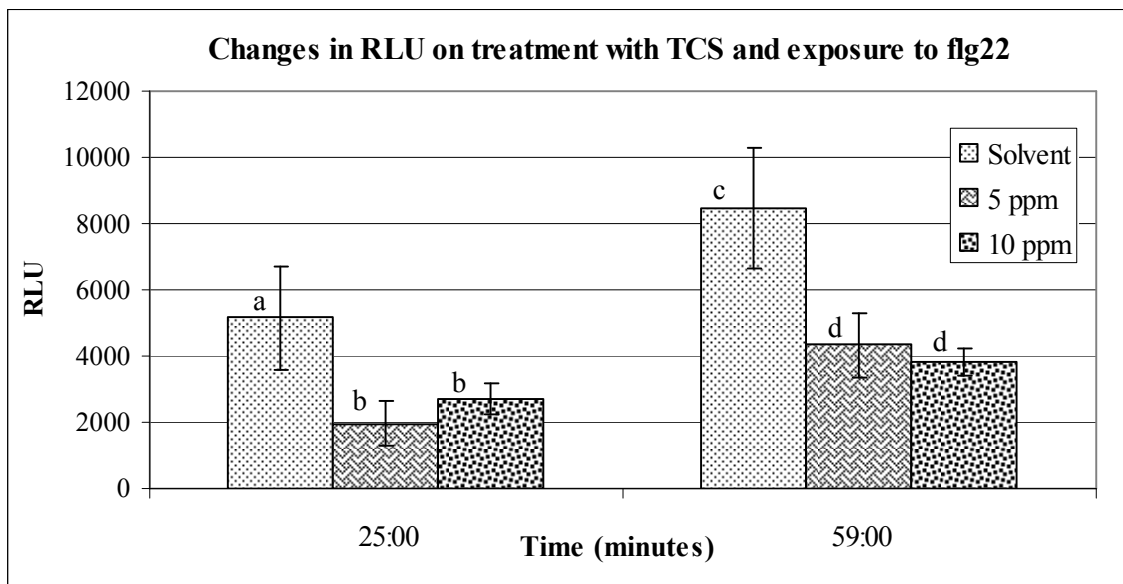


Fig. 7.4. Luminescence readings from 5-day Zat12 seedlings incubated with solvent (0.1% ethanol), 5 or 10 ppm TCS for 45 minutes. Subsequently all seedlings were transferred to fresh MQ water and exposed to 1 μ M flg22. Lowercase letters identify treatments/ time points where readings are significantly different (Tukey–Kramer $p < 0.05$) from solvent readings. Error bars indicate one standard deviation. $n = 6, 3, 3$ respectively.

7.3.4 Changes in ROS Measurement and Accumulation of TCS on Long-Term

Treatment of Seedlings to TCS Prior to Flg22 Exposure

7.3.4.1 TCS Accumulation

The effect of long-term TCS exposure on Zat12 5-day seedling response to treatment with flg22 was investigated by initiating exposure at 0.01, 0.1 and 1.0 ppm TCS at day 0 for germinating seeds. There was accumulation of TCS at all concentrations. All exposures resulted in significant accumulation of TCS in tissues at average concentrations from 0.06 to 6.65 μ g/g fresh mass (Fig. 7.5) representing bioconcentration factors (ratio of tissue concentration to exposure concentration) of 6, 8.2 and 6.6 for exposure concentrations 0.01, 0.1 and 1.0 ppm TCS respectively.

