METABOLISM AND ACTION OF POLYUNSATURATED N-ACYLETHANOLAMINES

IN Arabidopsis thaliana SEEDLINGS

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The lipoxygenase (LOX) pathway plays an important role in the oxidative metabolism of polyunsaturated N-acylethanolamines (PU-NAEs). The LOX pathway functions in conjugation with hydrolysis by fatty acid amide hydrolase (FAAH) and to produce oxidized NAEs during seed germination and early seedling development. When Arabidopsis seedlings were grown in low micromolar concentrations of lauroylethanolamide (NAE 12:0), growth retardation and elevated endogenous PU-NAE levels were observed due to the competitive inhibition of LOX by NAE 12:0. The elevated levels of endogenous PU-NAEs were more pronounced in genotypes with reduced NAE hydrolase capacity (*faah* knockouts), and less evident with overexpression of *FAAH*. Alterations in PU-NAE metabolism were studied in seedlings of various *lox* and *FAAH* mutants. The partitioning of PU-NAEs into oxylipin metabolites was exaggerated in the presence of exogenous linolenoyethanolamide (NAE18:3) and resulted in bleaching of cotyledons. The bleaching phenotype was restricted to a narrow developmental window (3-to-5 days after sowing), and was attributed to a reversible disruption of thylakoid membranes in chloroplasts. Biochemical and genetic evidence suggested that 9-hydro(peroxy)xy and 13-hydro(peroxy)xy octadecatrienoyethanolamides (9- and 13-NAE-H(P)OT), but not their corresponding hydro(peroxy)xy free fatty acids, induced cotyledon bleaching. The LOX-mediated metabolites of NAE18:3 shared some overlapping effects on seedling development with those of linoleoyethanolamide (NAE18:2) such as a reduction in seedling root growth. On the other hand, NAE18:3 oxylipin metabolites also exhibited distinct effects during seedling development such as
the inhibition of photomorphogenesis. Biochemical and genetic evidence indicated that a LOX-mediated metabolite of NAE18:2, 9-hydro(pero)xy octadecadienoylethanolamide (9-NAE-H(P)OD), acted as a potent negative regulator of seedling root development, and this depended on an intact abscisic acid (ABA) signaling pathway. Synergistic inhibition of root elongation between 9-NAE-H(P)OD and ABA was restricted to a narrow developmental window (3-to-5 d after sowing) of seedling development. Genetic evidence with Arabidopsis mutants in ABA synthesis (aba1, aba2), perception (pyr1, pyl2, pyl4, pyl5, pyl8) and transcriptional regulation (abi3-1) suggested that negative regulation of growth by 9-NAE-H(P)OD likely was mediated through an increase in ABA synthesis, and this was confirmed biochemically. Induction of a secondary dormancy program in Arabidopsis seedlings by environmental stresses also requires an intact ABA signaling cascade, and our study has shown that this regulatory seedling program is dependent, in large part, on NAE oxylipin formation. Together, results presented here indicated that LOX-mediated metabolites of NAE18:3 and NAE18:2 in Arabidopsis represent a newly-discovered group of bioactive metabolites, and their accumulation during the embryo-to-seedling transition of plant development may act to synchronize seedling establishment with environmental cues.
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<tbody>
<tr>
<td>ABA</td>
<td>abscisic acid</td>
</tr>
<tr>
<td>ABI</td>
<td>ABSCISIC ACID INSENSITIVE</td>
</tr>
<tr>
<td>AEA</td>
<td>arachidonoyl ethanolamide</td>
</tr>
<tr>
<td>AOC</td>
<td>allene oxide cyclase</td>
</tr>
<tr>
<td>AOS</td>
<td>allene oxide synthase</td>
</tr>
<tr>
<td>BSTFA</td>
<td>N,O-bis(trimethylsilyl)trifluoroacetamide</td>
</tr>
<tr>
<td>COX</td>
<td>cyclooxygenase</td>
</tr>
<tr>
<td>DES</td>
<td>divinyl ether synthase</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl Sulfoxide</td>
</tr>
<tr>
<td>EAS</td>
<td>epoxy alcohol synthase</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>FAAH</td>
<td>fatty acid amide hydrolase</td>
</tr>
<tr>
<td>FFAs</td>
<td>free fatty acids</td>
</tr>
<tr>
<td>GA</td>
<td>gibberellin</td>
</tr>
<tr>
<td>GC/MS</td>
<td>gas chromatography/Mass spectrometry</td>
</tr>
<tr>
<td>HETE-EA</td>
<td>hydroperoxyeicosatetraenoylethanolamide</td>
</tr>
<tr>
<td>H(P)OD</td>
<td>hydro(peroxy)xy-octadecadienoic acid</td>
</tr>
<tr>
<td>H(P)OT</td>
<td>hydro(peroxy)xy-octadecatrienoic acid</td>
</tr>
<tr>
<td>HPL</td>
<td>hydroperoxide lyase</td>
</tr>
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<td>HPLC</td>
<td>high performance liquid chromatography</td>
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JA  jasmonic acid
LOX  lipoxygenase
NAEs  N-acyl ethanolamines
NAE-H(P)OD  hydro(pero)xy-octadecadienoyl ethanolamide
NAE-H(P)OT  hydro(pero)xy-octadecatrienoyl ethanolamide
NAPE  N-acyl phosphatidylethanolamine
NDGA  nordihydroguaiaretic acid
OPDA  12-oxo phytodienoic acid
PEA  N-palmitoyl ethanolamine
PU  polyunsaturated
PXG  peroxygenase
SA  salicylic acid
SEA  N-stearoyl ethanolamine
SIM  single ion monitoring
TMS  trimethylsilyl
CHAPTER 1

INTRODUCTION

NAE occurrence

*N*-Acylethanolamines (NAEs) are bioactive ethanolamide-conjugated fatty acid derivatives first reported in the 1950s as constituents of soy lecithin and peanut meal (Kuehl et al., 1954). NAEs are ubiquitous throughout the plant kingdom and occur at the highest levels in desiccated seeds (Chapman, 2004; Venables et al., 2005; Kilaru et al., 2007; Blancaflor et al., 2014). The relative abundance of NAE species in seeds generally reflects the acyl groups present in the *N*-acylphosphatidylethanolamine (NAPE) precursor, a minor membrane lipid component of plant and animal cells (Schmid et al., 1996). Different molecular species of NAEs are identified by the chemical nature of the *N*-linked acyl chain, which, in plants, generally can be 12C-18C in length with up to 3 double bonds (Chapman, 2004; Blancaflor et al., 2014) (see also Figure 1.1). In seeds, the majority of NAEs are generally polyunsaturated species - acylethanolamides of linoleic and linolenic acids named *N*-linoleoylethanolamine (NAE 18:2) and *N*-linolenoylethanolamine (NAE 18:3). For instance, NAE 18:2 was shown to be the predominant species in seeds of several cultivated varieties of cotton (approximately 950 ng/g fresh weight) (Chapman et al., 1999). NAE 18:2 also was found to be the most abundant NAE type in leguminous seeds with only few exceptions observed. Selected species of *Medicago* also showed higher level of NAE 18:3 than NAE 18:2 (Venables et al., 2005). In *Arabidopsis thaliana*, the major NAE types that make up the total NAE content in desiccated seeds are the unsaturated 18C NAEs (NAE18:1, NAE18:2, and NAE18:3).
The first NAEs discovered in mammalian tissues were \(N\)-palmitoyl ethanolamine (PEA) and \(N\)-stearoyl ethanolamine (SEA) which are components in brains and peripheral tissues of rats and guinea pigs (Bachur et al., 1965). The accumulation of NAEs and NAPEs in infracted dog heart muscle was also reported later (Epps et al., 1979). The biological activities of NAEs in animals became clearer after the discovery of anandamide as an endogenous ligand for the cannabinoid (CB1) receptor (Devane et al., 1992). Anandamide has been reported to exhibit biological effects similarly to those of tetrahydrocannabinol (THC), a potent bioactive secondary metabolite in marijuana. The effects reported in animal models include antinociception, hypothermia, hypomotility, catalepsy, mood, cognitive, and emotional processes (Smith et al., 1994; Zanettini et al., 2011).

**NAE metabolism**

NAEs are lipid mediators in what is now termed the endocannabinoid signaling pathway of animal systems. Their bioactivity is typically terminated by fatty acid amide hydrolase (FAAH), a member of the amidase superfamily of proteins. NAEs are hydrolyzed by FAAH into their corresponding free fatty acids (FFAs) and ethanolamine (McKinney and Cravatt, 2005). Recent studies with FAAH-deficient mice showed that they contained higher levels of NAE 16:0, NAE 18:1 and NAE 20:4, especially in neuronal tissues, compared to those in wild-type mice (Kilaru et al., 2010), which indicates the important role of FAAH in regulating endogenous NAE levels in mammals. More recently other hydrolases have been identified which hydrolyze NAEs such as the NAE acid amidase (NAAA; (Sun et al., 2005; Wang et al., 2008)) or ceramidase.
(Tsuboi et al., 2005), although the physiological significance of these enzymes in the endogenous termination of NAE signaling is less understood.

In Arabidopsis, a homologue of the mammalian FAAH appears to be capable of modulating NAE levels as well. In desiccated seeds of AtFAAH T-DNA knock out (faah KO; TDNA SALK lines) lines, NAE content was approximately 30% higher than wild type, while AtFAAH overexpressing lines (AtFAAH OE) had substantially lower NAE levels than wild type (Wang et al., 2006; Kilaru et al., 2011). Even though the differences among genotypes were most obvious in desiccated seeds, 8d-old seedling of AtFAAH OE still showed 15% lower total NAE content while KO showed 10% higher total NAE content compared to wild type. Further, faahKO seedlings were hypersensitive to the negative growth effects of exogenous NAE. By contrast, FAAH OE lines were tolerant to exogenous NAE, indicating that indeed FAAH can act in planta to hydrolyze NAEs and inactivate their growth regulating activities (Wang et al., 2006). Still, there was significant depletion of NAEs even in faah KO seedlings during seedling establishment, indicating the need to look for alternative mechanisms or pathways for NAE metabolism.

Polyunsaturated fatty acids in plants typically undergo lipid peroxidation by lipoxygenase (LOX)-mediated pathway, which gives rise to various oxylipins with significant regulatory roles in plant systems (Feussner and Wasternack, 2002). Similar to free fatty acids, polyunsaturated N-acylethanolamines (PU-NAEs) were shown to serve as substrates for the lipoxygenase pathway (Van Der Stelt et al., 2000; Shrestha et al., 2002; Keereetaweep et al., 2010; Kilaru et al., 2011). It is likely that the LOX pathway acts on polyunsaturated NAEs in seedlings and that the resulting metabolites might have inherent effects on seedling physiology.
Studies with imbibed cottonseeds revealed that NAE 18:2 was subjected to both hydrolysis and oxidation during seed imbibition and germination. GC/MS analysis also confirmed that 12-oxo-13-hydroxy-N-(9Z)-octadecanoylthanolamine was produced from NAE 18:2 in isolated microsomal membranes (Shrestha et al., 2002) indicating action by both LOX and allene oxide synthase. These results suggested that these two competing pathways, hydrolysis by FAAH and oxidation by LOX, might cooperate to contribute to the marked decline in total PU-NAE levels during seed germination and post germinative growth. In fact, the decline of PU-NAEs content during seed germination and seedling development in Atfaah KO plants might be explained by LOX-mediated oxidation. However the identification and quantification of endogenous NAE oxylipins has not been firmly established, so while the enzymatic machinery in plants has been demonstrated to be active toward NAE18:2 and NAE18:3 in vitro (Van Der Stelt et al., 2000; Shrestha et al., 2002; Keereetaweep et al., 2010; Kilaru et al., 2011), further work is required to elucidate the oxidation pathway for PU-NAEs in planta and its physiological significance.

In animals, the oxidative products of polyunsaturated NAEs, including eicosanoid ethanolamides, prostaglandins and leukotrienes, were reported to be important signaling compounds that participate in diverse physiological processes (De Petrocellis et al., 2004). Recent studies showed that arachidonoyl ethanolamide (AEA) can be oxidized to prostaglandin ethanolamides (prostamide) by cyclooxygenase-2 (COX-2) (Kozak et al., 2002; Sang and Chen, 2006). The first prostamide discovered was PGE$_2$-ethanolamide (Yu et al., 1997). The pharmacological effects of prostamide F$_{2\alpha}$ and its analogue bimatoprost have been studied extensively. Several studies also focus on antiglaucoma properties of bimatoprost (Noecker et al., 2003; Parrish et al., 2003; Woodward et al., 2004). AEA has also been reported as a
substrate for 12-LOX and 15-LOX in human polymorphonuclear leukocytes and human platelets, generating 12- and 15-hydroperoxyeicosatetraenoyl ethanolamide (12-HETE-EA and 15-HETE-EA), respectively (Edgemond et al., 1998). Oxidative metabolites of AEA generated by 12-LOX were proposed to play roles in pain modulation (Craib et al., 2001). In addition to oxidation by COX-2 and LOXs, AEA also undergoes oxidation by several of human cytochrome P450 enzymes such as CYP3A4, CYP4F2, CYP4X1, resulting in various oxidized lipid species. Some of these metabolites exhibit important physiological roles as evidenced by a potent cannabinoid receptor-2 agonist - the P450-derived epoxide of anandamide (Snider et al., 2010).

**The Lipoxygenase Pathway in Plants**

Lipoxygenase (LOX) is a dioxygenase enzyme widely found in both plants and animal kingdoms. Fatty acids containing a series of cis-double bonds, including essential fatty acids in humans, serve as suitable substrates for the LOX pathway. The formation of oxylipins is catalyzed by lipoxygenase (Feussner and Wasternack, 2002) or α-dioxygenase (Hamberg et al., 2005). Plant LOXs can be classified based on the specificity of position of molecular oxygen introduced into linoleic acid (18:2) or linolenic acid (18:3). The introduction of molecular oxygen on the hydrocarbon backbone can take place at C-9 (9-LOX) or at C-13 (13-LOX) (Schneider et al., 2007). The unique specificities of LOXs give rise to the corresponding hydroperoxide derivatives – 9-hydroperoxy and 13-hydroperoxy derivatives with distinct enantiomeric configurations (S). The hydroperoxy derivatives can be further metabolized by allene oxide synthase (AOS), allene oxide cyclase (AOC), divinyl ether synthase (DES), epoxy alcohol synthase
(EAS), hydroperoxide lyase (HPL), peroxygenase (PXG) and reductase which collectively generate a broad range of various oxylipin species. In addition, polyunsaturated fatty acids can be converted into hydroperoxy- and hydroxy-derivatives by non-enzymatic reactions, but in the case of spontaneous oxidation, there is no stereospecificity, so this results in equal amounts of \( R \) and \( S \) enantiomers (Göbel and Feussner, 2009).

Oxylipins generated from PUFAs have diverse functions in cellular processes (Mosblech et al., 2009). Occurring in much lower levels in plants, PU-NAEs also were reported to be substrates for enzymes in lipoxygenase pathway (Van Der Stelt et al., 2000; Keereetaweep et al., 2010; Kilaru et al., 2011). Oxylipin products of PU-NAEs may potentially serve as mediators in cellular processes during plant growth and development.

**NAE metabolism in seedling establishment and secondary dormancy**

Seed dormancy is a complex process that allows mature seeds to delay germination until the optimal growth conditions are met. This process largely involves the balance between two major phytohormones, abscisic acid (ABA) and gibberellin (GA); while ABA promotes seed dormancy, GA promotes seed germination processes. Mediator molecules that interact with the ABA or GA signaling pathways can be positive or negative regulators which can enhance or suppress the signals, respectively (Finkelstein et al., 2008). In the event that germinating seeds are exposed to unfavorable growth conditions, they may enter secondary dormancy until the conditions allow them to resume growth. The process of secondary dormancy serves as a response and defense mechanisms to unfavorable environmental cues. ABA has been shown to
activate this process within a narrow developmental window after early seedling establishment (Lopez-Molina et al., 2001; Lopez-Molina et al., 2002).

Several lines of evidence suggest that NAEs may promote secondary dormancy via interaction with ABA signaling. First, NAEs occur at the highest levels in desiccated seeds of various plant species (Chapman, 2004; Venables et al., 2005; Kilaru et al., 2007), and their levels markedly decline a few hours after imbibition (Chapman et al., 1999), suggesting that the rapid metabolism of NAEs might be required for dormancy-breaking and subsequently successful germination. Further, the levels of endogenous NAEs and ABA during seed germination and early seedling development drop concurrently (Teaster et al., 2007). In addition, when the level of NAE12:0 was kept artificially elevated after germination, the germinating seeds/seedling can enter secondary dormancy (Teaster et al., 2007). Also, this growth arrest induced by NAE12:0 only occurs within a narrow developmental window (up to 6 days after sowing) and overlaps with reported ABA window of sensitivity (Lopez-Molina et al., 2001).
Figure 1.1. Structures of NAEs identified in various plant species (Adapted Blancaflor et al., 2014).

(a) Saturated NAEs

(b) Unsaturated NAEs
References


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anandamide and other N-acylethanolamines in macrophages. Biochim Biophys Acta 1736, 211-220.


CHAPTER 2

LAUROYLETHANOLAMINE IS A POTENT COMPETITIVE INHIBITOR OF LIPOXYGENASE ACTIVITY*

Abstract

The lipoxygenase (LOX) pathway was proposed to compete with hydrolysis and be partly responsible for the metabolism of polyunsaturated N-acylethanolamines (PU-NAEs). Treatment of Arabidopsis seedlings with lauroylethanolamide (NAE 12:0) resulted in elevated levels of PU-NAE species, and this was most pronounced in plants with reduced NAE hydrolase activity. Enzyme activity assays revealed that NAE 12:0 inhibited LOX-mediated oxidation of PU lipid substrates in a dose-dependent and competitive manner. NAE 12:0 was 10–20 times more potent an inhibitor of LOX activities than lauric acid (FFA 12:0). Furthermore, treatment of intact Arabidopsis seedlings with NAE 12:0 (but not FFA 12:0) substantially blocked the wound-induced formation of jasmonic acid (JA), suggesting that NAE 12:0 may be used in planta to manipulate oxylipin metabolism.

Introduction

N-Acylethanolamines (NAEs) are ethanolamide-conjugated fatty acid derivatives and their metabolism is a central part of the endocannabinoid signaling pathway in mammals (Egertová et al., 2008). As a consequence, NAEs regulate a broad range of physiological

processes in vertebrates including neurotransmission, satiety, immune function, embryo implantation, and apoptosis (Wasilewski and Wojtczak, 2005; Kilaru et al., 2007b; Huang et al., 2009; Pellegrini-Giampietro et al., 2009; Zhang et al., 2009). The biological activities of NAEs are controlled through a balance between their formation and degradation, although the precise machinery and molecular regulation of these processes is only partly understood.

In plants, the functional role(s) of NAE metabolism is (are) only recently being explored (Kilaru et al., 2007b). NAEs occur at their highest levels (ppm levels) in desiccated seeds and these levels decline with the progression of germination and seedling growth suggesting a possible role in seedling establishment (Chapman, 2004). NAE species in plants contain 12–18C acyl groups with the unsaturated 18C NAE types (NAE 18:1, NAE 18:2, NAE 18:3) being the most abundant (Venables et al., 2005; Blancaflor and Chapman, 2006). Polyunsaturated NAE (PU-NAE; e.g., 18:2, 18:3) species comprised more than 75% of the total NAE pool in Arabidopsis seeds, and their proportionate levels were depleted substantially during seedling establishment, in part by a fatty acid amide hydrolase (FAAH; (Shrestha et al., 2002; Shrestha et al., 2003; Wang et al., 2006)). However, more recent studies with faah T-DNA knockout plants (KO) that lacked FAAH activity showed that the PU-NAE content continued to decline with germination and post-germination growth, suggesting the likelihood of an alternative NAE metabolic pathway (Wang et al., 2006). Previously, activity of lipoxygenase (LOX) toward NAE 18:2 and NAE 18:3 substrates was demonstrated in vitro (Van Der Stelt et al., 2000). Further, Shrestha et al. (Shrestha et al., 2002) showed that both hydrolase and LOX pathways were able to metabolize NAEs in cotton seeds, proposing competing pathways that cooperated in the depletion of NAEs during seedling growth.
Recent studies showed that N-lauroylethanolamide (NAE 12:0) has potent growth inhibition properties when applied exogenously at low micromolar concentrations to Arabidopsis thaliana seedlings (Van Der Stelt et al., 2000; Blancaflor et al., 2003; Wang et al., 2006) (see also Figure. 2.1). Several possible mechanisms for this action have been suggested including inhibition of phospholipase D (PLDalpha) (Austin-Brown and Chapman, 2002), interaction with phytohormone signaling pathways (Kang et al., 2008), and modulation of ABI3 transcript levels (Teaster et al., 2007). However, NAEs have multiple targets in animal systems and perturbation of NAE levels is known to affect a broad range of processes in animals (Hansen et al., 2006). It is likely that NAE metabolism interacts with several different targets in plant systems, including inhibition of LOX enzymes.