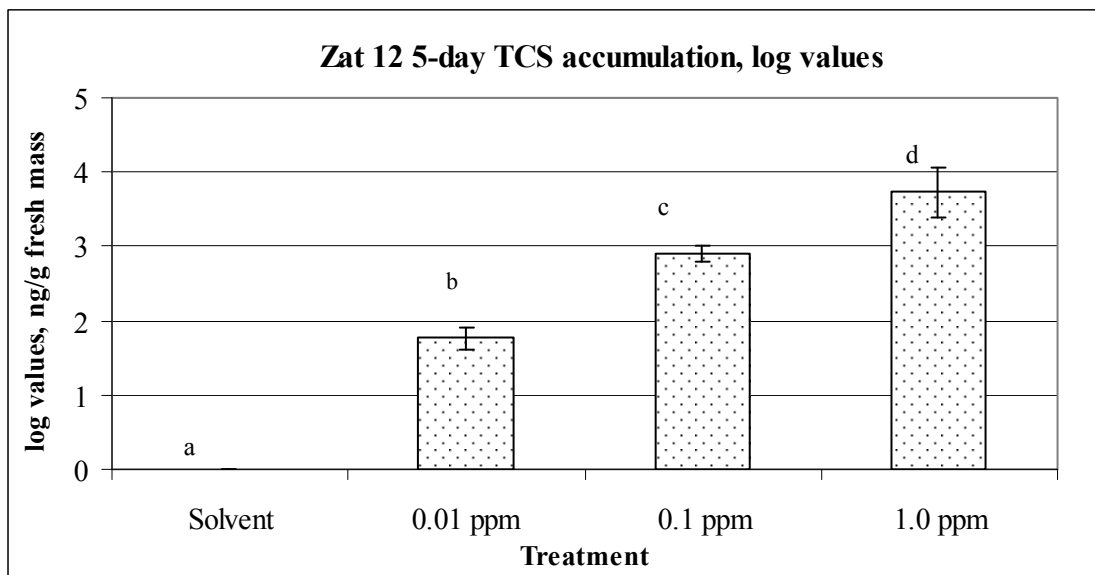


Fig. 7.5. TCS accumulation on long term exposure of Zat12 5-day seedlings to TCS concentrations of 0.01, 0.1 and 1.0ppm. All treatments were added at day 0. Similar lowercase letters identify treatment means that do not significantly differ (Tukey–Kramer $p < 0.05$). Error bars indicate one standard deviation. $n = 3$ for solvent and $n = 4$ for rest of the treatments.

7.3.4.2 ROS Analysis Discussion

Initial long-term exposure of the solvent, 0.01, 0.1 and 1.0 ppm TCS samples to flg22 indicated that there was a significant drop in luminescence responses of the 0.1 and 1.0ppm samples. Fig. 7.6 shows these differences based on dose response.

Fig. 7.7 shows responses of seedlings treated with solvent or 0.01, 0.1 and 1.0 ppm TCS and exposed to flg22 (1 μ M). There is no difference in ROS responses of solvent and 0.01 ppm treated samples. The ROS responses in 0.1 and 1.0 ppm samples were significantly lower. The initial responses in these treated samples did not show any trend based on exposure concentration, indicating no differences in *Zat12* expression as seen in high concentrations of short term TCS exposure.

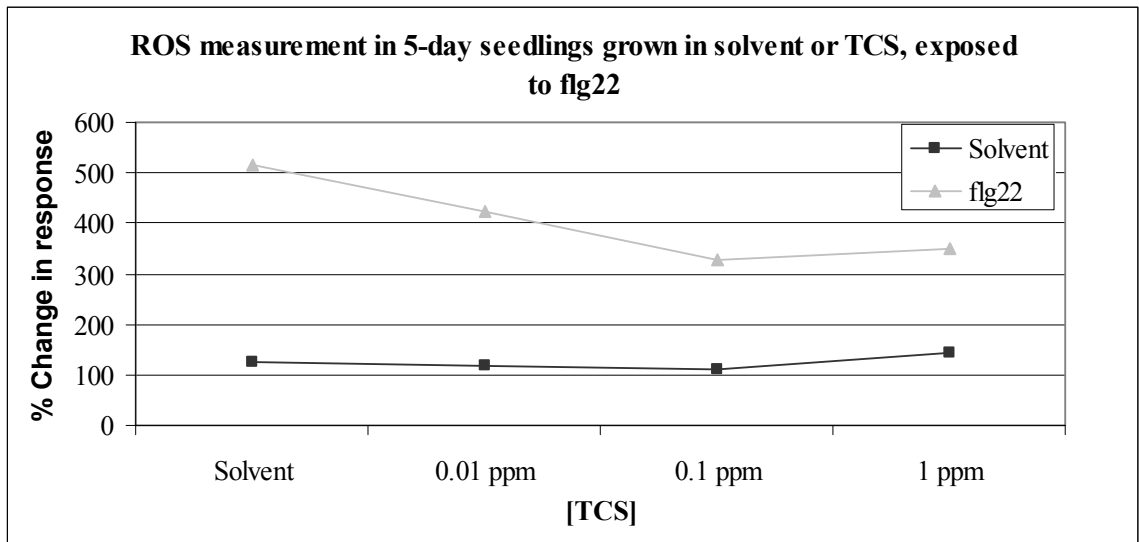


Fig. 7.6. Relative luminescence readings from 5-day Zat12 seedlings exposed to solvent (0.07% DMSO (s) or 1 μ M flg22 (f). The seedlings were incubated with a) solvent 0.1% ethanol (Solvent) or b) 0.01 ppm TCS (0.01) or c) 0.1 ppm TCS (0.1) or d) 1.0 ppm TCS (1) from day 0. Readings indicated are 60 minutes after addition of luciferin. Solvent and 0.01 ppm exposed samples showed higher luminescence readings than 0.1 and 1ppm exposures to flg22. DMSO only exposed samples showed relatively less increase in RLU. $n = 2$.

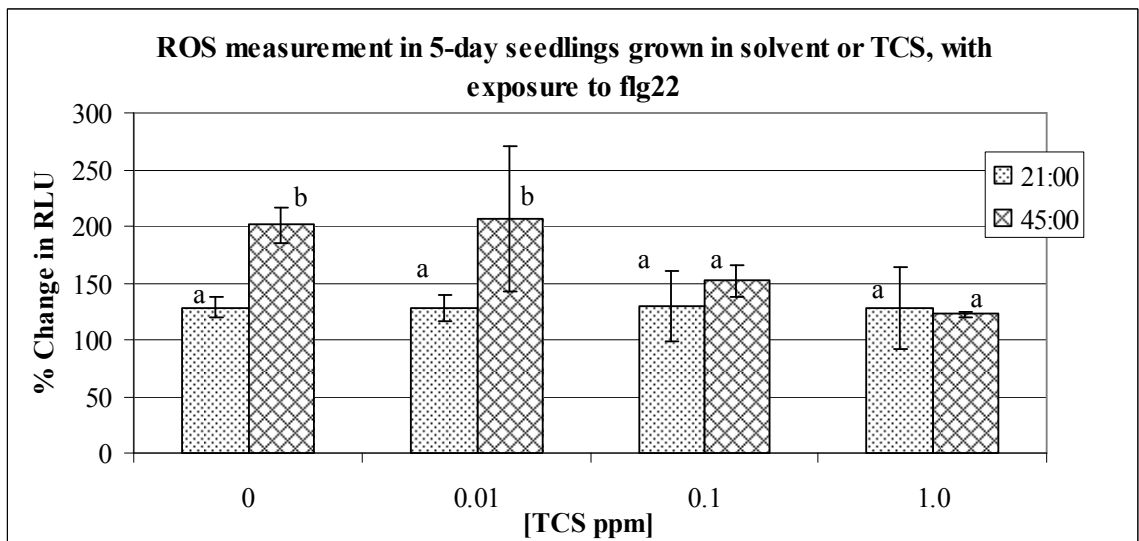


Fig. 7.7. Relative luminescence readings from 5-day Zat12 seedlings exposed to solvent 1 μ M flg22. The seedlings were incubated with a) solvent 0.1% ethanol (s) or b) 0.01ppm TCS (0.01) or c) 0.1 ppm TCS (0.1) or d) 1.0 ppm TCS (1) from day 0. Readings indicated are 21 and 45 minutes after addition of luciferin. Solvent and 0.01ppm exposed samples showed higher luminescence readings than 0.1 and 1ppm exposures. Similar lowercase letters identify treatment means that do not significantly differ (Tukey–Kramer $p < 0.05$). Error bars indicate one standard deviation. $n = 3$.

The null hypothesis H_{04} was that there will be no significant changes in ROS production due to long term exposure to TCS. My data indicate this null hypothesis should be rejected. At concentrations of 0.1 and 1 ppm, TCS depressed ROS production relative to solvent controls.

7.3.5 Discussion

Zat12 seedlings showed similar NAE profiles to WT seedlings and were used for analysis of short-term and long-term treatment effects of TCS on the ROS response to the bacterial elicitor flg22. TCS suppressed basal luminescence levels in the seedlings. Removal of TCS caused the seedlings to recover their response on short-term exposures. On long-term exposure responses were suppressed at even 0.1 ppm TCS. Thus there is a definite relation in the lipid signaling and synthesis and defense responses.

CHAPTER 8

CONCLUSIONS

Plants are the primary producers on Earth. There is a continuing need for increased food production by improving plant yields and decreasing plant susceptibility to disease. Plants have physical barriers, preformed and inducible chemical responses for defense immediately upon being attacked. Defense gene expression and systemic acquired resistance also contribute to defenses effective throughout the plant in the event of the failure of immediate and short-term responses.