LOX is a dioxygenase enzyme commonly found in plant and animal kingdoms and catalyzes the oxidation of polyunsaturated free fatty acids (PUFAs) to produce hydroperoxides (Andreou et al., 2009). In plants, LOX isoforms mostly exhibit substrate preferences for peroxidation at either the C9 or C13 position of 18C acyl chain and along with subsequent enzymes in the LOX pathway, generate a variety of oxylipin metabolites. Perhaps most widely studied is the 13-LOX pathway, which is ubiquitous in plants. Peroxidation, reduction and cyclization of 13-hydroperoxide of α-linolenic acid (FFA 18:3) followed by β-oxidation leads to the formation of jasmonic acid (JA). This pathway has been shown to be important in wound responses, defenses against herbivory and some pathogens, and in anther development (Vellosillo et al., 2007; Browse, 2009). Though 9-LOXs are less well characterized, their role in root development has recently been reported (Vellosillo et al., 2007). Further there is mounting evidence for oxidation of esterified or amide linked fatty acids in addition to the conventional
FFA precursors (Van Der Stelt et al., 2000; Kozak et al., 2004; Andreou et al., 2009). Collectively, the wide range of substrate preferences of LOX isoforms and pathway enzymes create the need to consider a broader contextual framework for oxylipin formation, metabolism and function.

Considering the extent of PU-NAE depletion in Arabidopsis seedlings, even in the absence of the majority of the hydrolase activity in faah knockouts, it is possible that LOX may oxidize NAE 18:2 and NAE 18:3 during seedling growth. Here we expand previous observations that LOX enzymes metabolize NAE 18:2 and NAE 18:3, to characterize and compare 9-LOX activity toward these ethanolamides with 13-LOX activity, and further demonstrate a novel, potent inhibition of LOX enzymes in general by a saturated medium-chain NAE (NAE 12:0). This phenomenon provides an explanation for the elevated levels of PU-NAEs in seedlings treated with this compound, especially exacerbated in faah knockouts, and also points to novel uses for this chemical inhibitor at micromolar concentrations to influence LOX metabolite levels, like JA, in planta.

Results

NAE 12:0 inhibits seedling growth and accumulates PU-NAEs

In plants, depletion of NAEs was shown to be important for normal growth and development, which is achieved by FAAH-mediated hydrolysis (saturated and unsaturated) and/or LOX-mediated oxidation [only PU species (Shrestha et al., 2002; Kilaru et al., 2007b)]. At the cellular level, exogenous NAE 12:0 was shown to affect cytoskeletal organization, endomembrane trafficking, cell wall and cell shape formation (Blancaflor et al., 2003; Motes et
al., 2005). At the physiological level, NAE action contributed to modulation of seedling growth, influenced responses of plants to biotic and abiotic stress, and interacted with salicylic acid (SA) and abscisic acid (ABA)-mediated signaling pathways (Wang et al., 2006; Kilaru et al., 2007b; Teaster et al., 2007; Kang et al., 2008). Here, we tested if NAE 12:0 might act as an inhibitor of LOX-mediated oxidation of PU-NAEs and how NAE 12:0 compared with that of NDGA, a potent but non-selective inhibitor of LOX (Blecha et al., 2007).

Arabidopsis seedlings that were grown in horizontally-oriented plates containing medium with either NAE 12:0 or NDGA showed substantial reduction in growth and abnormalities in seedling development (Figure. 2.1A). Quantification of seedling growth by root elongation in plates oriented vertically confirmed the reduction in seedling growth in the presence of NAE 12:0 and NDGA (Figure. 2.1B). The effect of NAE 12:0 (but not NDGA) was overcome by ectopic overexpression of FAAH (OE7, OE11), and was exacerbated by the loss of FAAH function (faah T-DNA knockout, KO), presumably related to the inherent ability of seedlings to catabolize the NAE 12:0 growth inhibitor and endogenous NAEs. In seedlings where growth was inhibited by NAE 12:0 or NDGA, the PU-NAE content in tissues was elevated substantially compared with untreated seedlings (Figure. 2.1C and D). In faah KO seedlings where hydrolysis activity was severely reduced, NAE 12:0 treatment appeared to block the ability to metabolize exogenously provided NAE 12:0 and to deplete endogenous PU-NAE levels. As a consequence, PU-NAE (both NAE 18:2 and NAE 18:3) content in NAE 12:0 treated faah KO seedlings was 10 times higher than that in the untreated seedlings and they remained severely stunted (Figure 2.1A and B), compared with WT and OEs. On the contrary, overexpression of FAAH facilitated the increased capacity for NAE hydrolysis, likely reducing the
NAE 12:0 inhibitor concentration over time as well as some endogenous PU-NAEs. In the case of NDGA, all of the FAAH-altered genotypes were affected similar to WT, in terms of development and at the metabolite level (Figure. 2.1); there was a 2–4-fold increase in NAE 18:2 levels and a 5–8-fold increase in NAE18:3 levels in NDGA-treated seedlings regardless of capacity for NAE hydrolysis (Figure. 2.1C and D). Overexpression of FAAH was unable to reduce these PU-NAE levels to levels in the untreated seedlings, suggesting no specific ability to overcome inhibition by NDGA.

**LOX-mediated oxidation of PU-NAEs**

To address the hypothesis that NAE 12:0 inhibits LOX-mediated oxidation of PU-NAE, we first compared the enzymatic properties of representative 13-LOX (soybean) and 9-LOX (potato) enzymes toward both PU-NAE and FFA substrates to estimate the relative ability of each enzyme to contribute to NAE oxidation. Both enzymes exhibited typical Michaelis–Menten kinetics when initial velocity measurements were made at increasing PU-NAEs/PUFAs concentrations. For the 13-LOX, the Km values were similar for NAE 18:2 and FFA 18:2 but were lower for NAE 18:3 (2-fold) when compared with FFA 18:3 (Table 2.1). The apparent maximum rates of reaction for 13-LOX was approximately 2-fold higher for FFA substrates, when compared with NAE substrates (Table 2.1). These results were generally consistent with those reported by Van der Stelt et al. (Van Der Stelt et al., 2000) where the utilization of NAE 18:2 and NAE18:3 by soybean 13-LOX was characterized; however, these earlier studies did not examine whether a 9-LOX type enzyme activity was capable of utilizing NAE substrates or not. Here we showed that indeed 9-LOX from potato was able to oxidize PU-NAEs, albeit at a lower maximal
rate than for corresponding FFAs (Table 2.1). Perhaps most notable was the affinity of 9-LOX for NAE 18:3, which was considerably higher than that for FFA 18:3, but this difference was not seen for the NAE 18:2 and FFA 18:2.

**NAE 12:0 inhibits LOX activity**

Following comparative kinetic studies, we tested the effect of including either NAE 12:0 (lauroylethanolamide) or FFA 12:0 in enzyme reactions to evaluate the influence of these non-substrate lipids on LOX activities toward their NAE and FFA substrates. Double-reciprocal plots of saturation kinetic measurements made with increasing concentrations of NAE 12:0 or FFA 12:0 showed that both types of lipids inhibited both 13- and 9-LOX activities in a competitive and concentration-dependent manner (Figures. 2.2 and 2.3). Inhibition characteristics were summarized in Table 2.2 In general, LOX enzymes had much lower estimated inhibition constants (Ki) for NAE 12:0 than for FFA 12:0, indicating that NAE 12:0 is a more potent inhibitor of LOX in vitro than FFA 12:0. This was evident for both 13- and 9-LOX activities toward either NAE or FFA substrates. NAE 12:0 was approximately 10–20 times more potent than FFA 12:0 in all cases, with inhibition in the low micromolar to submicromolar concentration range for NAE 12:0. The potent inhibition of 13- and 9-LOX activities by NAE 12:0 was consistent with our hypothesis that NAE 12:0 treatment of seedlings interferes with LOX-mediated oxidation of PU-NAEs in vivo.

To examine directly the influence of NAE 12:0 and FFA 12:0 on Arabidopsis (At)LOX activities toward PU-NAE and PU-FFA substrates, we used crude protein extracts from 4-day old Arabidopsis seedlings. Seedling homogenates likely possess both 13- and 9-LOX activities since
multiple LOX isoforms are expressed at these stages (per publicly available expression data).

Indeed inhibition of At-LOX activities by NAE 12:0 was observed in seedling homogenates (Figure. 2.4), but at these concentrations there was little or no inhibition by FFA 12:0. In general, the inhibition of At-LOX activities by NAE 12:0 in extracts of faahKO (Figure. 2.4A) was more pronounced than that in WT (Figure. 2.4B) likely due to a greater capacity for NAE hydrolysis by FAAH in WT seedlings allowing for some depletion of inhibitor from the reaction. Also, it is likely that the higher levels of lipid hydroperoxide detected in reactions of WT homogenates with PU-NAE substrates (compared to that for faahKO) were due to a combination of hydrolysis of PU-NAEs into PU-FFAs and subsequent oxidation by At-LOX activities.

The broad-based and potent inhibition of LOX activities by NAE 12:0 suggested that this compound might be generally used to influence oxylipin metabolism in vivo and perhaps even manipulate JA levels in plants. To address this possibility, we tested whether NAE 12:0 treatment of Arabidopsis seedlings could mitigate the characteristic wound-inducible formation of JA. When 8- day old seedlings were treated with NAE 12:0 (35 µM) for 24 h prior to the mechanical wounding, JA production 3 h after wounding was substantially lower than that without NAE treatment (Table 2.3). Inhibition was most effective with pre-incubation of tissues in NAE 12:0, and was only modestly affected by incubation with NAE 12:0 following wounding (post-incubation). By comparison, the same concentrations of FFA12:0 were ineffective in modulating JA levels, whether added pre- or post-wounding. Further, a time course of JA formation after wounding showed that NAE 12:0 treatment effectively reduced JA levels throughout the peak of accumulation (30–60 min; Figure. 2.5).
Discussion

*N*-Acylethanolamines (NAEs) are bioactive fatty-acid derivative whose levels rapidly decline during seed germination and seedling establishment (Shrestha et al., 2002; Kilaru et al., 2007b). Our results suggested that LOX activity toward other PUFAs is important for normal seedling growth and/or that the PU-NAE oxylipin metabolites may be required for normal seedling establishment. In any case, there was a clear elevation of PU-NAE species in NDGA-treated species and most dramatically in NAE 12:0-treated *faah* KO seedlings, consistent with our hypothesis that NAE 12:0 is a potent inhibitor of LOX activity. Moreover, these results support the concept of competing hydrolysis and oxidation pathways in the cooperative depletion of PU-NAEs during normal seedling establishment (Shrestha et al., 2002; Kilaru et al., 2007b). The results from *in vitro* studies using NAE12:0 as an inhibitor of LOX-mediated oxidation of PU-NAEs also suggested that FA ethanolamides could serve as plausible substrates for either 9- or 13-type LOXs in planta, although specific LOX isoforms may need to be examined in the future for additional enzymological or regulatory differences. The results from in vivo studies where seedlings were treated with NAE12:0 prior to mechanical wounding, NAE12:0 effectively mitigated wound-induced JA formation. Together, these results raise the possibility that NAE 12:0 and/or derivatives thereof might be used to manipulate plant processes that are dependent upon JA accumulation. In fact, NAE 12:0 was shown to delay lipid peroxidation, activity of LOX and superoxide anion production in carnations, thus improving the vase life of fresh-cut flowers (Zhang et al., 2007). Given the multitude of important processes in plants that depend upon accumulation of JA, lauroylethanolamide may find widespread commercial applications.
Caution should always be exercised when applying inhibitors to affect biological processes since non-target effects of compounds may complicate interpretation of results. Nonetheless, these studies provide a new, potent class of inhibitors for plant LOXs — the short/medium chain acylethanolamides. Moreover they provide further evidence for the cooperative metabolism of NAEs in plant seedlings and support the concept that PU-NAEs may be converted into ethanolamide oxylipins in planta. Future studies will be aimed at identification of new NAE-oxylipins and examination of their physiological relevance.

Methods

Chemicals

N-Linoleylethanolamide (NAE 18:2), N-linolenylethanolamide (NAE 18:3), linoleic acid (FFA 18:2), FFA 18:3, 9-lipoxygenase (potato), 13-lipoxygenase (soybean) and lauric acid (FFA 12:0) were purchased from Cayman Chemicals (Michigan). Nordihydroguaiaretic acid (NDGA) was purchased from Fluka. NAE 12:0 was synthesized from lauroylchloride and ethanolamine and purified by organic extraction as described previously (Austin-Brown and Chapman, 2002). Deuterated JA (D5-JA) was purchased from CDN Isotopes (Canada).

Plant materials

Arabidopsis seeds were surface-sterilized and then stratified for 3 days at 4°C in the dark for all experiments prior to sowing in liquid or solid MS medium (Teaster et al., 2007). Germination and growth was maintained in controlled conditions with 16-h-light/8-h-dark cycle (60 µmol/m²
at 20 to 22°C. Seedlings grown for four days in liquid medium were used for LOX activity assays. Seedlings grown for eight days in liquid medium in the presence of 35 µM NAE 12:0, 25 µM NDGA or DMSO (0.1% final) were used for PU-NAE quantification. Growth of the seedlings was monitored on solid nutrient media.

**Extraction and quantification of PU-NAEs**

Total lipids were extracted from 50 mg of seedlings with 2-propanol/chloroform/water (2/1/0.45 [v/v/v]) and PU-NAEs were separated by normal phase HPLC (Econosphere™ silica, 5 lm, 4.6 x 250 mm), as described previously (Venables et al., 2005). Both NAE 18:2 and NAE 18:3 were quantified against deuterated NAE 20:4 internal standard by GC–MS as their corresponding TMS ethers (derivatized by BSTFA; Sigma–Aldrich).

**LOX activity and inhibition assays**

Enzyme assays for kinetic characterization of 13-LOX and 9-LOX were conducted in 0.1 M borate (pH 9.0) and 0.1 M Tris–HCl (pH 7.2) buffers, respectively, with substrate concentrations ranging from 10 µM to 300 µM. Commercially available soybean and potato LOX enzymes (Cayman Chemicals) were chosen to represent a 13-LOX and 9-LOX activity, respectively. Continual changes in absorbance at 234 nm (Spectronic Genesys 5; Fisher Scientific) due to the formation of conjugated double bond system in hydroperoxides from PU-NAEs or FFAs (ε = 25,000 M⁻¹ cm⁻¹) were recorded. Absorbance changes at each substrate concentration without enzyme were subtracted to account for non-enzymatic oxidation. Auto-oxidation estimated in this way amounted to 0.4–2.4% of the enzyme-dependent oxidation for all lipid substrates. Rates of reaction (µmol of product h⁻¹ mg protein⁻¹) were determined by saturation kinetics.
(Michaelis–Menten) or by double reciprocal plots using Graphpad (Prism) software. For verification of reaction stereochemistry, enzyme-generated lipid oxidation products were distinguished from auto-oxidative products by chiral phase-HPLC (CHIRALCEL™ OD-H, 2.1 x 150 mm, Diacet Chemical Industries, Ltd, Osaka, Japan). For example, in the formation of N-linolenoyl(13S-hydroxy)ethanolamine from NAE18:3 catalyzed by soybean 13-LOX, the products contained 97.9% of the S enantiomer and 2.1% of the R enantiomer when the stereoisomers were separated by chiral-phase HPLC. Apparent $K_m$, $K_i$ and $V_{max}$ values were determined from the means of three replicates. For inhibition studies, inhibitors were pre-incubated with enzyme in a 1 ml cuvette for 10 min prior to substrate addition. Inhibition constants ($K_i$) were calculated from saturation-based enzyme assays at varying concentrations of either FFA12:0 or NAE 12:0. Arabidopsis (At)LOX enzyme activities were assayed in crude protein extracts of four-day old faahKO and wild type (WT) Arabidopsis seedlings grown in liquid media. Homogenates were prepared by grinding flash-frozen tissues in a mortar in 100 mM Kphosphate, pH 7.2, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM MgCl$_2$, 0.2 mM dodecylmaltoside, and 400 mM sucrose. Crude homogenates were clarified by centrifugation at 650xg in a Sorvall SS-34 rotor (4 °C) and used directly for enzyme assays. Protein concentration was determined against a bovine serum albumin standard curve (Bradford Protein Assay; BIO-RAD). Inhibitors (or solvent-only control) were pre-incubated with enzyme extracts in a 1 ml final volume for 10 min prior to substrate addition. Substrate-dependent At-LOX activity was determined by measuring production of hydroperoxides from appropriate polyunsaturated lipid substrates indirectly at 500 nm (Lipid Hydroperoxide Assay Kit; Cayman Chemicals). Assays with boiled enzyme extract were used to correct for spontaneous oxidation.
Acknowledgements

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Table 2. Kinetic parameters of 13- and 9-LOX with different substrates.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>( V_{\text{max}} ) (µmol/h/mg protein)</th>
<th>( K_m ) (µM)</th>
<th>( V_{\text{max}} ) (( K_m ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>13-LOX</td>
<td>NAE 18:2</td>
<td>311</td>
<td>27</td>
<td>11.7</td>
</tr>
<tr>
<td></td>
<td>NAE 18:3</td>
<td>312</td>
<td>15</td>
<td>20.8</td>
</tr>
<tr>
<td></td>
<td>FFA 18:2</td>
<td>619</td>
<td>21</td>
<td>29.6</td>
</tr>
<tr>
<td></td>
<td>FFA 18:3</td>
<td>712</td>
<td>32</td>
<td>22.4</td>
</tr>
<tr>
<td>9-LOX</td>
<td>NAE 18:2</td>
<td>27</td>
<td>27</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>NAE 18:3</td>
<td>11</td>
<td>3</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>FFA 18:2</td>
<td>62</td>
<td>14</td>
<td>4.4</td>
</tr>
<tr>
<td></td>
<td>FFA 18:3</td>
<td>81</td>
<td>30</td>
<td>2.7</td>
</tr>
</tbody>
</table>
Table 2. Inhibition constants ($K_i$) (µM) of NAE 12:0 and FFA 12:0 for 13- and 9-LOX on different substrates.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Inhibitor</th>
<th>Inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NAE 12:0</td>
<td>FFA 12:0</td>
</tr>
<tr>
<td>13-LOX</td>
<td>NAE 18:2</td>
<td>0.6 ± 0.1</td>
<td>8.4 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>NAE 18:3</td>
<td>0.7 ± 0.1</td>
<td>14.6 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>FFA 18:2</td>
<td>0.5 ± 0.1</td>
<td>12.1 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>FFA 18:3</td>
<td>0.9 ± 0.1</td>
<td>15.0 ± 1.5</td>
</tr>
<tr>
<td>9-LOX</td>
<td>NAE 18:2</td>
<td>1.2 ± 0.1</td>
<td>14.3 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>NAE 18:3</td>
<td>0.9 ± 0.1</td>
<td>9.5 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>FFA 18:2</td>
<td>0.9 ± 0.1</td>
<td>12.3 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>FFA 18:3</td>
<td>1.3 ± 0.2</td>
<td>11.9 ± 1.8</td>
</tr>
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</table>
Table 2. NAE 12:0, but not FFA 12:0, inhibits wound-induced JA synthesis \textit{in vivo}.

<table>
<thead>
<tr>
<th>Pre-incubation 24 h before wounding</th>
<th>Post-incubation 3 h after wounding</th>
<th>JA (ng/g FW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no wounding)</td>
<td>Media only</td>
<td>43 ± 4.4</td>
</tr>
<tr>
<td>Media only</td>
<td>Media only</td>
<td>597 ± 57</td>
</tr>
<tr>
<td>35 µM NAE 12:0</td>
<td>35 µM NAE 12:0</td>
<td>219 ± 14</td>
</tr>
<tr>
<td>35 µM NAE 12:0</td>
<td>Media only</td>
<td>230 ± 17</td>
</tr>
<tr>
<td>Media only</td>
<td>35 µM NAE 12:0</td>
<td>518 ± 32</td>
</tr>
<tr>
<td>35 µM FFA 12:0</td>
<td>35 µM FFA 12:0</td>
<td>625 ± 64</td>
</tr>
<tr>
<td>35 µM FFA 12:0</td>
<td>Media only</td>
<td>572 ± 71</td>
</tr>
<tr>
<td>Media only</td>
<td>35 µM FFA 12:0</td>
<td>659 ± 89</td>
</tr>
</tbody>
</table>
Figure 2. NAE 12:0 and NDGA increased the polyunsaturated NAE content.

Arabidopsis seedlings grown on solid media oriented horizontally containing either NAE 12:0 (35 µM) or NDGA (25 µM) showed significant retardation of seedling development when compared with DMSO solvent control (0.1%) (A). faah knockouts (KO) were hypersensitive to
NAE 12:0, whereas FAAH overexpressing lines, OE-7 and OE-11, were tolerant to NAE 12:0 compared with wild type (WT) or vector-only controls (VC). Quantification of primary root length of 8-d old seedlings in plates oriented vertically emphasized the marked inhibition of root elongation by NAE and NDGA (B). NAE quantification (C and D) indicated that NAE 12:0 and NDGA increased the polyunsaturated NAE content. In faah knockouts (KO), which were hypersensitive to NAE 12:0 (compared to WT or FAAH OE), levels of NAE 18:2 and NAE 18:3 were substantially higher than those of untreated or treated FAAH OE and WT seedlings. Seedlings with altered NAE metabolism showed similar growth responses on NDGA (A and B) compared with WT and their endogenous PU-NAE levels were similar to each other but higher than the untreated seedlings (C and D). Root lengths are the average and standard deviation of 30–32 seedlings, and results are representative of multiple experimental trials. PU-NAE quantitative data are the means ± S.D. of three biological replicates.
Figure 2.2. FFA 12:0 inhibited both 13- and 9-LOX activities in a competitive and concentration-dependent manner.

Different concentrations of FFA 12:0 were incubated with 9- or 13-LOX enzymes to test inhibition of peroxidation of PU-FFA (18:2 and 18:3) and PU-NAE (NAE 18:2 and NAE 18:3) substrates. Double-reciprocal plots of enzyme parameters (1/V and 1/[S]) were made using Graphpad Prism software. Competitive inhibition was evident and apparent $K_i$ values were determined from the means of three replicates over four inhibitor concentrations; values are summarized in Table 2.2.
Figure 2. NAE 12:0 inhibited both 13- and 9-LOX activities in a competitive and concentration-dependent manner.

Different concentrations of NAE 12:0 were incubated with 9- or 13-LOX enzymes to test inhibition of peroxidation of PU-FFA (18:2 and 18:3) and PU-NAE (NAE 18:2 and NAE 18:3) substrates. Double-reciprocal plots of enzyme parameters (1/V and 1/[S]) were made using Graphpad Prism software. Competitive inhibition was evident and apparent $K_i$ values were determined from the means of three replicates over four inhibitor concentrations; values are summarized in Table 2.2.
Figure 2.4. NAE 12:0 inhibited At-LOX activities in seedling homogenates.

NAE 12:0 (10 µM), FFA 12:0 (10 µM) or DMSO (0.2%) were incubated with homogenates of four-day old Arabidopsis seedlings of faah knockouts (A) or wild type (B) to test for inhibition of lipid hydroperoxide formation (hydroperoxide assay kit; Cayman Chemicals) from PU-FFA (18:2 and 18:3) and PU-NAE (NAE 18:2 and NAE 18:3) substrates. Assays each included 0.2 mg total protein and 100 µM substrate in 0.1 M Tris–HCl, pH 7.2. Reactions were pre-incubated for 10 min with inhibitor before adding substrate, and a further 10 min to assess oxidation. Pilot reactions were conducted to ensure conditions were saturating. Boiled enzyme controls were used to subtract for minimal non-enzymatic oxidation. Quantitative data are the means ± S.D. of three biological replicates.
Figure 2.5. NAE 12:0 treatment inhibited wound-induced JA formation.

Eight-day old Arabidopsis seedlings grown in liquid media were treated with either 35 µM NAE 12:0, 35 µM FFA 12:0 or DMSO equivalent (0.1%) for 24 h prior to mechanical wounding. Jasmonic acid levels were quantified against a D5-JA standard by GC–MS as methyl ester at 30 min, 1 h and 3 h after wounding. Inhibition by NAE 12:0 was evident at all time points compared with FFA 12:0 treatment or control group. Quantitative data are the means ± S.D. of three biological replicates.
References


CHAPTER 3

ETHANOLAMIDE OXYLIPINS OF LINOLENIC ACID NEGATIVELY REGULATE ARABIDOPSIS SEEDLING DEVELOPMENT*

Abstract

N-Acylethanolamines (NAEs) are fatty acid derivatives with potent biological activities in a wide range of eukaryotic organisms. Polyunsaturated NAEs are among the most abundant NAE types in seeds of Arabidopsis thaliana and they can be metabolized by either fatty acid amide hydrolase (FAAH) or by lipoxygenase (LOX) to low levels during seedling establishment. Here we identify and quantify endogenous oxylipin metabolites of N-linolenoylethanolamine (NAE 18:3) in Arabidopsis seedlings, and show that their levels were higher in faah knockout seedlings. Quantification of oxylipin metabolites in lox mutants demonstrated altered partitioning of NAE 18:3 into 9- or 13-LOX pathways, and this was especially exaggerated when exogenous NAE was added to seedlings. When maintained at micromolar concentrations, NAE 18:3 specifically induced cotyledon bleaching of light-grown seedlings within a restricted stage of development. Comprehensive oxylipin profiling together with genetic and pharmacological interference with LOX activity suggested that both 9-hydroxy and 13-hydroxy linolenoylethanolamides, but not corresponding free fatty acid metabolites, contributed to the reversible disruption of thylakoid membranes in chloroplasts of seedling cotyledons. We

suggest that NAE oxylipins of linolenic acid represent a newly-identified, endogenous set of bioactive compounds which may act in opposition to progression of normal seedling development, and must be depleted for successful establishment.

Introduction

*N*-Acylethanolamines (NAEs) are bioactive ethanolamide-conjugated fatty acid derivatives first reported in the 1950s as constituents of soy lecithin and peanut meal (Kuehl et al., 1954). NAEs are ubiquitous throughout the plant kingdom and occur at the highest levels in desiccated seeds (Chapman, 2004; Venables et al., 2005). The depletion of NAEs to very low levels is associated with normal seedling establishment, and this NAE regulatory pathway was shown to interact with ABA signaling through a key regulator of the embryo-to-seedling transition—ABSCISIC ACID INSENSITIVE 3 (ABI3)—and induce a “secondary dormancy” (Teaster et al., 2007; Kim et al., 2010). The relative abundance of NAE species in seeds generally reflects the acyl groups present in the *N*-acylphosphatidylethanolamine (NAPE) precursor, a minor membrane lipid component of plant and animal cells (Schmid et al., 1996). Different molecular species of NAEs are identified by the chemical nature of the *N*-linked acyl chain, which, in plants, generally is 12C-18C in length with up to 3 double bonds (Chapman, 2004). In most seeds, the majority of NAEs are polyunsaturated species - acylethanolamides of linoleic and α-linolenic acids named *N*-linoleoylethanolamine (NAE 18:2) and *N*-linolenoylethanolamine (NAE 18:3). NAE 18:2 was shown to be the predominant species in seeds of several cultivated varieties of cotton (approximately 950 ng/g fresh weight) (Chapman et al., 1999). NAE 18:2 also
was found to be the most abundant NAE type in leguminous seeds with only few exceptions observed. Selected species of *Medicago* also showed higher levels of NAE 18:3 than NAE 18:2 (Venables et al., 2005). In *Arabidopsis thaliana*, the major NAE types that make up the total NAE content in desiccated seeds are the unsaturated 18C NAEs (NAE 18:1, NAE 18:2, and NAE 18:3) (Wang et al., 2006).

In animal systems, NAEs are widely recognized for their role as lipid mediators in the endocannabinoid signaling pathway (Bachur et al., 1965; Howlett et al., 2004). Their bioactivity is typically terminated by fatty acid amide hydrolase (FAAH), a member of the amidase superfamily of proteins. NAEs are hydrolyzed by FAAH into their corresponding free fatty acids (FFAs) and ethanolamine (McKinney and Cravatt, 2005). In Arabidopsis, a homologue of the mammalian FAAH was identified (Shrestha et al., 2003), which we designated At-FAAH1, and it appears to partially regulate NAE levels as well. In desiccated seeds of At-FAAH1 T-DNA knockout (*faah1*) lines, NAE content is approximately 30% higher than wild type (Columbia ecotype-0, wild type), while At-FAAH1 overexpressing lines (*FAAH1 OE*) have substantially lower NAE levels than wild-type (Wang et al., 2006; Kilaru et al., 2011). Even though the differences among genotypes are most obvious in desiccated seeds, 8-d-old seedlings of *FAAH1 OE* still show ~15% lower total NAE content while *faah1* shows 10% higher total NAE content compared to wild type. Further, *faah1* seedlings are hypersensitive to the negative growth effects of exogenous NAE. By contrast, *FAAH1 OE* lines are tolerant of exogenous NAE, indicating that indeed FAAH can act in planta to hydrolyze NAEs and inactivate their growth regulating activities (Wang et al., 2006). Still, there is significant depletion of NAEs even in *faah1* seedlings
during seedling establishment, indicating that alternative mechanisms or pathways for NAE metabolism operate in plants.

Polyunsaturated fatty acids in plants typically undergo lipid peroxidation by the lipoxygenase (LOX)-mediated pathway (Brash, 1999; Feussner and Wasternack, 2002; Liavonchanka and Feussner, 2006; Mosblech et al., 2009; Schaller and Stintzi, 2009), which gives rise to various oxylipins with significant and diverse regulatory roles in plant systems (Vellosillo et al., 2007; Wasternack, 2007; Browse, 2009; Wasternack and Hause, 2013). Occurring in much lower levels in plants, polyunsaturated NAEs also were reported to be substrates for enzymes in the lipoxygenase pathway in vitro (Van Der Stelt et al., 2000; Shrestha et al., 2002; Keereetawee et al., 2010; Kilaru et al., 2011), suggesting that oxylipin products of polyunsaturated NAEs may potentially serve as mediators in cellular processes during plant growth and development.

It is likely that the LOX pathway acts on polyunsaturated NAEs in seedlings. Studies with imbibed cottonseeds revealed that NAE 18:2 is subjected to both hydrolysis and oxidation during seed imbibition and germination. GC/MS analysis also confirmed that 12-oxo-13-hydroxy-N-(9Z)-octadecanoyl ethanolamine is produced from NAE 18:2 in isolated microsomal membranes (Shrestha et al., 2002) indicating action by both 13-LOX and allene oxide synthase. These results suggested that these two competing pathways, hydrolysis by FAAH and oxidation by LOX, might cooperate to contribute to the marked decline in total polyunsaturated NAE levels during seed germination and post germinative growth. In fact, the decline of polyunsaturated NAE content during seedling development in faah1 knockout plants might be partially explained by LOX-mediated oxidation. Still, the identification and quantification of
endogenous NAE oxylipins has been difficult due to their low abundances, but it is emerging nevertheless. Kilaru et al. (2011) showed that endogenous NAE-oxylipins derived from NAE 18:2 are detected in 4-d-old Arabidopsis faah1 seedlings, but not in wild type, presumably due to higher flux through the oxidation pathway in the absence of FAAH1 expression. Identification of endogenous NAE 18:3 oxylipin metabolites remained elusive due to the lower levels of endogenous NAE 18:3 compared to NAE 18:2. On the other hand, feeding experiments with exogenous NAE 18:2 and NAE 18:3, populated LOX-derived metabolite pools to readily detectable levels. The levels of these metabolites are highest in faah1 and lowest in FAAH1 OE, confirming that the hydrolysis pathway competes with the oxidation pathway (Kilaru et al., 2011). So while seedlings have the capacity for LOX-mediated metabolism of NAE 18:2 and NAE 18:3 in vitro and in vivo (Van Der Stelt et al., 2000; Shrestha et al., 2002; Keereetawee et al., 2010; Kilaru et al., 2011), further work is required to indicate that these LOX-mediated pathway metabolites occur, especially for linolenoylethanolamides.

Here we detect and quantify endogenous NAE 18:3-oxylipins in Arabidopsis seedlings by single ion monitoring (SIM) gas chromatography/mass spectrometry (GC/MS). Through comprehensive metabolite profiling of NAE 18:3-oxylipins and 18:3 free fatty acid oxylipins in seedlings of many genotypes including faah and lox mutant lines, we were able to demonstrate partitioning of NAE 18:3 into hydrolysis, 9-LOX and 13-LOX pathways, yielding a host of ethanolamide oxylipin metabolites. These metabolite pools were particularly exaggerated in seedlings by “feeding” micromolar levels of NAE 18:3, where we noted a bleaching of cotyledons within a narrow developmental window—most pronounced at 4-to-7 days after sowing. Metabolite profiling studies with mutants as well as application of synthetic exogenous
lipids suggested that 9- or 13-hydroxides of NAE 18:3 were responsible for the specific bleaching effects on cotyledons. We propose that ethanolamide oxylipins of NAE 18:3 represent a previously uncharacterized class of bioactive metabolites in plants, and that their potential participation in the negative regulation of seedling development, specifically chloroplast biogenesis in cotyledons, represents an important new area for further investigation.

Results

Identification of LOX-mediated products by GC-MS

Although previous work had demonstrated the capacity for plant tissues to form oxylipins from exogenously applied NAE 18:3, there has been no clear evidence to date for the endogenous occurrence of LOX-derived metabolites of NAE 18:3. Here, the identity of endogenous NAE-oxylipins was confirmed by GC/MS as their TMS-derivatives by comparing their fragmentation patterns with those of synthesized standards (Kilaru et al., 2011). The molecular ions [M+] for TMS-derivatives of NAE-OPDA and 9/13NAE HOTs were m/z 407 and m/z 481, respectively. The characteristic ions used to distinguish between 9- and 13- hydroxide products were m/z 360 and m/z 412, respectively. A diagnostic fragment ion for all ethanolamide compounds is m/z 116. Additional diagnostic ions for NAE-HOTs were m/z 466, m/z 376 and m/z 391. MS characteristics of these metabolites are summarized in Figure 3.1 and Table 3.1. Identification of oxylipins in extracts was initially performed by comparing spectra to
those for standards (Figure 3.2), and later quantified using SIM mode to detect diagnostic ions for improved analytical sensitivity.

Quantification of endogenous NAE- and FFA-oxylipins in Arabidopsis seedlings

Hydroxides (as well as reduced hydroperoxides) of NAE 18:3 were present in low but detectable quantities (nmol/g fresh weight) in seedlings of Arabidopsis, and their levels did not change considerably over the time course of 5 to 11 days after sowing (Figure 3.3). These time points were selected for comparison based on phenotypic changes toward exogenous NAEs (described below). Both 9- and 13-hydroxides were identified and quantified in seedlings, indicating the activity of both 9- and 13-LOX enzymes toward endogenous NAE 18:3. A cyclized 12-oxo phytodienoic acid ethanolamide (NAE OPDA) was identified and quantified, indicating that endogenous Arabidopsis allene oxide synthase (AOS) and allene oxide cyclase (AOC) metabolized in vivo the ethanolamides of 13-hydroperoxy trienoic acid. These NAE 18:3 oxylipin metabolites were detectable mostly in seedlings, and were less abundant or below detectable limits in other Arabidopsis stages/parts (Table 3.2), suggesting that seedling development is most likely the stage at which these ethanolamide oxylipins are relevant physiologically.

Different genotypes were used to estimate the endogenous partitioning of NAE 18:3 through the FAAH- and LOX-mediated pathways. Clear increases in all LOX-derived metabolites were detected in seedlings with compromised FAAH activity (Figure 3.3). T-DNA insertional mutants of faah1 (Wang et al., 2006) and a double mutant with a second, suspected and related amidase candidate, faah2 (Kilaru et al., 2007b), both showed significantly higher levels
of NAE 18:3-derived oxylipins at all time points in seedlings. By contrast, seedlings over-expressing FAAH (FAAH1 OE) showed the lowest quantifiable amounts of all NAE 18:3-derived oxylipins. These results are consistent with previous speculation that the amidase and oxidase pathways compete for endogenous polyunsaturated NAEs and demonstrate that this competition can be manipulated in vivo by altering FAAH activity (see also below).

Arabidopsis LIPOXYGENASE 1 (LOX1) and LIPOXYGENASE 5 (LOX5) are 9-LOX enzymes (Kilaru et al., 2011). A double mutant lox1 lox5 still showed some formation of 9-hydroxides (Figure 3.3A). However, in these 9-LOX mutants, there was a small but significant increase (p< 0.05) in the endogenous amounts of 13-LOX derived NAE13-HOT (Figure 3.3B) relative to wild-type seedlings at all time points, suggesting that there may be some competition between 9- and 13-LOX enzymes for NAE 18:3 in situ. The corresponding 13-LOX mutant, lipooxygenase 2 (lox2), showed less obvious differences in endogenous LOX-derived metabolites. The lox2 seedlings had reduced NAE13-HOT relative to wild-type seedlings at only two time points (days 7 and 9), suggesting that other 13-LOX enzymes may be capable of acting on NAE18:3 in vivo (Chauvin et al., 2013). Clearer evidence for competition between LOX pathways came from experiments to increase oxylipin pool sizes (below).

_Ethanolamide oxylipins in seedlings with exogenous linolenoylethanolamide (NAE18:3)_

The low endogenous amounts of NAE 18:3-derived oxylipins made quantitative comparisons somewhat difficult among the different genotypes. Hence, pools of these polyunsaturated ethanolamide oxylipin metabolites were elevated more than 200-fold by exposing 4-d old seedlings to micromolar levels of exogenous NAE 18:3 for 3 days (Figure 3.4).
Levels of these metabolites were reduced after exogenous NAE 18:3 was removed from seedlings, indicating that Arabidopsis seedlings have the capacity to degrade these metabolites in vivo. Trends exhibited for endogenous metabolite profiles in different mutants were exaggerated by supplying additional NAE 18:3. Loss of FAAH expression (faah1 single and faah1 faah2 double mutants) consistently led to the highest levels of all NAE oxylipins, whereas FAAH OE showed little accumulation of any LOX-derived products (Figure 3.4), confirming that altering FAAH activity can manipulate the entry into the oxidation pathway. Moreover, competition between 9- and 13-LOX pathways was demonstrated. Mutants with compromised 9-LOX activity (lox1 lox5) showed reduced accumulation of 9NAE HOT relative to wild-type (Figure 3.4A) and markedly elevated levels of 13-LOX derived ethanolamide metabolites (Figure 3.4 B, C). On the other hand, lox2 mutants with compromised 13-LOX activity showed little accumulation of 13-LOX-derived ethanolamide oxylipins, but much higher proportions of 9NAE HOT. In fact, amounts of NAE 13-HOT and NAE-OPDA in lox2 mutants were similar to or less than levels in FAAH OE. Collectively, results with these mutants suggest a three-way competition in Arabidopsis seedlings for NAE 18:3—among FAAH, 13-LOX and 9-LOX enzymes. In addition, these results confirm that the formation of ethanolamide oxylipins from NAE 18:3 is mediated by LOXs as opposed to other oxidative enzymes.

**Free fatty acid oxylipins in Arabidopsis seedlings**

The relationship between the ethanolamide- and corresponding FFA oxylipin metabolites was investigated. The endogenous levels of FFA oxylipins (9-HOT, 13-HOT, and OPDA) were many times higher than those for ethanolamide oxylipins (Figure 3.5, compare to
Figure 3.3). Unlike the ethanolamide oxylipins, there was little difference in steady-state levels of these FFA oxylipins in seedlings regardless of genotype (Figure 3.5), and these levels generally were consistent with other reports of free oxylipins in Arabidopsis seedlings (Przybyla et al., 2008; Lopez et al., 2011; Kim et al., 2012). In sharp contrast to steady state levels, there were marked differences in these FFA oxylipins among the mutants when supplied with exogenous NAE 18:3 (Figure 3.6). Four-d-old seedlings were supplied with NAE 18:3 for 3 days and then removed to fresh media without NAE. Mutants disrupted in FAAH expression (faah1 and faah1 faah2) showed the lowest levels of FFA oxylipins derived from NAE 18:3, whereas FAAH OE (with the highest capacity to release FFA 18:3 from NAE 18:3) showed markedly high levels of all FFA oxylipins (Figure 3.6). Even more dramatic were the differences in FFA oxylipins in the different lox mutants. The lox1 lox5 mutants showed much lower levels of 9-HOT and higher proportions of 13-HOT and OPDA. By contrast, lox2 mutants showed higher levels of 9-HOT and markedly lower levels of 13-HOT and OPDA. These results indicate that the application of NAE 18:3 to seedlings can influence the FFA oxylipin metabolite pools in a predictable manner based on loss (faah and lox) or gain (FAAH1 OE) of function in metabolic mutants. It is possible that there is some hydrolysis of the ethanolamide oxylipins (after action of LOX enzymes) that would contribute to the free fatty acid oxylipin pools, but at present this has not been resolved.