Plant lipids, present in physical barriers (cutin), as part of signaling cascades (disruption, modification, in signaling) and as substrates for synthesis of new molecules, play important roles in plant defense. *N*-Acyl ethanolamines (NAEs) are a class of lipids recently recognized as signaling molecules which are controlled, in part, by their degradation by fatty acid amide hydrolase (FAAH). On the basis of increased NAE levels in a tobacco cell suspension-xylanase elicitor exposure system and the availability of *FAAH* mutants, OE and KO genotypes in *A. thaliana*, my research sought to further investigate the role of NAEs in plant defense.

With the increasing urban contamination of sediment and water systems, the role of broadly used antimicrobials in compromising plant defense responses is of increasing concern. TCS has been shown to be a potent suppressor of elicitor-triggered immune responses activated on perception of PAMP molecules such as flg22, and has also been

demonstrated to reduce the activity of a key enzyme involved in lipid synthesis. Thus, the examination of the potential role of TCS in inhibiting lipid signaling mediated defense responses, such as ROS production, via NAE metabolism became a second objective of my research.

The goals and conclusions from my work are summarized below.

8.1 Adult Plant Exposure to Pathogenic Bacteria: NAE Analyses

The goal of this study was to begin to evaluate the role of NAEs in plant defense responses to the non-host pathogens by use of WT and *FAAH* altered *A. thaliana* genotypes. Work conducted in collaboration with Dr. Mysore's lab, at Samuel Roberts Noble Foundation, Ardmore, OK demonstrated that endogenous SA concentrations were at least two-fold lower in *AtFAAH* OE (OE7) when compared to WT (ecotype Columbia) plants. OE7 plants were hyper-susceptible to non-host bacterial pathogens and addition of an SA analogue, benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH) reduced this susceptibility. SA biosynthesis and SA-mediated defense signaling genes were down-regulated in OE7 plants. Transcript profiling also revealed that several other plant defense related genes, including R genes, were less abundant in the OE7 plants when compared to WT plants. Transcripts of some R genes were less abundant in the *AtFAAH* OE7 only after inoculation with a non-host pathogen.

Based on earlier experiments demonstrating a role for NAEs in plant defense response and the altered susceptibility of OE7 to bacterial challenge, I hypothesized that NAEs might play a role in this altered susceptibility. NAE quantification, which was initially assessed 6 and 24 hrs post-inoculation, showed that there was no increase in

NAE levels as was seen with the previous tobacco cell culture and leaf studies. NAEs profiles were also assessed at two earlier time points of 0.5 and 1.5 h post-inoculation. Once again there was no demonstrable effect of pathogen infection on NAE profiles. Thus, no role of NAE signaling associated with defense response of *A. thaliana* to *Pseudomonas syringae* pv. *syringae* was demonstrated.

8.2 Seedlings Exposure to Bacterial Elicitor: NAE and ROS Analyses

The goal of this study was to determine if there were changes in NAE levels in 5-day seedlings on exposure to the bacterial PAMP elicitor flg22 and to validate the efficacy of the elicitor in production of defense response by measuring the ROS generated using a luminol based assay for measuring H₂O₂.

Range finding experiments, using WT 5-day seedlings with the bacterial elicitor, flg22, were conducted at 2 time points (1.5 and 24 h) and 2 concentrations (100 nM and 1 μ M) to determine changes in steady state NAE levels. Optimal conditions determined for exposure were 24 h, with elicitor concentration at 1 μ M. There was no significant difference in NAE levels due to elicitor treatments in WT, KO or OE2 seedlings. Overexpressor genotypes used in the experiments in this work include OE7 and OE2 which show a similar phenotypic characteristics and lower overall NAE levels when compared to WT.

Elicitation of ROS production was verified by exposure of WT 5, 7 and 10-day seedlings to flg22 at an optimal concentration of 1 μ M which resulted in elevated ROS production. However due to the large range of responses obtained, it was not possible to determine if there were genotypic (WT, KO and OE) differences in ROS responses.

Conclusions drawn from these results were that there were no significant changes in NAE levels on exposure to the PAMP-associated bacterial elicitor, whose effectiveness was confirmed by increased ROS production.