Seedling phenotypes in exogenous linolenoyl ethanolamide (NAE 18:3)

Addition of NAE 18:3 induced a dramatic phenotype in wild-type Arabidopsis seedlings (Figure 3.7). Seedling cotyledons bleached in 3 days following addition of NAE 18:3 in wild type,
faah mutants and lox mutants, but not in FAAH OE lines (Figure 3.7A). This visible phenotype was quantified at the biochemical level as a reduction in extractable chlorophyll a (Chl a) and chlorophyll b (Chl b) (Figure 3.7B), compared to seedlings without NAE 18:3 (Figure 3.7C). The lack of bleaching in FAAH1 OE seedlings indicated that the bleaching was unlikely to be due to an FFA metabolite, since these seedlings had the highest capacity for NAE hydrolysis. Instead it could be that the NAE 18:3 itself or perhaps a LOX-derived metabolite may be responsible for triggering cotyledon bleaching. In addition to bleaching of cotyledons, NAE 18:3, like other NAEs (e.g., NAE 12:0) reduced primary root elongation in Arabidopsis seedlings in a dose-dependent manner (Figure 3.8) (Blancaflor et al., 2003; Teaster et al., 2007).

Effects of NAE 18:3 on seedling development were stage specific (Figure 3.9). Sensitivity to NAE 18:3 was observed up to about six days after sowing. However, only seedlings treated with NAE 18:3 on day 4 showed severe bleaching of cotyledons. The bleaching appeared to be specific for cotyledonary tissues, since emerging true leaves of seedlings were green in the presence of NAE 18:3 (Figure 3.10), indicating a strict organ/stage specific sensitivity toward this acylethanolamide.

**NAE 18:3-specific effects on cotyledon chloroplasts**

Bleaching of cotyledons also was visualized at the cellular level as a reduction in chlorophyll autofluorescence (Figure 3.11). These effects of chlorophyll degradation were specific for NAE 18:3 (Figure 3.11B). Neither NAE 18:2 nor FFA 18:3 added to 4-d-old seedlings caused this reduction in mesophyll chlorophyll autofluorescence of cotyledons after 3 days exposure (Figure 3.11C, D). Here, bleaching of cotyledons was not observed in FFA 18:3 as
might be expected from studies by others of mutants impaired in fatty acid turnover (\textit{pxa1} and \textit{kat2} ; (Kunz et al., 2009), indicating that a functional beta oxidation pathway likely accommodated the treatment of these seedlings with FFA. This also suggests that the mechanism for bleaching of leaves in older plants reported by Kunz and coworkers (Kunz et al., 2009) - due to accumulation of free fatty acids-- was likely not responsible for the NAE 18:3-specific bleaching observed here in cotyledons of seedlings.

Seedling bleaching by NAE 18:3 was reversible (Figure 3.12). When seedlings were transferred to growth medium without NAE 18:3, cotyledons re-greened, and chlorophyll content and autofluorescence were restored over a period of 4 days (Figure 3.12). This indicates that NAE 18:3 did not induce a toxic, irreversible effect. Even \textit{faah1} knockout seedlings, which showed a more rapid and severe response to NAE 18:3 relative to wild-type, were able to recover at the cellular and whole seedling levels when NAE 18:3 was withdrawn (Figure 3.12). Consistent with the decline in autofluorescence, the morphology of chloroplasts in NAE 18:3-treated cotyledons (observed by transmission electron microscopy) showed disruption of thylakoid membrane organization and accumulation of visible aggregated materials in the chloroplast stroma (Figure 3.13). These subcellular defects were reversed over a 4 d period after withdrawal of NAE 18:3, such that normal chloroplast ultrastructure was observed in cotyledons after re-greening.

\textit{Seedling phenotypes are due to a LOX-derived ethanolamide oxylinp}

We tested whether the effects on cotyledon bleaching were attributable to NAE 18:3 itself or a LOX-mediated metabolite by taking advantage of a recently identified inhibitory
effect of saturated NAEs on LOX activity. Saturated NAEs were shown to inhibit LOX activity (9- and 13-LOX) in a potent, competitive manner (Keereetawee et al., 2010). Inhibition was highest for NAE 12:0, which was substantially more potent than FFA 12:0. We reasoned that seedlings incubated with NAE 12:0 in addition to NAE 18:3 would show reduced bleaching if the action was via a LOX-derived metabolite. Alternatively, if the bleaching were due to NAE 18:3 itself, then the addition of NAE 12:0 would not influence bleaching. Figure 3.14 shows that NAE 12:0 and to a lesser degree NAE 16:0, but not FFA 12:0, were able to prevent the bleaching induced by NAE 18:3. Further studies with NAE 12:0 showed that the inhibition of bleaching was dependent on the amount of NAE 12:0 added (Figure 3.15), and that bleaching was reduced significantly even by adding 10 µM NAE 12:0, a concentration that has minimal effects on seedling growth (Blancaflor et al., 2003). However, higher concentrations of NAE 12:0 were not as effective at reversing inhibition of root elongation by NAE 18:3, because NAE 12:0 itself inhibited root elongation (Figure 3.15C). Quantification of ethanolamide- and FFA oxylipins confirmed the inhibition of LOX in vivo by NAE 12:0 under these conditions (Figure 3.16). There indeed was a dose-dependent reduction in NAE 18:3 LOX-derived metabolites in the presence of NAE 12:0, confirming that cotyledon bleaching was associated with a LOX-derived product of NAE 18:3 in situ.

Ethanolamide oxylipins and FFA oxylipins were synthesized or purchased and added to seedlings to test which species were most effective at inducing bleaching (Figure 3.17). Either 9- or 13-ethanolamide hydro(pero)xides, but not the corresponding FFA oxylipins, was most effective in bleaching cotyledons (quantified as reduction in extractable chlorophylls). There was an additive effect between 9- and 13-ethanolamide hydro(pero)xide derivatives. Due to the
spontaneous reduction of the peroxides to hydroxides, there was an unavoidable combination of both hydroxides and peroxides together in the peroxide samples, so completely reduced hydroxides were tested for comparison. NAE 18:3 itself was more potent than the oxylipin metabolites when added to seedlings, but this is likely due to its greater accessibility (it is less polar than the oxylipins) to internal plant tissues.

*Endogenous ethanolamide oxylipins were elevated during ABA-mediated growth arrest*

Since much higher levels of ethanolamide oxylipins than were measured *in vivo* (under normal seedling establishment) were required to observe seedling growth arrest, we asked if there were conditions in which seedling growth could be arrested where a substantial increase in ethanolamide oxylipins could be observed. Chua and co-workers (Lopez-Molina et al., 2001; 2002) identified an ABA-dependent, developmentally-sensitive, so-called “secondary dormancy” stage in which seedling growth can be arrested in Arabidopsis. The stage of sensitivity (within the first few days after germination) for this secondary dormancy overlapped with the timing of sensitivity of Arabidopsis seedlings to NAE 18:3 (Figure 3.9), and we hypothesized that ABA-treated seedlings would show both growth arrest and associated elevation in endogenous concentrations of NAE 18:3-derived oxylipins. Indeed, application of ABA to seedlings at 3 d after sowing showed a dose-dependent and profound increase in endogenous 9- and 13- NAE-HOT levels measured after 4 additional days of exposure (Figure 3.18). This was accompanied by reductions in growth such that at the highest ABA levels tested, the arrested embryos look somewhat similar in morphology to those treated with NAE 18:3, except cotyledons were not quite as bleached. While these results do not provide
conclusive cause- and- effect relationships between ABA and LOX-derived NAE oxylipins, they clearly indicate that established conditions and pathways by which seedling establishment can be arrested, indeed do feature a marked, endogenous 50-to-60-fold increase in NAE oxylipin levels.

Discussion

A growing body of evidence in mammalian systems indicates that the oxidative products of polyunsaturated NAEs, including eicosanoid ethanolamides, prostaglandins and leukotrienes, are important signaling compounds that participate in diverse physiological processes (De Petrocellis et al., 2004). Studies showed that arachidonoyl ethanolamide (AEA) can be oxidized to prostaglandin ethanolamides (prostamide) by cyclooxygenase-2 (COX-2) (Kozak et al., 2002; Kozak et al., 2004; Sang and Chen, 2006). AEA also has been demonstrated to be a substrate for 12-LOX and 15-LOX in human polymorphonuclear leukocytes and human platelets, generating 12- and 15-hydroperoxyeicosatetraenoylethanolamide (12-HETE-EA and 15-HETE-EA), respectively (Edgemond et al., 1998). Oxidative metabolites of AEA generated by 12-LOX were proposed to play roles in pain modulation (Craib et al., 2001). In addition to oxidation by COX-2 and LOXs, AEA also undergoes oxidation by several human cytochrome P450 enzymes such as CYP3A4, CYP4F2, and CYP4X1, resulting in various oxidized lipid species (Snider et al., 2010). In plant tissues, the oxidation of polyunsaturated NAEs and the action(s) of their oxidative metabolites have received far less attention. Here we demonstrate the occurrence of endogenous LOX-derived NAE 18:3-oxylipins in Arabidopsis seedlings, and quantify their levels.
in seedlings during early seedling growth (Figure 3.3). A complex pathway for the depletion of endogenous NAE 18:3 during seedling establishment that involves competition between FAAH, 9-LOX, and 13-LOX enzymes was demonstrated in vivo using selected mutants and transgenic seedlings. Figure 3.19 summarizes the results taken together from steady state metabolite profiling experiments for both ethanolamide and FFA oxylipins (Figures 3.3-3.6, 3.16) and illustrates the relative partitioning and metabolic directions in the various mutant backgrounds. Whereas these pathway metabolites were identified during normal seedling growth (Figures 3.3, 3.5), their steady state levels could be exaggerated by the addition of exogenous NAE 18:3 (Figures 3.4, 3.6) helping to clarify these conclusions. Pharmacological inhibition of LOX activity by the addition of the competitive inhibitor, NAE 12:0, demonstrated further the metabolism in planta of NAE 18:3 by LOX pathway enzymes (Figure 3.16; see also Keereetaweep et al., 2010), and showed that the phenotypic changes in seedlings were attributed to a LOX-derived NAE 18:3 metabolite.

Manipulation of FAAH activity in Arabidopsis results in substantial alterations in seedling growth (Wang et al., 2006), and negative regulation of the embryo-to-seedling transition was proposed for NAEs in conjunction with ABA signaling pathways during seedling establishment (Teaster et al., 2007; Cotter et al., 2011). Previous pharmacological experiments with seedlings mostly have been performed with lauroylethanolamide (NAE 12:0; (Blancaflor et al., 2003; Motes et al., 2005) due to its stability, but this NAE is among the least abundant NAE types in desiccated Arabidopsis seeds, especially compared to the polyunsaturated NAEs- NAE 18:2 and NAE 18:3 (Wang et al., 2006). Here, when seedlings were grown in the presence of elevated NAE 18:3, a specific visible effect was observed that was not seen with other NAE types.
Seedling cotyledons treated with NAE 18:3 at 4-to-5 days after sowing, bleached and lost chlorophyll. This effect was specific for NAE 18:3, and was organ (cotyledon) and stage specific (Figure 3.10). FAAH1 OE did not show the bleaching phenotype (Figure 3.7A), suggesting that removal of NAE 18:3 was important for the normal development of cotyledons and that the formation of FFA oxylipins from NAE 18:3 did not contribute to the bleaching phenotype (see Figure 3.6 for FFA oxylipin profiles of FAAH1 OE during bleaching). Subsequent experiments provided additional clarity in that it was the accumulation of LOX-derived hydro(pero)xides of NAE 18:3 that induced cotyledon bleaching, and this could be reversed by preventing their accumulation (i.e. by FAAH1 overexpression, Figures 3.4, 3.7; or with a general LOX inhibitor, Figures 3.14-3.16) or by removing NAE 18:3 and depleting these ethanolamide oxylipins during a recovery period (Figures, 3.4, 3.12).

The ethanolamide oxylipin metabolite profiles of lox2 mutants or the lox1 lox5 double mutants indicated that NAE 18:3 could be partitioned into either pathway (Figures 3.3, 3.4). Since both sets of lox mutants, compromised in either 13-LOX activity (lox2) or 9-LOX activity (lox1 lox5), exhibited bleaching in the presence of exogenous NAE 18:3, it seemed likely that metabolites from both pathways might be active in disrupting chloroplast development. Indeed adding either 9- or 13-ethanolamide hydro(pero)xides to seedlings could induce bleaching (Figure 3.17), consistent with visible phenotypes (Figure 3.7) and metabolite profiles (Figure 3.4) of mutants treated with NAE 18:3. On the other hand, none of the FFA oxylipins induced chloroplast disruption in cotyledons (Figure 3.17), pointing to specific metabolism of NAE 18:3 by LOX enzymes as a means of disrupting thylakoid membrane organization (Figure 3.13). Perhaps the disruption of thylakoids in chloroplasts of cotyledons and the reduction in root
elongation, both induced by elevated levels of NAE 18:3, are suggestive of a regulatory network that is out of balance, and that normally functions in the progression of root/shoot development to synchronize the embryo-to-seedling transition and ensure that chloroplast development in cotyledons proceeds in conjunction with overall seedling growth. In other words, it may be that seedling development normally progresses with the metabolic depletion of these negative lipid regulators through a complex set of competing reactions. Here, the gross pharmacological or genetic manipulation of metabolite levels in these pathways disrupted the balance of processes required for normal seedling establishment resulting in seedling growth inhibition and even “de-greening” of cotyledons.

In summary, we propose that NAE metabolites function as negative regulators of seedling growth and development. Considerable evidence indicates that NAE metabolism begins with seed imbibition and progresses through seedling establishment and that this is accomplished through both hydrolysis and oxidation pathways (described above and summarized in Figure 3.19). The NAEs in desiccated seeds are depleted to low levels and NAE oxylipins do not accumulate substantially under normal conditions of seedling development (Figure 3.3). On the other hand, at early stages of establishment, seedlings retain the capacity to arrest growth and re-activate embryo-specific programs until conditions are more favorable for establishment. This reversible window is narrow—only a few days in Arabidopsis-- and it can be demonstrated through seedling sensitivity to ABA or abiotic stress (Lopez-Molina et al., 2001; Lopez-Molina et al., 2002). We suggest that NAE metabolism is part of this “back-up” mechanism of secondary dormancy that can negatively regulate seedling development through its interaction with various phytohormone pathways during the critical, early stages of seedling
establishment. The growth effects that are visible following the external application of high concentrations of NAE 18:3 are most likely a manifestation of an exaggerated physiological process that is induced only when environmental conditions are unfavorable for establishment, and so under normal conditions, levels of NAE oxylipins would be low. This is analogous to the depletion of ABA levels and embryo-specific regulators (like ABI3) that normally accompany post-germinative growth. As pointed out, however, there is a small developmental window when these processes can be reversed. Indeed treatment of seedlings with osmotic stress, ABA (Lopez-Molina et al., 2002), or here with NAE 18:3 (Figures 3.7-3.10, 3.12, 3.14), can arrest development and growth in the first few days of seedling establishment. Here, we also show that when seedling growth is arrested by treatment with ABA, that levels of endogenous 9- and 13-NAE-HOT metabolites increase in a dose-dependent fashion (Figure 3.18). At 50 μM ABA when seedling growth was arrested dramatically, NAE-HOT levels were more than 50 times higher than in untreated seedlings that developed normally (Figure 3.18). In other words, there was an inverse association of higher endogenous NAE-HOTs with reduced seedling growth precisely at the developmental stage at which seedlings were sensitive to NAE 18:3. We recognize that these endogenous NAE-HOT levels were still lower than what was used exogenously to arrest growth, but this is often the case when applying bioactive compounds to plant tissues (e.g. often high micromolar concentrations of phytohormones are added to elicit visible effects). This difference between effective exogenous amounts versus endogenous concentrations is likely due to the necessity of getting the appropriate amounts of mediators to the correct endogenous location (in spite of mitigating factors like tissue uptake, competing pathways, sequestration, etc.). Nonetheless, based on the higher levels of exogenous NAEs
required to elicit growth reduction and cotyledon bleaching, we make our interpretation that NAEs are negative regulators of seedling establishment with some caution. Whereas additional work is required to further explore these possibilities, our results point to a profound influence of NAE metabolism on seedling development, providing evidence that endogenous ethanolamide oxylipins have potent biological activities during seedling establishment.

Methods

Chemicals

9S-hydroxy-10E,12Z-octadecadienoic-9,10,12,13-d4 acid (9(S)-HODE-d4), 9S-hydroxy-10E,12Z,15Z-octadecatrienoic acid (9(S)-HOT), 13S-hydroxy-9Z,11E,15Z-octadecatrienoic acid (13(S)-HOT), 12-oxo phytodienoic acid (OPDA), α-linolenylethanolamine (NAE 18:3), 13-LOX from soybean (13-GmLOX) and 9-LOX from potato (9-StLOX) were purchased from Cayman Chemicals (Ann Arbor, MI).

Synthesis and separation of NAE oxylipins

NAE-oxylipin standards were synthesized to establish fragmentation patterns of these compounds for detection and identification of endogenous NAE-oxylipins. These standards were synthesized by LOX-mediated enzyme reactions using NAE 18:3 as substrates and 13-GmLOX or 9-StLOX enzymes as representative 13- and 9-LOX enzymes, respectively. Hydroperoxide products from 13-LOX and NAE 18:3 were incubated with 13-AOS and 13-AOC (both enzymes were from Arabidopsis thaliana) to generate corresponding 12-oxo-
phytodienoic acyl ethanolamide (NAE OPDA). NAE-oxylipins were separated on reverse phase HPLC (RP-HPLC). The fraction collector was set to collect samples from 6-11 min (1 min/fraction). The first NAE oxylipin to be eluted around 6.2 min was NAE OPDA, 9 and 13NAE HOT were eluted together around 7.5 min. Normal phase HPLC (NP-HPLC) was used to further separate 13 and 9NAE HOT which were eluted around 26 and 34 min, respectively.

LOX-mediated oxidative reactions with 13-GmLOX and 9-StLOX were carried out in 100 mM sodium borate buffer pH 9.2 and 100 mM Tris-HCl buffer pH 7, respectively. Substrate (250 μg of NAE 18:3) was incubated with enzyme at room temperature for 30 min. Corresponding hydroperoxides were converted into hydroxides by adding sodium tetrahydroborate. Hydroperoxide product from 13-GmLOX and NAE 18:3 were further incubated with Arabidopsis enzymes AOC2 (recombinant plasmids in E. coli provided by C. Wasternack) and AOS (recombinant plasmids in E. coli (Hughes et al., 2006)) in 0.1 M potassium phosphate buffer pH 7.0 for 30 minutes to generate NAE OPDA (Van der Stelt et al., 2000).

Extraction of oxylipins

Oxylipins were extracted as described previously (Kilaru et al., 2011; Christensen et al., 2013) with some modifications as follows. Approximately 5 g fresh weight of Arabidopsis seedlings were ground and homogenized in 4 ml n-hexane/isopropyl alcohol (3:2 with 0.0025% w/v 2-butyl-6-hydroxytoluene). 100 ng 9-HOD-d₄ was added as internal standard in each sample prior to extraction. Screw-cap test tubes (Pyrex® 16 x 125 mm) were immediately capped under nitrogen stream. The extract was shaken at 4 °C for 30 min and then centrifuged at 4500 X g for 10 min. The hexane-rich phase was collected and combined with 2 ml 6.6% potassium
sulfate in aqueous solution. To reduce all peroxides to hydroxides for analysis, approximately 10 mg of sodium tetrahydoborate was added and the extract was then centrifuged at 4500 X g for 10 min. The hexane-rich phase was recovered, dried under nitrogen and re-suspended in 200 μl hexane/isopropyl alcohol (100:5). After samples were vortexed vigorously, they were transferred into 2 ml amber vials (Fisher Scientific), dried under nitrogen and resuspended in 100 μl methanol/water (80:20 v/v). Samples were transferred into 100 μl glass inserts (Grace Davison Discovery Science) for separation by RP-HPLC.

**Separation of oxylipins**

Extracted lipids were suspended in methanol and separated by HPLC on an Agilent 1100 HPLC coupled to a UV diode array detector. The conjugated diene system was monitored at 234 nm. Reverse phase HPLC was carried out on a EC 250/2 nucleosil 120-5 C18 column (250 x 25 mm, 5 μm particle size, ID 2 mm; Macherey & Nagel, Düren, Germany) using a binary gradient system (solvent A: methanol/water/acetic acid (80:20:0.1, v/v/v), solvent B: methanol/acetic acid (100:0.1, v/v)) at a flow rate of 0.18 ml/min with 3 min of 100% solvent A and a linear increase to 25% solvent B in 18 min, then to 100% solvent B in 2 min followed by isocratic post-run of 100% solvent A for 10 min. For NAE-oxylipin standards, NAE-oxylipins collected from RP-HPLC were further separated by normal phase HPLC (NP-HPLC) carried out on Zorbax Rx-SIL column (150 x 2.1 mm, 5 μm particle size; Agilent, Waldbronn, Germany) using an isocratic system of n-hexane:2-propanol:trifluoroacetic acid (100:12.5:0.02, v/v/v) at a flow rate of 0.125 ml/min for 50 min (Kilaru et al., 2011). To verify the reaction stereochemistry, oxidative products generated by enzymatic reaction were distinguished from products formed by auto-
oxidation by chiral phase-HPLC (CHIRALCEL™ OD-H, 2.1x 150 mm, Diacel Chemical Industries, Ltd, Osaka, Japan)

*Identification of LOX-mediated products by GC-MS*

Identity of oxylipins as TMS derivatives was confirmed by GC/MS carried out with an Agilent GC 7890A /MSD 5975C system using a capillary HP-5MS column (30 m x 0.250 mm, 0.25 mm coating thickness; Agilent technologies) full mass scan mode. The oven temperature gradient was 40 °C for 1 min, 40 °C – 200 °C at 50 °C/min, 200 °C – 285 °C at 5 °C/min, followed by 300 °C for 15 min. Ultra-pure helium was used as a carrier gas at a constant flow of 0.5 ml/min and the transfer line was kept at 280 °C during the runs. Identification was based on identification of characteristic molecular and fragment ions in comparison with known, synthetic standards.

*Quantification of NAE- and FFA-oxylipins*

Oxylipins were quantified by SIM as TMS-derivatives by GC/MS using the same instrument, column and conditions described above. Standard curves were generated using 9-HOD-d₄, FFA 9-HOT, FFA 13-HOT and free OPDA. Ethanolamide-derivatives were quantified against corresponding FFA standard curves using the same deuterated standard, 9-HOD-d₄. Fragmentation patterns, diagnostic and quantitative ions (m/z) of oxylipins identified by GC/MS are presented in Figure 3.1, Figure 3.2, and Table 3.1. Full mass scans were performed to confirm identities of each compound.
Plant materials and tissue collection

FAAH1 overexpressing (FAAH1 OE) and faah1 T-DNA knockouts (SALK_095108) are described in Wang et al. (2006). The faah1 (At5g64440) mutant was crossed with a T-DNA insertional mutant in a second predicted FAAH locus (At5g07360; Kilaru et al., 2007), which we have designated as faah2 (SALK_011213). Progeny from the crosses were genotyped for the presence of T-DNA disruptions in both genes to generate faah1 faah2 double mutants. T-DNA knockouts of lox1 (SALK_038475) and lox5 (SALK_012188) were crossed to obtain the double mutant lox1 lox5 and were genotyped to verify the disruption of the respective genes. The loss-of-function lox2 (CS57949) mutant was kindly provided by E. Farmer (Glauser et al., 2009).

Desiccated seeds of Arabidopsis were surface-sterilized with 70% v/v ethanol and 30% commercial bleach for three minutes each, rinsed with sterile deionized water three times and subsequently stratified in sterile deionized water for 3 days at 4°C in the dark prior to sowing in liquid or solid MS (0.5X) (Murashige and Skoog; Teaster et al., 2007). Seeds were germinated and grown in controlled conditions with 16-h-light/8-h-dark cycle (60 µmol/m²/s) at 20 to 22 °C. For many experiments, Arabidopsis seedlings were grown for 4 days prior to treatment with 100 μM NAE 18:3 (or 0.07% DMSO as solvent control). Treated seedlings were collected at 24 h, 48 h and 72 h after treatment. For experiments to examine the reversibility of NAE 18:3 effects, seedlings were transferred into fresh media and collected at 48 h and 96 h after the transfer. For experiments with ABA treatment, ABA (concentration based on cis-isomer) was added to seedlings at d 3, and seedlings were collected for analysis at d 7. Collected tissues were immediately flash frozen in liquid nitrogen, and stored at -80 °C until further analysis.
Chlorophyll extraction and quantification

To quantify chlorophyll content in Arabidopsis seedlings, 5-10 seedlings were collected and weighed, then homogenized in 1 ml 80% acetone. The extracts were centrifuged at 3000 X g for 2–3 min. The supernatant was collected and absorbance was measured at 647 nm and 664.5 nm using 1.00 cm cuvettes (Spectronic Genesys 5; Fisher Scientific). The amounts of chlorophyll a and b were calculated as follows: Chl b (mg/L) = 20.47 A₆₄₇ – 4.73 A₆₆₄.₅, Chl a = 12.63 A₆₆₄.₅ - 2.52 A₆₄₇ (Inskeep and Bloom, 1985). Chlorophyll content was normalized to fresh weight.

Confocal laser scanning microscopy of chlorophyll autofluorescence

For observation of chloroplast disassembly/assembly, cotyledons from control and NAE 18:3-treated seedlings were wet-mounted onto glass slides. Images of chlorophyll autofluorescence from the mesophyll layer of the cotyledon were acquired using a Zeiss Axiovert 200M optical microscope equipped with a 63x water immersion objective (1.25 Numerical Aperture), and coupled to a Yokogawa CSU10 confocal scanner. Images were obtained by projecting z-stacks of 50 optical sections taken at 1 µm intervals. Chlorophyll autofluorescence from mesophyll cells was detected by illuminating cotyledons with the 488 nm line of the argon laser and emission captured at 692/40 nm.

Transmission electron microscopy

Cotyledon pieces from wild-type and NAE 18:3-treated seedlings were fixed in 3% glutaraldehyde (Electron Microscopy Science, PA, USA) in 10 mM potassium phosphate buffer
(pH 7.0) under vacuum for 5 min (3 times), and subsequently maintained in fixative at 4°C overnight. Cotyledon pieces were washed 6 times (15 min each) with potassium phosphate buffer followed by post-fixation by 1% osmium tetroxide 2 h on ice in the dark. Tissue pieces were dehydrated through a graded series of ethanol – 30%, 60%, 70%, 80%, 90%, 95%, 95%, 95%, 100%, 100% and 100% (15 min each), and embedded in Spurr’s epoxy resin (Electron Microscopy Science, PA, USA) using flat embedding molds. Ultra-thin sections (less than 100 nm) were cut in cross-section from polymerized blocks by using an ultramicrotome (RMC MT6000) equipped with a glass knife. Sections were mounted onto carbon coated, 100-mesh copper grids and stained with 1% uranyl acetate and 0.1% lead citrate. Sections were viewed in a Philips EM420 transmission electron microscope at 80 KeV.

Accession Numbers

The Arabidopsis Genome Initiative numbers for the genes mentioned in this article are as follows: FAAH1 (At5g64440), FAAH2 (At5g07360), LOX2 (At3g45140), LOX1 (At1g55020), and LOX5 (At3g22400).

Acknowledgements

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Author contributions

Table 3.1. Retention times and diagnostic/quantitative ions of NAE-oxylipins and corresponding FFA as detected by GC-MS.

<table>
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<th>Q&lt;sub&gt;ion&lt;/sub&gt;</th>
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<th>[M&lt;sup&gt;+&lt;/sup&gt; - CH&lt;sub&gt;3&lt;/sub&gt;]</th>
<th>R&lt;sub&gt;t&lt;/sub&gt;</th>
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<td>FFA HOD D4</td>
<td>318</td>
<td>444</td>
<td>429</td>
<td>14.195</td>
</tr>
<tr>
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<td>438</td>
<td>423</td>
<td>14.201</td>
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<td>364</td>
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<td>14.035</td>
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<td>466</td>
<td>18.917</td>
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<td>466</td>
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<td>149</td>
<td>407</td>
<td>392</td>
<td>18.835</td>
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</table>
Table 3.2. Quantification of NAE 18:3 and LOX-derived metabolites from different parts of Arabidopsis plants (Col 0).

<table>
<thead>
<tr>
<th></th>
<th>Desiccated seeds</th>
<th>4d seedlings</th>
<th>8d seedlings</th>
<th>rosette leaves</th>
<th>roots (2wk old)</th>
<th>siliques (6wk old plants)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAE 18:3 (pmol/g FW)</td>
<td>1223.93 ± 99.38</td>
<td>422.28 ± 50.65</td>
<td>106.32 ± 14.28</td>
<td>24.95 ± 3.24</td>
<td>17.02 ± 2.58</td>
<td>6.25 ± 0.95</td>
</tr>
<tr>
<td>9NAE HOT (pmol/g FW)</td>
<td>ND*</td>
<td>6.57 ± 0.78</td>
<td>6.97 ± 0.96</td>
<td>2.24 ± 0.19</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>13NAE HOT (pmol/g FW)</td>
<td>ND</td>
<td>5.82 ± 0.84</td>
<td>5.44 ± 0.68</td>
<td>1.96 ± 0.12</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>NAE-OPDA (pmol/g FW)</td>
<td>ND</td>
<td>1.96 ± 0.24</td>
<td>1.68 ± 0.18</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Means +/- standard deviation; n = 3; *ND, not detected.
**Figure 3.1.** Structures of TMS derivatives of ethanolamide and free oxylipin metabolites with molecular ions and several key diagnostic fragment ions indicated for each species.

Representative spectra from GC-MS are shown in Figure 3.2.

(A) TMS derivatives of 9-hydroxy-10,12,15-octadecatrienylethanolamide (9-NAE-HOT).

(B) TMS derivatives of 13-hydroxy-9,11,15-octadecatrienylethanolamide (13-NAE-HOT).

(C) TMS derivatives of (13S,9Z,10E,15Z)-12-oxo-10,15-phytodienylethanolamide.

(D) TMS derivatives of 9-hydroxy-10,12,15-octadecatrienoic acid (9-HOT).

(E) TMS derivatives of 13-hydroxy-9,11,15-octadecatrienoic acid (13-HOT).

(F) TMS derivatives of (13S,9Z,10E,15Z)-12-oxo-10,15-phytodienoic acid.
Figure 3.2. Identification of endogenous NAE 18:3 and FFA 18:3 oxylipins.
Figure 3.3. Endogenous ethanolamide oxylipin metabolites derived from NAE 18:3.

Arabidopsis seedlings (the wild type, faah1, faah1 faah2, FAAH1 OE, lox1 lox5, and lox2) were grown for 11 d. Seedling tissues (~5 g) were sampled at days 5, 6, 7, 9, and 11 after sowing and oxylipins were quantified. A single asterisk indicates a significant difference compared with wild-type seedlings, which was determined by Student’s t test (P < 0.05). A double asterisk indicates a significant difference compared with wild-type seedlings, which was determined by Student’s t test (P < 0.01). Values are presented as means ± SD of three biological replicates. FW, fresh weight.
Figure 3.4. Ethanolamide oxylipins in seedlings after addition of exogenous NAE 18:3.

Arabidopsis seedlings (the wild type, faah1, faah1 faah2, FAAH1 OE, lox1 lox5, and lox2) were grown for 4 d, transferred into media containing 100 μM NAE 18:3, and continued to grow for 72 h. Seedlings were subsequently transferred into fresh media as a recovery period without NAE for an additional 96 h. Seedlings (~2 g) were collected at 24 h (day 5), 48 h (day 6), and 72 h (day 7) after addition of NAE 18:3 or 48 h (day 9) and 96 h (day 11) after removal to fresh medium without NAE 18:3. Values are presented as means ± SD of three biological replicates.
A single asterisk indicates a significant difference compared with wild-type seedlings, which was determined by Student’s t test (P < 0.05). A double asterisk indicates a significant difference compared with wild-type seedlings, which was determined by Student’s t test (P < 0.01). FW, fresh weight.
Figure 3.5. Endogenous free oxylipin metabolites.

Arabidopsis seedlings (the wild type, faah1, faah1 faah2 knockout, FAAH1 OE, lox1 lox5, and lox2) were grown for 11 d. Seedling tissues (~5 g) were sampled at days 5, 6, 7, 9, and 11 after sowing and oxylipins were quantified. Values are presented as means ± SD of three biological replicates. FW, fresh weight.
Figure 3.6. Free oxylinps in seedlings after addition of exogenous NAE 18:3.

Arabidopsis seedlings (the wild type, faah1, faah1 faah2, FAAH1 OE, lox1 lox5, and lox2) were grown for 4 d, transferred into media containing 100 µM NAE 18:3, and continued to grow for 72 h. Seedlings were subsequently transferred into fresh media without NAE for 96 h. Seedlings (~2 g) were collected at 24 h (day 5), 48 h (day 6), and 72 h (day 7) after addition of NAE 18:3 or 48 h (day 9) and 96 h (day 11) after removal to fresh medium without NAE 18:3. Values are presented as means ± SD of three biological replicates. A single asterisk indicates a significant
difference compared with wild-type seedlings, which was determined by t test (P < 0.05). A double asterisk indicates a significant difference compared with wild-type seedlings, which was determined by Student’s t test (P < 0.01). FW, fresh weight.
Figure 3. 7. NAE 18:3 induces cotyledon bleaching in Arabidopsis seedlings except in FAAH1 OE. Arabidopsis seedlings (the wild type [WT], faah1, faah1 faah2, FAAH1 OE, lox1 lox5, and lox2) were grown for 4 d, transferred into media containing 100 μM NAE 18:3 (or 0.07% DMSO as a
solvent-only control), and allowed to grow for 72 h. Total chlorophyll contents were quantified spectrophotometrically after extraction in 80% acetone.

(A) Representative images of 7-d-old Arabidopsis seedlings treated with 100 μM NAE 18:3.

(B) Chlorophyll contents of seedling exposed to 100 μM NAE 18:3 for 72 h. Chlorophyll contents of FAAH1 OE were not affected by exposure to elevated NAE 18:3, while other genotypes show substantial reduction in their total chlorophyll contents. FW, fresh weight.

(C) Chlorophyll (chl) contents of seedlings exposed to 0.07% DMSO (control). Data are the means ± SD of three biological replicates.
Figure 3.8. NAE 18:3 inhibits primary root elongation in a dose-dependent manner.

Four-day-old wild-type seedlings were transferred onto media containing NAE 18:3 at varying concentrations (with 0.07% DMSO as solvent control). Primary root length data are the means ± SD of 20 seedlings. Three replicate experiments showed similar results.
Figure 3. 9. NAE 18:3 negatively regulates growth and induces bleaching of cotyledons within a narrow window of developmental sensitivity.

*Arabidopsis* seedlings were treated with 100 μM NAE 18:3 at different time points (from day 0 to day 9). Only 10d old seedlings treated with NAE 18:3 on day 4 showed bleaching of cotyledons.
Figure 3.10. Arabidopsis seedlings show organ-specific sensitivity to exogenous NAE 18:3. Four-day old wild type seedlings grown in liquid media for 4 days were transferred into media containing 100 μM NAE 18:3 for 12 additional days. Cotyledons remained bleached but the emerging true leaves were not affected but NAE 18:3.
Figure 3. NAE 18:3 specifically disrupts chloroplasts in cotyledons of Arabidopsis seedlings. Four-day-old wild-type seedlings were transferred into media containing 0.07% DMSO (solvent control) (A), 100 μM NAE 18:3 (B), 100 μM NAE 18:2 (C), or 100 μM FFA 18:3 (D) for 72 h. Chloroplast disruption was specific for NAE 18:3; neither free linolenic acid (FFA 18:3) nor NAE 18:2 showed the same effect. Images are z-stacks of mesophyll cells visualized by confocal fluorescence microscopy showing chlorophyll autofluorescence in chloroplasts.
**Figure 3.12.** NAE 18:3 bleaching/chloroplast disruption was reversible.

Effects were more severe in faah1 knockouts (right) than in the wild type (WT; left). NAE 18:3 (100 μM) was applied to 4-d-old wild-type and faah1 seedlings for 72 h. Seedlings were subsequently removed from treatment to fresh media for 4 d. Not only was there a marked reduction in seedling growth noted after 3 d of treatment (insets), but a profound loss of chlorophyll in the seedlings also was observed.

(A) Four-day-old seedling before addition of NAE 18:3 (control).

(B) to (D) Chloroplast autofluorescence 1, 2, and 3 d after transfer to media containing NAE 18:3.
(E) and (F) Recovery of chloroplasts after seedlings had been removed to fresh media. Microscopy images are z-stacks of mesophyll cells visualized by confocal fluorescence microscopy showing chlorophyll autofluorescence in chloroplasts.

(G) Bottom panels are chlorophyll quantification at different stages, consistent with bleaching of seedlings and the disappearance and reappearance of chloroplasts (left, the wild type; right; faah1). Data are means and SD of three replicate samples (of three to five seedlings each). FW, fresh weight.
Figure 3. Representative electron micrographs of chloroplasts in cotyledons of wild type seedlings treated with NAE 18:3.

(A) Ultrastructure of chloroplasts in 4-d old seedling before addition of 100 μM NAE 18:3 (control)

(B), (C), (D) Ultrastructure of chloroplasts 1, 2 and 3d after transfer to media containing NAE 18:3.

(E) and (F) Ultrastructure of chloroplasts after seedlings were transferred to fresh media without NAE18:3.
**Figure 3. 14.** NAE12:0, a general LOX inhibitor can prevent seedling bleaching.

Four-day-old Arabidopsis wild-type seedlings grown in liquid media for 4 d were transferred to media for three additional days containing 100 μM NAE 18:3 alone or with NAE 12:0, NAE 16:0, or FFA 12:0 at 20 μM. The bottom panels show NAE 12:0, NAE 16:0, and FFA 12:0. At these concentrations, NAE 12:0 and partially NAE 16:0, but not FFA 12:0, prevented seedling bleaching.
Figure 3.15. NAE 12:0 reversed the effects of NAE 18:3 in a dose-dependent manner.

Four-day-old wild-type seedlings were treated with 100 μM NAE 18:3 for an additional 72 h in the presence of NAE 12:0 at 20, 30, 40, or 50 μM. chl, chlorophyll; FW, fresh weight.

(A) Total chlorophyll contents were quantified spectrophotometrically following extraction with 80% acetone. Data are the means ± SD of three biological replicates.

(B) Primary root lengths of seedlings treated with 100 μM NAE 18:3 in presence of NAE 12:0 were measured. Data are the means ± SD of n = 40.

(C) Primary root lengths of NAE 12:0-treated seedlings were measured. Data are the means ± SD of n = 40.
Figure 3.16. NAE12:0, a general LOX inhibitor, blocks the endogenous formation of oxylipins.

Quantification of oxylipins in 7-d-old wild-type seedlings treated with 100 μM NAE 18:3 for 72 h in the presence of NAE 12:0 at 20, 30, 40, and 50 μM (cf. Figure 3.15). Data are means and SD of three replicate samples. A single asterisk indicates a significant difference compared with seedlings treated with NAE 18:3 alone, which was determined by Student’s t test (P < 0.05). A double asterisk indicates a significant difference compared with seedlings treated with NAE 18:3 alone, which was determined by Student’s t test (P < 0.01). FW, fresh weight.

(A) Ethanolamide oxylipins

(B) FFA oxylipins
*Figure 3. 17.* Synthetic ethanolamide oxylipins, but not free oxylipins, bleached seedling cotyledons.

Chlorophyll content in 7-d-old wild-type seedlings treated for 72 h with 100 μM NAE 18:3, 9-NAE-HOT, 13-NAE-HOT, a combination of 9- and 13-hydro(pero)xy octadecatrienylethanolamide (H[P]OT) (not fully reduced or fully reduced), or corresponding FFA oxylipins. Either 9- or 13-NAE-H(P)OT, but not corresponding free oxylipins, elicited bleaching in cotyledons. Data are means and SD of three replicate samples. chl, chlorophyll; FW, fresh weight.
**Figure 3.18.** Exogenous ABA-induced accumulation of NAE 18:3-derived oxylipins.

Quantification of oxylipins in 7-d-old seedlings (wild type) treated with DMSO (control) or 0.5, 5, or 50 μM ABA on day 3. Concentrations of ABA were calculated based on the cis-isomer. Data are means and SD of three replicate samples. A single asterisk indicates a significant difference compared with untreated seedlings (DMSO), which was determined by Student’s t test (P < 0.05). A double asterisk indicates a significant difference compared with untreated seedlings, which was determined by Student’s t test (P < 0.01). FW, fresh weight.
Figure 3. 19. Summary of NAE 18:3 metabolism via hydrolysis, the 9-LOX or the 13-LOX pathways, and the relative partitioning through these pathways in the various mutants used in these studies.