8.3 Seedlings Exposure to Bacterial Elicitor and TCS: NAE, TCS, ENR Activity

Analyses

The goal of this study was to determine if the antimicrobial stressor TCS affects the steady state NAE levels on 24 h exposure and changes in levels, if any, when exposed to an elicitor, and if there is any difference in responses based on different genotypes. TCS and its metabolite effects were measured by changes in enoyl-ACP reductase enzyme activity in a concentration dependent manner.

Range finding experiments were conducted using various concentrations of TCS (0.01 to 10 ppm) and exposure times (2 and 12 h) to determine optimal exposure conditions based on the inhibition of ENR enzyme activity. The ENR enzyme activity showed that though the protein extract of seedling samples was sensitive down to 0.05 ppm of TCS, intact WT seedlings ENR activity was inhibited only after exposure at 0.1 and 1 ppm for a 12 h exposure, and only at 10 ppm after a 2 h exposure. All three genotypes showed a similar significant drop in enzyme activity when compared at an exposure concentration of 10 ppm. There was no effect of the TCS metabolite MTCS on ENR activity at the highest concentration tested (50 ppm). Assessment of NAE profiles with exposure to TCS and flg22 + TCS treatments showed no significant differences among all three genotypes. TCS readily bioconcentrated in *A. thaliana* in 24 h time with a 3-order of magnitude increase in tissue concentration over exposure concentration at 10

ppm in all three genotypes. There was also significant accumulation (one order of magnitude) of MTCS in the 10 ppm TCS exposed samples. Long term exposure to TCS at 10 ppm showed a drastic inhibition in seedling growth, indicating suppression in fatty acid biosynthesis (figure not shown).

Conclusions drawn from this experiment were that there were no significant differences in NAE levels with 10 ppm TCS exposure of 24 h despite significant inhibition of ENR enzyme activity. Thus there was no evidence that TCS inhibition of ROS production is mediated by modulation of NAE signaling.

8.4 Zat12 Seedlings Exposure to Bacterial Elicitor and TCS: NAE, ROS and TCS Analyses

The goal of this study was to quantify changes in ROS based on short/long-term exposures to TCS by measuring the expression of *Zat12* gene in 5-day seedlings, using a genotype in which the *Zat12* promoter is fused with the reporter gene luciferase.

With the availability of *Zat12*: luciferase genotype, which showed similar NAE profiles to WT seedlings, experiments were more reproducible with RLU readings relatively higher and more consistent than seen with earlier experiments in which ROS production was evaluated on the basis of production of H₂O₂ in WT *A. thaliana*.

At TCS concentration of 10 ppm there was a drastic drop in ROS. However, on removal of TCS, the production of ROS rapidly recovered. Long term exposure at low concentrations of TCS showed that at 0.01 ppm there was no effect on production of ROS. However higher concentrations of 0.1 and 1.0 ppm showed a decrease in the ROS response to flg22. Long term exposure to TCS caused a decrease in ROS response at low

concentrations of 0.1 ppm. The recovery on removal of TCS indicates that the effect is short-lived and probably of little environmental consequence unless exposure is continuous.

8.5 Summary and Future Work

NAE levels play an important role in plant growth indicated by a rapid decrease in levels at germination, which is affected by exogenous application of NAE 12:0. Earlier studies had indicated that NAEs play a role in plant defense responses, with change in levels within 10 minutes of elicitor exposure in tobacco-xylanase elicitor system. I saw no change in steady state NAE profiles (on individual and total overall levels) in *A. thaliana-pseudomonas syringae* pv. *syringae* and *A.thaliana-flg22* challenge systems despite evidence that defense responses were activated in these systems. The overexpressor plants showed overall lower NAE levels compared to WT and KO showed higher NAE levels compared to WT. There was a significant drop in FASII ENR enzyme activity on exposure of the seedlings to TCS at 10 ppm for 24 h and decrease in ROS production due to flg22 in long term exposure of 0.1 ppm and short term exposure of 5 ppm. However these responses were not accompanied by significant changes in steady state NAE profiles.

Future work would involve the analysis of long term changes in NAE concentration profiles based on longer (>24 h) TCS exposures. Analysis of short-term time points of 5 to 20 minutes for immediate changes in NAE concentration profiles in PAMP challenge. The use of slightly older leaves from seedlings observed to germinate at the same time may also eliminate effects of variations in NAE levels based on age. Further

analysis of uptake of TCS into seedlings in concurrent exposure with pathogen challenge can be done to determine if there is inhibition of uptake by pathogen.

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