NAE 18:3 undergoes 13- and/or 9-LOX–mediated oxidation to generate corresponding hydroperoxides that are further reduced to hydroxides. Hydroperoxides and hydroxides of NAE 18:3 may also undergo hydrolysis and enter the larger pool of corresponding free hydroperoxides and hydroxides (as shown in blue). 13-NAE-HPOT is also metabolized by AOS.
and/or AOC to generate the unstable epoxide and NAE-OPDA, respectively. Corresponding FFA oxylipins are generated in the same LOX pathways (dashed lines). 9NAE-HPOT, (9S,12Z,10E,15Z)-9-hydroperoxy-10,12,15-octadecatrienoylthanolamide; 13-NAE-HPOT,(13S,9Z,11E,15Z)-13-hydroperoxy-9,11,15-octadecatrienoylthanolamide; 9NAE-HOT, (9S,12Z,10E,15Z)-9-hydroxy-10,12,15-octadecatrienoylthanolamide; 13-NAE-HOT, (13S,9Z,11E,15Z)-13-hydroxy-9,11,15-octadecatrienoylthanolamide; 12,13S-epoxy-NAE 18:3, (13S, 9Z, 11E, 15Z)-12,13-epoxy-9,11,15-octadecatrienoylthanolamide; NAE-OPDA, (13S,9Z,10E,15Z)-12-oxo-10,15-phytodienoylthanolamide (summarized from data in this article and consistent with previous data and speculation in Van Der Stelt et al., 2000; Shrestha et al., 2002; Kilaru et al., 2011). WT, the wild type.
References


The maize lipoxygenase, ZmLOX10, mediates green leaf volatile, jasmonate and herbivore-induced plant volatile production for defense against insect attack. Plant J 74, 59-73.


CHAPTER 4

LIPOXYGENASE DERIVED 9-HYDRO(PERO)XIDES OF LINOLEOYLETHANOLAMIDE INTERACT WITH

ABA SIGNALING TO ARREST ROOT DEVELOPMENT DURING ARABIDOPSIS SEEDLING

ESTABLISHMENT*

Abstract

Ethanolamide-conjugated fatty acid derivatives, also known as N-acylethanolamines (NAEs), occur at low microgram per gram levels in desiccated seeds, and endogenous amounts decline rapidly with seedling growth. Linoleylethanolamide (NAE18:2) is the most abundant of these NAEs in nearly all plant seeds including Arabidopsis thaliana. In Arabidopsis, NAE18:2 can be oxidized by lipoxygenase (LOX) or hydrolyzed by fatty acid amide hydrolase (FAAH) during normal seedling establishment and this contributes to the normal progression of NAE depletion that is coincident with the depletion of abscisic acid (ABA). Here we provide biochemical, genetic and pharmacological evidence that a specific 9-LOX metabolite of NAE18:2-- 9-hydro(pero)xy linoleylethanolamide (9-NAE-H(P)OD)-- has a potent negative influence on seedling root elongation, and it acts synergistically with ABA to modulate the transition from embryo to seedling growth. Genetic analyses with mutants in ABA synthesis (aba1, aba2), perception (pyr1, pyl2, pyl4, pyl5, pyl8) or transcriptional activation (abi3-1) indicated that arrest of root growth by 9-NAE-H(P)OD requires an intact ABA signaling pathway, and likely operates to elevate ABA synthesis as part of a positive feedback loop to modulate seedling growth.

establishment in response to adverse environmental conditions. These results identify a specific, bioactive ethanolamide oxylipin metabolite of NAE18:2, different from those of ethanolamide-conjugated linolenic acid (NAE18:3), as well as a molecular explanation for its inhibitory action, continuing to point to the oxidative metabolism of NAEs as an important feature of seedling development.

Introduction

*N*-Acylethanolamines (NAEs) have been found in desiccated seeds of all plant species examined, and their metabolic depletion has been associated with successful seedling establishment (Blancaflor et al., 2014). Over-expression of the NAE hydrolase, fatty acid amide hydrolase (FAAH), resulted in accelerated Arabidopsis seedling growth, while the addition of exogenous NAEs, especially to *faah* knockouts, markedly inhibited seedling development (Wang et al., 2006). Most of the activities of NAEs have been attributed to lauroylethanolamide (NAE12:0) (Blancaflor et al., 2003; Teaster et al., 2007; Cotter et al., 2011; Teaster et al., 2012), a medium chain NAE of generally minor concentration in seeds. However, polyunsaturated NAEs are consistently the most prevalent of the NAEs in seeds and application of NAE12:0 was shown to elevate the endogenous levels of the polyunsaturated NAEs, NAE18:2 and NAE18:3, during seedling growth arrest (Kilaru et al., 2012). It is possible that some of the reported inhibitory effects of exogenous NAE12:0 on seedlings may be attributed to an “entourage effect” of other NAEs as has been shown for mammals (Ho et al., 2008; Grillo et al., 2013), and
it is likely that these inhibitory effects would be mitigated by over-expression of FAAH to reduce the overall concentrations of endogenous NAEs.

Recent evidence indicates that hydrolysis by FAAH and oxidation by lipoxygenase (LOX) compete for the metabolism of polyunsaturated NAEs to generate a range of free fatty acid and oxylipin metabolites (Kilaru et al., 2011; Keereetaweep et al., 2013) (see also Figure 4.1), and these results have prompted a more detailed examination of the metabolism and function of polyunsaturated ethanolamide oxylipins in seedling development. Oxylipin metabolites of NAE18:3 induced a marked bleaching of seedling cotyledons, and this was attributed to stage-specific disruption of chloroplasts in cotyledon cells (Keereetaweep et al., 2013). The regulation of chloroplast disruption was specific for the ethanolamide conjugates of 9- or 13-hydro(pero)xides, and not free fatty acid oxylipins. Chloroplast disruption was specific to cotyledons (did not occur in true leaves), and was reversible when NAE18:3-derived oxylipin levels were reduced. Interestingly, cotyledon bleaching could be partially simulated by exogenous application of abscisic acid (ABA), a well characterized inducer of seedling growth arrest and secondary dormancy (Lopez-Molina et al., 2001; Lopez-Molina et al., 2002), and this growth arrest was accompanied by a marked increase in endogenous NAE18:3-derived ethanolamide oxylipins (Keereetaweep et al., 2013), suggesting a potential link between NAE metabolism and ABA action.

In experiments with NAE12:0, NAE metabolism and ABA signaling have been implicated to interact to inhibit seedling growth in a synergistic manner (Teaster et al., 2007). However, it now seems clear that the most marked changes in NAE content during seedling establishment are associated with the complex hydrolysis and oxidation of NAE18:2 and NAE18:3 (Kilaru et al.,
and that application of NAE12:0 elevates the levels of NAE18:2 and NAE18:3 (and their phospholipid precursors) in plant tissues (Kilaru et al., 2012). This suggests that perhaps a more direct action of NAEs might be via ethanolamide oxylipins derived from NAE18:2 and NAE18:3, and the evidence with NAE18:3 and cotyledon bleaching supports this concept.

NAE18:2 is the most abundant NAE in desiccated seeds of Arabidopsis (and most plant species), and yet there is little known regarding its function, if any, in seed or seedling development. Elevation of NAE18:2 levels during seedling establishment did not elicit cotyledon bleaching, but did inhibit seedling growth of germinated seeds (Wang et al., 2006). Here, we test if the inhibition of seedling growth by NAE18:2 is attributed to a LOX-derived metabolite and if this process interacts with ABA signaling to induce secondary dormancy. Taken together, our results suggest that a specific 9-LOX product derived from NAE18:2 increases ABA synthesis and sensitivity in young seedlings through a positive feedback loop that involves LOX1 and LOX5, two genes that encode redundant 9-LOX enzymes in Arabidopsis for 9-LOX activity (Feussner and Wasternack, 2002; Liavonchanka and Feussner, 2006). It is possible that the oxidative metabolism of both NAE18:3 and NAE18:2 act to generate signals to synchronize seedling development between cotyledons and emerging roots, and provide an internal metabolic cue to influence hormone pathways and arrest growth if environmental conditions are unfavorable for seedling establishment.
Results

*Exogenous NAE 18:2 inhibits root elongation in seedlings*

The sensitivity of Arabidopsis seedlings to NAE18:2 is shown in Figure 4.2. Seedling root elongation accompanied with normal seedling establishment is reduced in the presence of NAE18:2, and this is enhanced in mutants that are compromised in their ability to hydrolyze NAEs to ethanolamine and free fatty acids (*faah* knockouts, KOs (Wang et al., 2006)). By contrast, plants with increased capacity for NAE hydrolysis (FAAH over-expressors, OE) are resistant to the inhibitory action of NAE18:2. On the surface, it would seem then that NAE18:2 itself inhibits seedling growth, but the situation is not so straightforward. LOX double mutants (*lox1 lox5*) with compromised ability to introduce peroxy groups into the C9 position of polyunsaturated acyl groups were as tolerant to NAE18:2 as FAAH over-expressors. By contrast, *lox2* mutants that are severely compromised in 13-LOX activity (Glauser et al., 2009), were sensitive to NAE18:2. These results raised the possibility that a 9-LOX derivative of NAE18:2, rather than NAE18:2 itself, might inhibit seedling root elongation in Arabidopsis.

*Genetic and biochemical evidence point to 9-NAE-HO(P)D as bioactive inhibitory molecules*

Oxylipin metabolite profiling in these genotypes was performed to provide additional clues as to the inhibitory metabolite(s) derived from NAE18:2 (Figure 4.3). Ethanolamide and free fatty acid hydroxides were quantified in seedlings without (solvent controls, DMSO) or with NAE18:2 (peroxy groups were chemically reduced to hydroxyl groups to quantify these lipids as the more stable hydroxides). Profiles of ethanolamide 9- and 13-hydroxides in untreated seedlings were altered in these mutants in a manner consistent with partitioning of NAE18:2
through hydrolysis (FAAH) or oxidation (9-LOX or 13-LOX) pathways. Ethanolamide oxylipin levels were quite low in untreated seedlings, but were especially exaggerated in seedlings treated with NAE18:2 so that metabolic partitioning was much more evident (Figure 4.3, left panels). For example, faah knockouts had higher amounts of ethanolamide oxylipins, whereas FAAH over-expressors had considerably less ethanolamide oxylipins. Mutants disrupted in 9-LOX activity (lox1 lox5) showed comparatively more 13-NAE-HOD, and similarly, mutants compromised in most of their 13-LOX activity (lox2) showed much more relative amounts of 9-NAE-HOD. The free fatty acid oxylipins did not vary among genotypes in untreated seedlings, but did differ in concentration when seedlings were supplied with NAE18:2 presumably serving as a source of 18:2 free fatty acids following hydrolysis (Figure 4.3, right panels). When comparing the metabolite profiles of the two genotypes that were tolerant to NAE18:2—FAAH overexpressors and lox1 lox5 double mutants—one common change was observed, and this was reduced 9-NAE-H(P)OD amounts (arrows), compared to other genotypes treated with NAE18:2 (Figure 4.3, left column, row 3), suggesting that 9-NAE-H(P)OD was a key inhibitory metabolite derived from NAE18:2. These two genotypes had markedly different free fatty acid (FFA) oxylipin profiles, so it is unlikely that FFA oxylipins were responsible for the observed seedling growth inhibition.

Exogenous 9-NAE-HOD inhibited seedling root elongation in lox1 lox5, but not in FAAH overexpressors

Further evidence that 9-NAE-HOD inhibits seedling root growth was obtained with synthetic ethanolamide hydroxides (Figure 4.4). 9-NAE-HOD inhibited Arabidopsis (Col-0)
seedling root growth even more than NAE18:2. FAAH overexpressors were not inhibited by 9-NAE-HOD, suggesting that FAAH may hydrolyze this ethanolamide in planta. The double mutant, lox1 lox5, although insensitive to NAE18:2 (Figure 4.4) and compromised in 9-NAE-HOD formation (Figure 4.3), retained sensitivity to root growth inhibition by 9-NAE-HOD (Figure 4.4). None of the genotypes were sensitive toward 13-NAE-HOD, indicating the specificity of the response for 9-hydroxy linoleylethanolamide. The lack of root growth inhibition in FAAH overexpressors by any of these ethanolamides suggests that inhibition is not through FFAs or FFA oxylipins. Seedling root elongation was inhibited by micromolar concentrations of 9-NAE-HOD in a dose-dependent manner (Figure 4.5). While endogenous concentrations of 9-NAE-HOD were considerably lower than those required to inhibit growth during normal progression of seedling growth (Figure 4.3), supplying higher exogenous levels clearly resulted in dramatic reductions in seedling root growth.

The Synergistic inhibition between NAE 18:2 and ABA occurs within a narrow developmental window

Previous research indicated that ABA and NAE12:0 could synergistically arrest seedling growth within a strict developmental window (Teaster et al., 2007), and because NAE12:0 treatment was shown to elevate endogenous NAE18:2 levels (Kilaru et al., 2012), we asked whether NAE18:2 also interacts with ABA to arrest seedling root growth. Low levels of NAE18:2 and ABA, that by themselves did not arrest growth, were combined and this combination showed a marked reduction in seedling root growth (Figure 4.6). Seedlings were sensitive to the synergistic inhibition of growth by ABA and NAE18:2 on 3 or 4 d after sowing, but not at 5 d
(Figure 4.6, right column) or later (not shown). These results indicate that NAE18:2/ABA action in primary roots of seedlings occurs within the same developmental window (3-4 days after sowing) as that for other NAEs (Teaster et al., 2007; Keereetawee et al., 2013) and for ABA (Lopez-Molina et al., 2001), and this stage overlaps with the developmental period when seedlings are capable of secondary dormancy (Lopez-Molina et al., 2002).

**Growth arrest by NAE18:2 and 9-NAE-HOD requires an intact ABA signaling pathway**

The synergistic interaction with ABA in the inhibition of seedling development suggested that ABA signaling was likely to be involved in the response of seedlings to NAE18:2, and this was confirmed with genetic evidence showing that ABA mutants were tolerant to NAE18:2 and to 9-NAE-HOD (Figure 4.7). Arabidopsis mutants in ABA perception (pentuple receptor mutant, pyr1 pyl2 pyl4 pyl5 pyl8 (Gonzalez-Guzman et al., 2012) or ABA-mediated transcriptional regulation (abi3-1) were tolerant to NAE18:2 and 9-NAE-HOD, not exhibiting the severe growth reduction of their corresponding wild-type backgrounds (Figure 4.7). An ABA synthesis mutant (aba1-4), in the Landsberg erecta (Ler) background (Koornneef et al., 1982), also was somewhat tolerant to exogenous NAE18:2 and 9-NAE-HOD, but these seedlings retained sensitivity to exogenously supplied ABA where growth was more severely inhibited. These results indicate that an intact ABA signaling pathway is required for NAE18:2 or 9-NAE-HOD action in seedlings, and may be influenced partially through ABA synthesis.

**Higher ABI3 transcript levels were directly associated with more pronounced growth inhibition**

In support of the involvement of ABA signaling in NAE18:2 and 9-NAE-HOD action, transcript levels of *ABI3* were increased in response to treatment with either NAE18:2 or 9-
NAE-HOD in 4-d-old seedlings (Table 4.1). The increase in ABI3 transcript abundance induced by NAE18:2 was markedly reduced in the lox1 lox5 mutants (Table 4.1); however, these mutants retained sensitivity to 9-NAE-HOD, indicating that the dramatic increase in ABI3 expression (like root growth inhibition) was dependent on the 9-LOX product of NAE18:2 and not NAE 18:2 itself. Increases in ABI3 transcripts were nearly exclusively restricted to root tissues of seedlings, suggesting that this response to NAE-18:2 oxylipin metabolites is tissue specific. Additional experiments confirmed that the marked increase in several ABA responsive genes that were induced by 9-NAE-HOD (and NAE18:2) were stage specific—much more elevated when seedlings were treated at 4 d after sowing compared to just after stratification at 0 d (Table 4.2).

Exogenous NAE18:2/9-NAE-HOD affects endogenous levels of ABA

The partial tolerance of the ABA synthesis mutant aba1-4 to NAE18:2 and 9-NAE-HOD (Figure 4.7), suggested that the NAE18:2-derived oxylipin may inhibit seedling growth through the activation of ABA synthesis. Indeed treatment of wild-type Arabidopsis seedlings with 9-NAE-HOD (or NAE18:2) was associated with an increase in endogenous ABA levels during the “sensitive” developmental window when seedling growth is capable of arrest (Figure 4.8). The 9-NAE-HOD induced an increase in ABA content in seedlings of wild-type and lox1 lox5 double mutants. By contrast, treatment of the lox1 lox5 double mutant with NAE18:2 (unable to produce as much 9-NAE-HOD from the NAE18:2) did not show an increase in endogenous ABA levels, indicating specificity of the ethanolamide oxylipin for ABA induction. Consistent with ABA quantification, transcripts of ABA synthesis genes, ABA1 and ABA2, were increased in
response to 9-NAE-HOD (Figure 4.9). The increase in ABA1 and ABA2 transcripts was especially notable in root tissues, and was not observed to a substantial degree in lox1 lox5 mutants treated with NAE18:2 (with compromised ability to produce 9-NAE-HOD and insensitive to NAE18:2), again pointing to a specific role for 9-NAE-HOD in the induction of ABA synthesis.

*Exogenous ABA is associated with the increase of endogenous 9-NAE-HOD and seedling root growth inhibition*

During normal seed germination and seedling establishment, NAE18:2 levels declined dramatically, and this was accompanied by a transient increase in 9-NAE-HOD levels at about 3-to-6 days after sowing (Figure 4.10). By contrast 13-NAE-HOD levels increased over a slightly later time course and did not decrease during seedling establishment. The transient increase in endogenous 9-NAE-HOD may be a metabolic “mark” for the developmental window in which seedlings are capable of arrest by ABA. Interestingly, application of ABA to seedlings at concentrations that reduced seedling growth, was associated with an elevation of 9-NAE-HOD in seedlings in a dose-dependent manner (Figure 4.11). The ABA-induced increase in 9-NAE-HOD was reduced somewhat, but not eliminated, in the lox1 lox5 mutants, pointing to LOX1/LOX5 as important but not essential for this increase in 9-NAE-HOD, suggesting that other LOX enzymes might be capable of oxidizing NAE18:2. Previous studies *in vitro* suggested that other Arabidopsis LOX enzymes (i.e. LOX4 and LOX6) could utilize NAE18:2 as a substrate (Kilaru et al., 2011), albeit with less specificity than LOX1 and LOX5. Indeed treatment of seedlings with ABA, increased transcript levels of LOX genes in lox1 lox5 mutants and this was especially true for LOX4 and LOX6 transcripts in seedlings treated with NAE18:2 and ABA (Figure 4.12). In
any case, collectively, our results suggest that ABA acts to increase endogenous NAE18:2 oxylipin levels, and vice versa. Perhaps this affords a positive reinforcement of seedling growth arrest that can rapidly accommodate adverse conditions known to induce secondary dormancy (Lopez-Molina et al., 2002; Finkelstein et al., 2008).

*Salinity and simulated drought stress suggested ABA-dependent secondary dormancy by ABA and 9-NAE-HOD working together*

To ask whether the interaction between 9-NAE-H(P)OD formation and ABA-dependent growth inhibition is important in a physiological stress context, we subjected seedlings to two abiotic stresses previously shown to inhibit seedling establishment via an ABI3-dependent ABA signaling pathway (Lopez-Molina and Chua, 2000; Lopez-Molina et al., 2001). Seedlings were subjected to drought stress (Figure 4.13) or salt stress (Figure 4.14) (Lopez-Molina et al., 2001). Seedlings were subjected to simulated drought on d 3 or subjected to salt stress for 3 d due to the observed timing of the transient rise of endogenous 9-NAE-HOD (Figure 4.10) and the narrow window of synergistic action between ABA and NAE 18:2 during day 3 and 4 (Figure 4.6). Seedling root growth responses to these abiotic stresses were quantified, and, as expected, the quintuple ABA receptor mutant and the *abi3-1* loss-of-function mutant were significantly less inhibited by stresses compared with respective wild-type seedlings (Col-0 or Ler backgrounds) (Figure 4.13 a, b; Figure 4.14 a, b). By comparison, primary roots of *lox1 lox5* mutants, although not as tolerant to stress as ABA mutants, were significantly less inhibited by both drought (Figure 4.13 a, b) and salt stress (Figure 4.14 a, b) during seedling establishment.
consistent with their elevations during seedling growth inhibition (Figure 4.13c, d and Figure 4.14c, d). In wild-type seedlings (Col-0 and Ler), both 9-NAE-HOD and ABA levels increased substantially in drought- and salt-treated seedlings in association with reduced seedling root growth. The involvement of NAE oxylipin metabolism in root growth inhibition was evident in results with mutants; the lox1 lox5 mutants with reduced capacity for 9-NAE-HOD, also had significantly lower levels of ABA (and less growth inhibition), suggesting that NAE oxylipin formation at least partially participates in modulation of ABA signaling in seedling stress, likely by influencing ABA formation. ABA levels in lox1 lox5 mutants were still elevated somewhat compared with unstressed controls, indicating that lox1 and lox5 were not entirely responsible for inducing ABA formation, but rather could be part of a feed forward pathway to magnify the responses of seedlings to abiotic stress. As anticipated, primary roots of ABA signaling mutants were insensitive to elevated ABA content despite elevation of both 9-NAE-HOD and ABA levels in these seedlings, consistent with the concept that ABA signaling is downstream from NAE18:2 oxylipin formation.

Discussion

Polyunsaturated NAEs are the most dominant types of acylamides found in desiccated seeds, and their levels are depleted during normal seedling establishment (Kilaru et al., 2011; Keereetawee et al., 2013; Blancaflor et al., 2014) along a time course similar to ABA (Teaster et al., 2007). Addition of exogenous polyunsaturated NAEs reduces seedling growth in a developmentally sensitive window that overlaps with the ABA-induced secondary dormancy
and seedling growth arrest (Lopez-Molina et al., 2002). Here we show that a specific 9-LOX product of NAE18:2 mediates the inhibition of root elongation through an interaction with ABA signaling, suggesting that 9-NAE-HOD may be part of a metabolic process that can invoke secondary dormancy in conjunction with ABA during seedling development. A summary diagram describing the interaction of ABA signaling and NAE18:2 metabolism to modulate seedling establishment is shown in Figure 4.15.

Several lines of evidence support the interaction of 9-NAE-HOD with ABA signaling during seedling development. First, NAE18:2 and ABA appeared to arrest seedling growth in a synergistic manner (Figure 4.6). Second, NAE18:2 and 9-NAE-HOD treatment elevated ABA-dependent gene expression in seedlings during the ABA-sensitive window of seedling development (Table 4.1, Table 4.2). Third, transcript levels for ABA synthesis genes, ABA1 and ABA2, were elevated in response to 9-NAE-HOD (Figure 4.9), and ABA content in seedlings was similarly increased (Figure 4.8). Fourth, mutants in ABA signaling, perception and synthesis (Figure 4.7) were insensitive to 9-NAE-HOD, indicating that ethanomide oxylipin action requires an intact ABA signaling pathway. Further, ABA treatment during the sensitive window of seedling development, increased endogenous levels of 9-NAE-HOD concomitant with growth inhibition (Figure 4.11), and vice versa (Figure 4.8). Finally, abiotic stresses during seedling establishment, known to involve ABA signaling, showed an association between root growth inhibition and increased levels of endogenous 9-NAE-HOD (Figures 4.13, 4.14); a reduction in 9-NAE-HOD formation in lox1 lox5 mutants showed a reduced elevation of ABA content and a partial reversal of stress-induced growth inhibition, providing direct evidence for the
involvement of 9-LOX activity, endogenous 9-NAE-HOD formation and ABA signaling during seedling establishment.

The inhibition of root elongation by 9-NAE-HOD was dose dependent to low micromolar concentrations when added exogenously (Figure 4.5). While these concentrations were higher than those measured in vivo, it should be emphasized that synergistic interactions with ABA (Figure 4.6) point to the likelihood that elevated endogenous, lower levels of 9-NAE-HOD combined with endogenous phytohormones, may effectively arrest growth during conditions that are unfavorable for seedling establishment. Indeed, treatment of seedlings with ABA alone induced increases in endogenous levels of 9-NAE-HOD and vice versa (Figures 4.8, 4.11). Further, abiotic stress (drought and salt) treatments of seedlings resulted in increased endogenous levels of both 9-NAE-HOD and ABA and this was sufficient to reduce seedling growth (Figures 4.13, 4.14). Seedlings of LOX double mutants with reduced capacity for 9-NAE-HOD formation, had lower endogenous levels of stress-induced ABA and the root growth of these seedlings was less inhibited by salt/drought compared with wild-type seedlings (Col-0) (Figure 4.13, 4.14). Overall, the results suggest that these two small molecules, ABA and 9-NAE-HOD, can influence the accumulation of each other, and may together participate to regulate the capacity of seedlings for ABA-dependent secondary dormancy. This represents a potential positive feedback where elevated levels of 9-NAE-HOD support transcriptional increases in ABA synthesis, signaling and response genes. We noted previously that NAE-LOX activity increases during immediate post-germinative seedling growth (Kilaru et al., 2011), consistent with the measured rise in NAE18:2 oxylipin levels. Others showed that OPDA (12-oxo-phytodienoic acid) could act synergistically with ABA to inhibit seed germination in Arabidopsis (Dave et al., 2011).
While there are some similarities to our studies in that oxylipins interact with ABA to influence seed physiology, there are differences that indicate the effects of OPDA and 9-NAE-HOD are distinct. Our studies here demonstrate the bioactivity of 9-LOX metabolites of NAE 18:2 (not 13-LOX metabolites of 18:3 like OPDA), and involve inhibition of root growth several days after germination, later than the stages characterized by Dave et al (2011). Nonetheless, in both cases oxylipin metabolites inhibit seed/seedling physiological processes, highlighting the need to consider the complexity of oxylipin metabolism in the modulation of seed germination and seedling growth. While more work needs to be done to identify the mechanism that integrates LOX metabolites of NAE with endogenous ABA signaling, our results demonstrate clearly that this interaction can be manipulated in a reciprocal and synergistic manner to dramatically influence the rate of seedling establishment.

The root-specific activity of 9-NAE-HOD (Table 4.1, Figure 4.9) is consistent with its targeted reduction in root elongation. In fact, shoot development was not nearly as affected as roots by NAE18:2 or the 9-NAE-HOD, suggesting that there are likely tissue specific differences in the regulation of secondary dormancy. Unlike here with NAE18:2, previous studies with NAE18:3 demonstrated a dual effect on seedling development, arresting the development of both roots and shoots—bleaching cotyledons and preventing chloroplast development even in the light. These effects of NAE18:3 were reversible and were attributed to LOX-mediated metabolites of NAE18:3—9- and 13-NAE-H(P)OT. Similar to 9-NAE-HOD, ABA treatment dramatically increased the accumulation of NAE18:3 oxylipin metabolites, but since the effects of NAE18:3 oxylipins were both on root elongation and cotyledon development, this suggests that a complex combination of LOX isozyme activities toward polyunsaturated NAEs may act
differentially in organs/tissues of the developing seedling in conjunction with ABA signaling to accomplish coordinated arrest of seedling establishment. Arabidopsis has six LOX genes (Feussner and Wasternack, 2002), and the proteins have different and sometimes overlapping peroxidation activities on polyunsaturated acyl substrates (Bannenberg et al., 2009; Kilaru et al., 2011). Other work has shown that various free fatty acid oxylipin metabolites can influence growth patterns of seedling roots, but it was not clear if these are related to ABA signaling (Vellosillo et al., 2007). Still, other, more recent research has suggested possible interactions of LOX1-mediated free fatty acid oxylipin formation and ABA signaling in plant defense (Vicente et al., 2012) or in stomatal regulation (Montillet et al., 2013). While ethanolamide conjugates were not implicated in these studies, the results may suggest that lipid oxidation pathways may more generally interact with or modulate ABA signaling pathways, providing for enhanced ways that plants can modulate their physiology than perhaps previously appreciated. While much future work will be required to sort out the complex role(s) of LOX isoforms and their metabolites in seedling establishment, our results point to an important activity for this family of enzymes and a group of minor acylamide substrates (NAEs) that can profoundly affect the phytohormone-mediated progression of seedling establishment.

Materials and methods

Chemicals

9S-hydroxy-10E,12Z-octadecadienoic-9,10,12,13-d4 acid (9-HOD d4), 9S-hydroxy-10E, 12Z-octadecadienoic acid (9-HOD), 13S-hydroxy-9Z,11E-octadecadienoic acid (13-HOD), NAE18:2, 9-
LOX from potato (Solanum tuberosum; 9-StLOX), and 13-LOX from soybean (Glycine max; 13-GmLOX) were purchased from Cayman Chemicals. Cis,trans-Abscisic Acid was purchased from Sigma-Aldrich. Cis,trans-Abscisic Acid -d6 was purchased from Santa Cruz Biotechnology.

**Plant Materials and Growth Assays**

FAAH1 OE and faah1 T-DNA knockouts (SALK_095108) were described in Wang et al. (2006). T-DNA knockouts of lox1 (SALK_038475) and lox5 (SALK_012188) were crossed to obtain the double mutant lox1 lox5, and the lox2 mutant was provided by E. Farmer (Glauser et al., 2009) as indicated previously (Keereetaweep et al., 2013). The pyr1 pyl2 pyl4 pyl5 pyl8 pentuple mutant was provided by P. Rodriguez. The ABA insensitive mutant, abi3-1 and ABA synthesis mutant, aba1-4, were obtained from the ABRC. Prior to sowing in liquid Murashige and Skoog medium (0.5x), Arabidopsis seeds were surface-sterilized, stratified for 3 d, germinated and grown in a 16-h-light/8-h-dark cycle (60 μmol m⁻² s⁻¹) at 20 to 22°C as previously described (Teaster et al., 2007). For growth inhibition assays and metabolite profiling, Arabidopsis seedlings were treated with NAE18:2, NAE-HODs or 0.07% DMSO as solvent control after 4 days. Seedling tissues were collected over a 7-d time course, flash-frozen in liquid nitrogen, and stored at -80°C until further analysis. To study the effects of exogenous NAE18:2/9-NAE-HOD on endogenous ABA level and effect of exogenous ABA on endogenous NAE18:2/9-NAE-HOD levels, seedlings were treated with NAE18:2, 9-NAE-HOD, ABA or 0.07% DMSO as solvent control on day 3. ABA concentrations were calculated based on the active cis-isomer. Images of seedlings were captured by using a Nikon digital camera D5100. Primary root lengths were measured using ImageJ.
Drought and salt treatments

Seedlings were subjected to drought and salt stresses at the specific developmental window when seedlings were sensitive to both 9-NAE-HOD and ABA. Conditions were similar to those described by Lopez-Molina et al, 2001 that induce secondary dormancy, but with a few modifications so that root growth after stress and metabolite and hormone levels could be quantified. For drought studies, seeds of wild-type (Col-0, Ler), lox1 lox5, pyr1, pyl2, pyl4, pyl5, pyl8 and abi3-1 were stratified in sterile deionized water at 4°C in the dark for 3 d and subsequently grown in controlled conditions as above for 3 d in liquid media. Three-d-old seedlings were then transferred onto filter paper (Fisher Scientific, PA) for 1 hour to simulate drought stress. Seedlings that were subjected to simulated drought stress or control seedlings (no drought treatment) were then transferred back to liquid media and continued to grow for additional 2 d as a recovery period. Seedlings were collected on d 3 (after simulated drought) and d 5 (the end of recovery period) for 9-NAE-HOD and ABA analysis and quantification of growth. For salt stress treatments, seeds of wild-type (Col-0, Ler), lox1 lox5, pyr1, pyl2, pyl4, pyl5, pyl8 and abi3-1 were stratified in sterile deionized water with or without 100 mM NaCl at 4°C in the dark for 3 d and subsequently grown in controlled conditions as above for 3 d in liquid media with or without 100 mM NaCl. Seedlings were then transferred to fresh media without NaCl to recover from salt stress and continued to grow for an additional 2 d. Seedlings were collected on d 3 (the end of salt treatment) and d 5 (the end of recovery period) for 9-NAE-HOD and ABA analysis and growth measurements.
Extraction and quantification of NAE18:2

Approximately 50 mg of desiccated seeds or 250 mg of seedlings were ground in warm isopropanol using bead beater. Deuterated NAE standards (1 ppm of D₄-NAE16:0 and D₄-NAE20:4) were added prior to the extraction. Total lipids were extracted into chloroform and further purified by solid phase extraction (SPE). Silica SPE cartridges (100 mg, 1.5 mL; Grace Davison Discovery Sciences) were conditioned with 2 mL methanol and followed by 4 mL chloroform. Samples suspended in 1 ml chloroform were loaded onto the column and then washed with 2 ml chloroform. NAE18:2 were eluted with 2 ml of ethyl acetate:acetone (1:1 v/v). The eluate was dried under nitrogen stream and derivatized with 50 µl BSTFA at 55°C for 30 min (Fisher Scientific, PA) (Kilaru et al., 2010). NAE18:2 was quantified as TMS derivatives against deuterated NAE16:0 standard. The quantification was carried out on Agilent GC 7890A/MSD 5975C system with a capillary HP-5 MS column (30 m x 0.250 mm, 0.25 mm coating thickness; Agilent technologies) in SIM mode as described previously (Venables et al., 2005).

Synthesis of NAE oxylipins

NAE-oxylipin standards were prepared by enzymatic synthesis using either and 13-GmLOX (13-LOX reactions) or 9-StLOX (9-LOX reactions) with NAE 18:2 as substrate. Enzyme reactions and identification of oxylipin products by GC-MS were carried out as previously described (Keereetaweep et al., 2013).
**Extraction and separation of oxylipins**

Ethanolamide-conjugated- and free oxylipins were extracted as described previously (Kilaru et al., 2011; Christensen et al., 2013) with some modifications (Keereetaweep et al., 2013). Using approximately 5 g fresh weight of Arabidopsis seedlings and deuterated internal standard (100 ng of 9-HOD-d₄) for quantification. Extracted oxylipins were separated by reverse-phase-HPLC carried out on 150 x 4.6 mm, 5 µm particle size, C18 Nucleosil 120-5 column (Macherey-Nagel, PA) as described previously (Keereetaweep et al., 2013). The conjugated diene system was monitored by UV detection at 234 nm. The eluate was collected from 9-12 min and 14-17 min for NAE-HODs and free HODs, respectively. In case of NAE-oxylipin standards, fractions collected from reverse phase-HPLC were further separated by normal phase-HPLC (Kilaru et al., 2011; Keereetaweep et al., 2013). The stereochemistry of the reactions were verified using chiral phase (CP)-HPLC (CHIRALCEL™ OD-H, 2.1x 150 mm, Diacel Chemical Industries, Ltd, Osaka, Japan) and oxidative products generated by enzymatic reaction were distinguished from products formed by auto-oxidation.

**Identification and quantification of NAE- and free oxylipins**

NAE- and free oxylipins were identified by GC-MS as TMS derivatives using Agilent GC 7890A/MSD 5975C system with a capillary HP-5 MS column (30 m x 0.250 mm, 0.25 mm coating thickness; Agilent technologies) in full mass scan mode (Keereetaweep et al., 2013). Identification was based on characteristic molecular and fragment ions in comparison with known and synthetic standards. NAE- and free oxylipins were quantified by GC-MS in SIM mode. Standard curves were generated from 9-HOD-d₄, 9-HOD, 13-HOD. NAE-oxylipins were
quantified against corresponding FFA standard curves using 9-HOD-d4 as the internal standard.

The characteristic fragmentation patterns along with the diagnostic and quantitative ions (m/z) of oxylipins are summarized in Figure 4.16 and Table 4.3.

**ABA quantification**

Approximately 100 mg of desiccated Arabidopsis seeds or seedlings were extracted in imidazole buffer (30 mM, pH 7 in 70% isopropanol). The extracts were pre-purified by solid phase extraction (NH₂-SPE columns, Grace Davison Discovery Science, IL) and fractionated by reverse-phase HPLC using a 150x4.6 mm C18, Nucleosil 120-5 (Macherey-Nagel, PA). ABA was quantified against a D6-ABA standard by GC–MS as methyl ester (derivatized in ethereal diazomethane) (Kilaru et al., 2007a).

**Real Time Quantitative RT-PCR**

Total RNA from seedling, shoots or roots was isolated using an RNeasy Mini Kit (Qiagen, CA).

Quantification of transcripts were carried out on Smart Cycler II (Cepheid, CA) instrument using a One Step Ex Taq™ qRT-PCR Kit (Takara-Bio, CA) with SYBR Green I (Life technologies, NY).

Gene-specific primers used are as follows; ABA1 (At5g67030) (F) 5’-

TCGAAGATGATGCTCGTGT-3’ (R) 5’-TGTCGGACGATCTAAACCGC -3’, ABA2 (At1g52340) (F) 5’-

GTGAGGCACATACGAGGGA-3’ (R) 5’-CGTCTCCTTGGACTCACCCAC-3’, ABI3 (At3g24650) (F) 5’-

AGAAACCGATGGCGAGACAG -3’ (R) 5’- CCACCGGGTTAAGTTGTGGA -3’, HVA22B (At5g62490) (F) 5’-

5’-ACGAGCCTATATGTGCGTAC-3’ and (R) 5’ GCCGCACCAGGAATCCCTA-3’, ATEM1 (At3g51810) (F) 5’-

5’-CCTGCTTGTAGGTTCCGTGT-3’ (R) 5’- ACGGTACAACACTGCCGTTA-3’, and CRA1 (At5g44120) (F) 5’-CGTTCAGGCTGAGGAG-3’ (R) 5’-TCCCATAAGACCTCGTCCCT-3’. Relative
transcript levels were normalized using 18S (F) 5’-AAGGGCGAACACTCATCGTT-3’ (R) 5’-TGGAATTTCGTGTGGAGGG-3’ (Dean Rider et al., 2003). Relative expression levels were calculated using cycle threshold (Ct) method (Livak and Schmittgen, 2001).

Acknowledgments

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Table 4. 1. Comparison of fold changes in ABI3 transcript abundance in 50 μM NAE18:2 or 25 μM 9-NAE-HOD treated Col-0 wild-type (or lox1 lox 5) seedlings at 7 d relative to untreated seedlings.

<table>
<thead>
<tr>
<th></th>
<th>ABI3 transcript</th>
</tr>
</thead>
<tbody>
<tr>
<td>4d + 3d NAE18:2 (seedlings, Col-0)</td>
<td>25.2 ± 2.8</td>
</tr>
<tr>
<td>4d + 3d 9-NAE-HOD (seedlings, Col-0)</td>
<td>49.5 ± 4.3</td>
</tr>
<tr>
<td>4d + 3d NAE18:2 (seedlings, lox1 lox5)</td>
<td>7.0 ± 0.6</td>
</tr>
<tr>
<td>4d + 3d 9-NAE-HOD (seedlings, lox1 lox5)</td>
<td>54.9 ± 6.0</td>
</tr>
<tr>
<td>4d + 3d NAE18:2 (shoots, Col-0)</td>
<td>1.5 ± 1.1</td>
</tr>
<tr>
<td>4d + 3d NAE18:2 (roots, Col-0)</td>
<td>18.2 ± 2.3</td>
</tr>
<tr>
<td>4d + 3d 9-NAE-HOD (shoots, Col-0)</td>
<td>2.1 ± 2.2</td>
</tr>
<tr>
<td>4d + 3d 9-NAE-HOD (roots, Col-0)</td>
<td>31.5 ± 4.1</td>
</tr>
</tbody>
</table>

Values for transcripts were presented as means ± S.D. of three biological replicates (normalized to 18S rRNA). Higher ABI3 transcript levels were directly associated with more growth inhibition.
Table 4.2. Comparison of fold changes in transcript abundance in 9-NAE HOD/NAE18:2-treated wild-type seedlings at 7 days relative to untreated seedlings.

<table>
<thead>
<tr>
<th></th>
<th>Fold Changes in Transcript Abundance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>added on d0</td>
</tr>
<tr>
<td></td>
<td>50 μM NAE 18:2 25 μM 9-NAE-HOD</td>
</tr>
<tr>
<td>ABI3</td>
<td>3.2 ± 0.5 4.5 ± 0.6</td>
</tr>
<tr>
<td>HVA22B</td>
<td>3.7 ± 0.7 2.3 ± 0.3</td>
</tr>
<tr>
<td>ATEM1</td>
<td>2.9 ± 0.5 4.6 ± 0.7</td>
</tr>
<tr>
<td>CRA1</td>
<td>4.2 ± 0.8 3.1 ± 0.7</td>
</tr>
</tbody>
</table>
Table 4.3. Diagnostic ions of NAE18:2-oxylipins and corresponding FFA as detected by GC/MS.

<table>
<thead>
<tr>
<th>Qion</th>
<th>[M⁺]</th>
<th>[M⁺ - CH₃]</th>
<th>Rₜ</th>
</tr>
</thead>
<tbody>
<tr>
<td>9-HOD D4</td>
<td>318</td>
<td>444</td>
<td>429</td>
</tr>
<tr>
<td>9-HOD</td>
<td>317</td>
<td>438</td>
<td>423</td>
</tr>
<tr>
<td>13-HOD</td>
<td>369</td>
<td>438</td>
<td>423</td>
</tr>
<tr>
<td>9-NAE-HOD</td>
<td>360</td>
<td>481</td>
<td>466</td>
</tr>
<tr>
<td>13-NAE-HOD</td>
<td>412</td>
<td>481</td>
<td>466</td>
</tr>
</tbody>
</table>
Figure 4.1. Summary of NAE 18:2 metabolism via hydrolysis, the 9-LOX or the 13-LOX pathways.

NAE18:2 undergoes 13- and/or 9-LOX mediated oxidation to generate corresponding hydroperoxides and further reduced to hydroxides. 13-NAE-HPOD is also predicted to be further metabolized by allene oxide synthase (AOS) to generate unstable epoxide which further converted to more stable ketol. 9-NAE-HPOD, (9S,12Z,10E)-9-hydroperoxy-10,12-octadecadienoylethanolamide; 13-NAE-HOPD, (13S,9Z,11E)-13-hydroperoxy-9,11-octadecadienoylethanolamide; 9-NAE-HOD, (9S,12Z,10E)-9-hydroxy-10,12-octadecadienoylethanolamide; 13-NAE-HOD, (13S,9Z,11E)-13-hydroxy-9,11-octadecadienoylethanolamide; 12,13S-epoxy-NAE 18:2, (13S, 9Z, 11E)-12,13-epoxy-9,11-octadecadienoylethanolamide.
Figure 4. 2. Exogenous NAE 18:2 inhibits root elongation in seedlings.

Arabidopsis seedlings (wild-type Col-0, faah1 KO, FAAH1 OE, lox1 lox5 and lox2) grown for 4 days in liquid media were transferred into media containing 50 μM NAE 18:2 or DMSO and continued to grow for 72 hr. (a) shows phenotypes observed with exogenous NAE18:2 treatment. (b) shows %reduction of primary root length of seedling treated with NAE 18:2 compared with control (n=30).
Figure 4.3. Dramatic growth inhibition was observed in NAE18:2-treated genotypes that accumulate 9-NAE-HOD.

Arabidopsis seedlings grown for 4 days in liquid media were transferred into media containing DMSO or 100 μM NAE 18:2 and continued to grow for 72 hr. Tissues were collected at 72 hr after addition of NAE18:2 (or DMSO) for oxylipin analysis. NAE and FFA oxylipins were quantified as TMS-derivatives by GC/MS and presented as means ± S.D. of three biological replicates.
* indicates a significant difference compared with wild-type seedlings determined by student’s t test ($P < 0.05$).

** indicates a significant difference compared with wild-type seedlings determined by student’s t test ($P < 0.01$).
Figure 4. Primary root elongation was affected by 9-NAE-HOD, but not 13-NAE-HOD.

Arabidopsis seedlings grown for 4 days in liquid media were transferred into media containing 50 μM NAE18:2, 25 μM 9-NAE-HOD, 25 μM 13-NAE-HOD or DMSO and continued to grow for 72 hr. Effect of NAE 18:2 and 9-NAE-HOD was overcome by overexpression of FAAH in FAAH1 OE.

* indicates a significant difference compared with wild-type seedlings determined by Student’s t test (P < 0.05).

** indicates a significant difference compared with wild-type seedlings determined by Student’s t test (P < 0.01). Values are presented as means of n=40 ± SD.
Figure 4.5. 9-NAE-HOD inhibits primary root elongation in concentration-dependent manner.

Arabidopsis seedlings (wild-type Col-0) grown for 4 days in liquid media were transferred into media containing different micromolar concentration of 9-NAE-HOD or DMSO and continued to grow for 72 hr. Primary root lengths were measured and presented as means ± S.D. of n = 40.
Figure 4.6. NAE18:2 and ABA negatively regulates seedling growth.

Wild-type Arabidopsis seedlings at different age were treated with 10 μM NAE18:2, 0.5 μM ABA or combination of NAE18:2 and ABA or DMSO as control. ABA concentration is calculated from cis-isomer. Images represent 10d-old seedling with treatment added on different days after sowing. The synergistic inhibition between NAE18:2 and ABA occurs within a narrow developmental window.
Figure 4. 7. Growth arrest by NAE18:2 and 9-NAE-HOD requires an intact ABA signaling pathway.

Arabidopsis seedlings were treated with 50 μM NAE18:2/25 μM 9-NAE-HOD/5 μM ABA or 0.07% DMSO (control) on d3. ABA synthesis mutants (aba1-4; in Landsberg background), ABA receptor mutant (pyr1 pyl2 pyl4 pyl5 pyl8; in Columbia background) and ABA insensitive mutant (abi3-1; in Landsberg background) showed tolerance to NAE18:2 and 9-NAE-HOD.

(a) shows phenotypes observed on d7. (b) shows primary root length of 7d-old seedlings (n=30).
Figure 4. Exogenous NAE18:2/9-NAE-HOD affects endogenous levels of ABA.

Wild-type and lox1 lox5 Arabidopsis seedling grown for 3 days in liquid media were treated with 25 µM 9-NAE-HOD/50 µM NAE18:2 or DMSO and continued to grow for 7 days. ABA levels are presented as means ± S.D. of three biological replicates. The higher concentration of ABA is associated with more pronounced growth retardation.
Figure 4. 9-NAE-HOD induced ABA1 and ABA2 expression in both wild-type and the lox1 lox5 mutant.

Comparison of fold changes in ABA1, ABA2 transcript abundance in 9-NAE-HOD/NAE18:2 treated Col-0 wild type and lox1lox5 seedlings at 7 d relative to untreated seedlings. Values for transcripts were presented as means ±S.D. of three biological replicates (normalized to 18S rRNA).
Figure 4. NAE18:2 levels decreased, accompanied by a transient increase in 9-NAE-HOD during normal seed germination and seedling establishment.

(a) NAE18:2 levels declines during seed germination and seedling development.

(b) NAE18:2-oxylipins levels rise and fall during seed germination and seedling development.

NAE18:2, 9- and 13-NAE-HOD in desiccated seeds and seedlings grown in liquid media were quantified as TMS-derivatives by GC/MS. Values are presented as means ± S.D. of three biological replicate.
Figure 4.11. The exogenous ABA is associated with the increase of endogenous 9-NAE-HOD and growth retardation.

Wild-type and lox1 lox5 Arabidopsis seedling grown for 3 days in liquid media were treated with 5 µM ABA or DMSO and continued to grow for 7 days. 9-NAE-HOD levels are presented as means ± S.D. of three biological replicates.
Figure 4.12. Comparison of fold changes in LOX transcript abundance in 7-day-old lox1 lox5 seedlings treated with 0.5 μM ABA, 10 μM NAE18:2 or both.

Values for transcript levels were presented as means ±S.D. of three biological replicates.
Simulated drought stress suggested ABA-dependent secondary dormancy by ABA and 9-NAE-HOD working together.

Wild-type (Col and Ler), lox 1 lox5, pyr1 pyl2 pyl4 pyl5 pyl8 and abi3-1 seedlings grown for 3 days in liquid media were dried on filter paper for 1 h and then transferred to liquid media and continue to grow for 2 days. (a) shows phenotypes observed in lox1 lox5 mutant and ABA mutants compared with their respective wild-type (b) shows primary root length (n=30) (c) 9-NAE-HOD levels are presented as means ± S.D. of three biological replicates (d) ABA levels are presented as means ± S.D. of three biological replicates.

* indicates a significant difference compared with wild-type seedlings determined by student’s t test (0.01 < P < 0.05).
** indicates a significant difference compared with wild-type seedlings determined by student’s t test (0.001 < P < 0.01).

*** indicates a significant difference compared with wild-type seedlings determined by student’s t test (P < 0.001).
Figure 4. 14. Salinity suggested ABA-dependent secondary dormancy by ABA and 9-NAE-HOD working together.

Wild-type (Col and Ler), lox1 lox5, pyr1pyl2pyl4pyl5pyl8 and abi3-1 seeds were kept in the dark with or without NaCl for 3 days at 4°C and then grown for 3 days. Three day seedlings were then transferred to fresh media and continue to grow for 2 days. (a) shows phenotypes observed in lox1 lox5 mutant and ABA mutants compared with their respective wild-type (b) shows primary root length (n=30) (c) 9-NAE-HOD levels are presented as means ± S.D. of three biological replicates (d) ABA levels are presented as means ± S.D. of three biological replicates * indicates a significant difference compared with wild-type seedlings determined by student’s t test (0.01 < P < 0.05).
** indicates a significant difference compared with wild-type seedlings determined by student’s t test (0.001 < P < 0.01).

*** indicates a significant difference compared with wild-type seedlings determined by student’s t test (P < 0.001).
**Figure 4.15.** Highly simplified model for the interaction of NAE metabolism with ABA signaling in the control of secondary dormancy and seedling establishment.

NAEs are hydrolyzed by FAAH to free fatty acids or oxidized by lipoxygenases (LOX1 and LOX5 are 9-LOXes introducing a hydro(per)oxy group into NAE18:2 to generate 9-NAE-H(P)OD).

Elevated levels of ethanolamide oxylipin intermediates within a narrow window of seedling development can arrest growth via ABA-dependent mechanism. The cooperative metabolic depletion of NAEs leads to successful seedling establishment, but provides for fine regulation of development depending upon favorable environmental conditions. This hypothetical model is derived from evidence presented in this manuscript.
**Figure 4.16.** TMS derivatives of free and ethanolamide-conjugated oxylipin metabolites.

(a) TMS derivative of 9-hydroxy-10,12-octadecadienoylethanolamide (9-NAE-HOD).

(b) TMS derivative of 13-hydroxy-9,11-octadecadienoylethanolamide (13-NAE-HOD).

(c) TMS derivative of 9-hydroxy-10,12-octadecadienoic acid (9-HOD).

(d) TMS derivative of 13-hydroxy-9,11-octadecadienoic acid (13-HOD).
References


CHAPTER 5

SUMMARY AND SIGNIFICANCE

The actions of N-acylethanolamines (NAEs) have most often been attributed to the acylethanolamide molecule itself, and a fatty acid amide hydrolase has been presumed to be the major means of terminating lipid mediator function in both animal and plant systems (Wang et al., 2006; Ahn et al., 2008). However, several reports on the oxidative metabolism of PU-NAEs in mammalian systems have suggested that oxidized metabolites may include various signaling compounds that can participate in several physiological processes (Edgemond et al., 1998; Kozak et al., 2002; Kozak et al., 2004; Sang and Chen, 2006). In plant systems, the oxidative metabolism of PU-NAEs, especially its formation of bioactive metabolites, has received far less attention to date. In plants, NAEs occur at the highest levels in desiccated seeds of various plant species, and their rapid depletion is required for normal seedling establishment (Chapman, 2004; Kilaru et al., 2007; Blancaflor et al., 2014). The decrease of NAEs observed during seed germination and seedling development was significantly higher with the over-expression of FAAH. However, in seedling of faah knockouts, although the hydrolysis activity was compromised, the levels of NAEs still declined dramatically throughout seedling establishment. This indicated that NAE depletion is not entirely dependent on hydrolysis, and that there may be alternative pathways that support depletion of NAEs.

Markedly higher levels of PU-NAEs were measured in seedlings when grown NAE12:0 or NDGA, a potent but non-selective LOX inhibitor. The reduction of seedling growth was more pronounced in faah KO seedlings (compared to wild type seedlings), and growth inhibition was
reversed by FAAH over-expression. At the time, this was interpreted as FAAH mediated hydrolysis being important for NAE depletion. On the other hand, commercially available 9-LOX (potato) and 13-LOX (soybean) enzymes utilized PU NAEs as substrates, and exhibited typical Michaelis-Menten kinetics when PU-NAEs and PUFAs were used as substrates. These in vitro results pointed to the plausibility that PU-NAEs may serve as substrates for LOXs in vivo. Following the kinetics studies, we tested the effects of NAE12:0 and FFA12:0 on the LOX activities. Double-reciprocal plots of saturation kinetics with low micromolar concentrations of both lipids showed that they inhibited LOX activities in competitive manner. In general, NAE12:0 was a more potent inhibitor than FFA for both types of LOX enzymes, and also inhibited LOX activity in Arabidopsis in vitro and in vivo. Collectively these results, along with previous reports from Shrestha et al., 2002, and Van Der Stelt et al., 2000, suggested that oxidation of PU-NAEs may be more important than previously appreciated.

Although the results showed that NAE12:0 can inhibit LOX activities and formation of lipid hydroperoxide, it should be emphasized that LOX activities are inhibited but not eliminated. Other than increasing endogenous PU-NAEs, exogenous NAE12:0 was also shown to increase endogenous PE and NAPE in Arabidopsis seedlings to the level observed in desiccated seeds and 2-3 times higher than desiccated seeds, respectively (Kilaru et al., 2012). Thus, the effects of 12:0 on seedling growth documented here can be a combination of various pathways it influences.

While PU-NAEs are the major species of NAEs in desiccated seeds, previous experiments of NAE action were mostly performed with exogenously applied NAE12:0. Thus, much of our knowledge and understanding of how NAEs affect physiological processes in plants came from
NAE12:0 with a focus on FAAH-mediated hydrolysis. Here, it is clear that an important aspect of NAE metabolism features the endogenous formation of LOX-mediated metabolites of NAE18:2 and NAE18:3 in Arabidopsis seedlings. The complexity of PU-NAE metabolism which involves the competition between hydrolysis by FAAH and oxidation by 9- or 13-LOX was illustrated through the use of various faah and lox mutants. Relative partitioning of PU-NAEs into different branches of oxylipin metabolites was also associated with stage specific regulation of seedling development, and showed for the first time that bioactive NAE oxylipins are formed in plants.

When seedlings were grown in elevated level of NAE18:3, a distinct visible phenotype was observed. Cotyledons of the seedlings bleached and lost their extractable chlorophyll and visible, organized thylakoid membranes. This phenotype was only specific to NAE18:3 and was not observed in either NAE18:2 or FFA18:3 treated seedlings. In addition, these effects of NAE18:3 were organ- and stage-specific; Bleaching was evident in cotyledons only between three and five days after sowing, and did not occur in emerging true leaves.

Among the selected FAAH and lox mutants, FAAH OE was the only genotype that did not show any abnormality in cotyledon development in elevated NAE18:3. While hydro(pero)xides levels of NAE18:3 were lower in resistant FAAH OE seedlings than other genotypes, the corresponding hydro(pero)xides of FFA were significantly higher than other seedlings, indicating that the bleaching phenotype was unlikely associated with FFA or FFA oxylipin metabolites. When NAE12:0 was added to the liquid media at the same time as NAE 18:3 as a general LOX inhibitor, the hydro(pero)xide formation and level of bleaching of cotyledons of wildtype seedlings in elevated NAE18:3 became less with higher concentrations of NAE12:0. This inverse relationship between the hydro(pero)xide of NAE18:3 levels and severity of bleaching of
cotyledon supported that the effects of NAE18:3 on seedling cotyledons was likely associated with the hydro(pero)xides of NAE18:3. LOX mutants that were compromised in either 9-LOX activity (lox1 lox5) or 13-LOX activity (lox2) still showed visible bleaching phenotypes pointing to the possibility that the active molecules that are responsible for NAE18:3 effects on seedling cotyledon might be metabolites from both of the 9- and 13-LOX pathways. Indeed addition of either 9- or 13-NAE-HOTs to seedlings showed bleaching, although not quite as severely as NAE18:3 itself, which was interpreted as due to differences in uptake or transport of these LOX metabolites. Still, it was clear that acylethanolamide hydro(pero)xides derived from NAE18:3 were potent negative regulators of cotyledon greening.

Other inhibitory effects of 9- and 13-NAE-HOTs were observed in seedlings such as marked reductions in root elongation. The addition of ABA to developing seedlings also arrested root development (as expected), and ABA induced a dose-dependent increase in the endogenous levels of both 9- and 13-NAE-HOT (with progressively more severe inhibition of root elongation), suggesting that at least part of the inhibitory effects of NAE 18:3 oxylipins on seedlings might be explained by an association with ABA regulation. On the other hand, the lack of cotyledon bleaching in ABA, suggests that other, as yet undiscovered pathway(s), may interact with NAE hydro(pero)xides to regulate chloroplast development. Nevertheless, my data collectively support the conclusion that LOX-derived oxylipins of NAE18:3 can negatively influence different aspects of seedling development, and may suggest that NAE metabolism is part of mechanism to synchronize events during Arabidopsis seedling establishment to assure that chloroplast development in cotyledons and the overall seedling growth are coincident.
The most abundant specie of NAEs in desiccated seeds of Arabidopsis, NAE18:2, also shares overlapping effects on seedling growth with NAE18:3 within a narrow developmental window. Unlike NAE18:3, the effects of NAE18:2 are more specific to seedling roots. When seedling were subjected to elevated NAE18:2, seedling roots of lox1 lox5 and FAAH OE were less affected compared to those of wild type. The metabolite profiles of these two genotypes (lox1 lox5 and FAAH OE) both showed a reduction in the accumulation of 9-NAE-H(P)OD in the presence of elevated NAE18:2. The NAE18:2-tolerant phenotype and the reduction of 9-NAE-H(P)OD suggested that 9-NAE-H(P)OD was the key metabolite that might be attributed to inhibition of seedling root growth by NAE18:2.

NAE18:2 also was shown to arrest seedling growth in synergistic manner with ABA which was most visible during d 3-4 after sowing. The synergistic action between NAE18:2 and ABA could potentially result from the induction of their biosynthesis as evident in the increased endogenous levels of 9-NAE-HOD when seedlings were subjected to elevated ABA during the narrow developmental window of sensitivity and vice versa. Indeed, transcript levels of ABA synthesis genes, ABA1 and ABA2, were increased in response to 9-NAE-HOD where endogenous ABA levels were also elevated. In addition, NAE18:2/9-NAE-HOD treatment resulted in the induction of ABA-dependent gene expression, supporting interaction between NAE18:2/9-NAE-HOD and ABA signaling pathway.

When the mutants in ABA signaling, perception and synthesis were grown in elevated NAE18:2/9-NAE-HOD, no marked inhibition of seedling root growth was observed, indicating that an intact ABA signaling pathway is required for inhibition by NAE18:2 metabolites. Further, when seedlings were subjected to abiotic stress conditions known to activate the ABA signaling
pathway, salinity and drought (Lopez-Molina et al., 2001), the severity of seedling root growth inhibition was directly associated with increasing levels of endogenous 9-NAE-HOD. In both salt stress and simulated drought stress, 9-NAE-HOD formation in lox1 lox5 mutant, where 9-LOX activity was compromised, was lower than wild type seedlings, resulting in a reduced increase in endogenous ABA a partial tolerance to the stress conditions. Together, the evidence of interactions between 9-NAE-HOD and ABA suggests the potential positive feedback mechanism where these two bioactive molecules induce the formation of each other and influence the seedling capacity for ABA-dependent secondary dormancy.

To conclude, the rapid depletion of PU-NAEs during seedling growth is, in part, accomplished by LOX-mediated pathway(s). Under normal seedling establishment, NAEs in desiccated seeds are substantially decreased and NAE oxylipin levels are kept low, or increase only transiently at the early stages of seedling development. When levels of these NAEs or their oxylipin metabolites are artificially elevated in a narrow window of seedling development, visible seedling abnormalities are observed, which overall result in a delay of seedling establishment. Here we showed that while NAE18:2 mainly arrested seedling root development, NAE18:3 affects both root and shoot development. Seedlings have the ability to arrest growth in the early stages of their establishment if the growth conditions are unfavorable. We propose that LOX-mediated NAE metabolism is part of the activation of secondary dormancy which negatively regulates seedling establishment through the interaction with various phytohormones, like ABA signaling pathways. The fact that 9-NAE-H(P)OD only arrested seedling root growth while 9-, 13-NAE-H(P)OT affected both seedling root and chloroplast development also suggests that perhaps different LOX isozymes are also associated
with organ-specific activities of PU-NAEs. While several aspects of NAE metabolism require further studies, our results suggested that the LOX-mediated products of PU-NAEs represent an additional set of bioactive metabolites that act in opposition normal seedling establishment by influencing seedling root and chloroplast development during the embryo-to-seedlings transition.
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