Ethanolamide Oxylipins of Linolenic Acid Can Negatively Regulate Arabidopsis Seedling Development

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N-Acylethanolamines (NAEs) are fatty-acid derivatives with potent biological activities in a wide range of eukaryotic organisms. Polysaturated 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 lipooxygenase (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 iox 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 that 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 (Glycine max) lecithin and peanut (Arachis hypogaea) 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 abscisic acid (ABA) signaling through a key regulator of the embryo-to-seedling transition, ABSCISIC ACID INSSENSITIVE3 (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 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 three double bonds (Chapman, 2004). In most seeds, the majority of NAEs are polyunsaturated species: acylethanolamides of linoleic and a-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 (Gossypium hirsutum; ~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 homolog of the mammalian FAAH was identified (Shrestha et al., 2003), designated 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 ~30% higher than the wild type (Columbia-0), while At-FAAH1 overexpressing lines (FAAH1 OE) have substantially lower NAE levels than the 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 with the wild type. Furthermore,
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 (Velillosio 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 LOX pathway in vitro (Van Der Stelt et al., 2000; Shrestha et al., 2002; Keereetaweep 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. Gas chromatography–mass spectrometry (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 (AOS). 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 postgerminative 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 the wild type, presumably attributable to higher flux through the oxidation pathway in the absence of FAAH1 expression. Identification of endogenous NAE 18:3 oxylipin metabolites remained elusive because of the lower levels of endogenous NAE 18:3 compared with 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 (Kilaru et al., 2011). 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; Keereetaweep et al., 2010; Kilaru et al., 2011), further work is required to indicate that these

Figure 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 Supplemental Figure 1 online.
(A) TMS derivatives of 9-hydroxy-10,12,15-octadecatrienoyl ethanolamide (9-NAE-HOT).
(B) TMS derivatives of 13-hydroxy-9,11,15-octadecatrienoyl ethanolamide (13-NAE-HOT).
(C) TMS derivatives of (13S,9Z,10E,15Z)-12-oxo-10,15-phytodienoyl ethanolamide.
(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.
[See online article for color version of this figure.]
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) GC-MS. Through comprehensive metabolite profiling of NAE 18:3 oxylipins and 18:3 FFA 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 d 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 trimethylsilyl (TMS) derivatives by comparing their fragmentation patterns with those of synthesized standards.

Figure 2. 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.

(A) 9-NAE-HOT.
(B) 13-NAE-HOT.
(C) NAE-OPDA.
The molecular ions [M+] for TMS derivatives of NAE-OPDA (for 12-oxo phytodienoic acid) and 9/13-NAE hydroxy-octadecatrienylethanolamides (HOTs) were mass-to-charge ratio (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. Mass spectrometry characteristics of these metabolites are summarized in Figure 1 and Supplemental Table 1 online. Identification of oxylipins in extracts was initially performed by comparing spectra to those for standards (see Supplemental Figure 1 online) 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 d after sowing (Figure 2). These time points were selected for comparison based on phenotypic changes toward exogenous NAEs (described below). Both 9- and 13-hydroxides were identified

Figure 3. 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.
(A) 9-NAE-HOT.
(B) 13-NAE-HOT.
(C) NAE-OPDA.
and quantified in seedlings, indicating the activity of both 9- and 13-LOX enzymes toward endogenous NAE 18:3. A cyclized (13S,9Z,10E,15Z)-12-oxo-10,15-phytodienoic acid (OPDA) ethanamide (NAE OPDA) was identified and quantified, indicating that endogenous Arabidopsis AOS and allene oxide cyclase (AOC) metabolized in vivo the ethanamides 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 (see Supplemental Table 2 online), suggesting that seedling development is most likely the stage at which these ethanamide 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 2). 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., 2007), both showed significantly higher levels of NAE 18:3–derived oxylipins at all time points in seedlings. By contrast, seedlings overexpressing 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 LIPOXYGENASE1 (LOX1) and LOX5 are 9-LOX enzymes (Kilaru et al., 2011). A double mutant lox1 lox5 still showed some formation of 9-hydroxides (Figure 2A). 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 2B) 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, lox2, showed less obvious differences in endogenous LOX-derived metabolites. The lox2

Figure 4. 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 ± so of three biological replicates. FW, fresh weight.

(A) 9-HOT.
(B) 13-HOT.
(C) OPDA.
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 (NAE 18:3)**

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 d (Figure 3). 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), 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 9-NAE HOT relative to the wild type (Figure 3A) and markedly elevated levels of 13-LOX derived

![Image](image_url)

**Figure 5.** Free 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 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 Students t test (P < 0.05). A double asterisk indicates a significant difference compared with wild-type seedlings, which was determined by Students t test (P < 0.01). FW, fresh weight.

(A) 9-HOT.
(B) 13-HOT.
(C) OPDA.
ethanolamide metabolites (Figures 3B and 3C). 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 9-NAE 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.

**FFA 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 (cf. Figures 4 and 2). Unlike the ethanolamide oxylipins, there was little difference in steady-state levels of these FFA oxylipins in seedlings regardless of genotype (Figure 4), and these levels generally were consistent with other reports of free oxylipins in Arabidopsis seedlings (Przybyla et al., 2008; López et al., 2011; Kim et al., 2012). In sharp contrast with steady-state levels, there were marked differences in these FFA oxylipins among the mutants when supplied with exogenous NAE 18:3 (Figure 5). Four-day-old seedlings were supplied with NAE 18:3 for 3 d 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 5). 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 FFA oxylipin pools, but at present this has not been resolved.

**Seedling Phenotypes in Exogenous Linolenoylethanolamide (NAE 18:3)**

Addition of NAE 18:3 induced a dramatic phenotype in wild-type Arabidopsis seedlings (Figure 6). Seedling cotyledons bleached in 3d following addition of NAE 18:3 in the wild type, faah muts, and lox mutants, but not in FAAH OE lines (Figure 6A). This visible phenotype was quantified at the biochemical level as a reduction in extractable chlorophyll a and chlorophyll b (Figure 6B), compared with seedlings without NAE 18:3 (Figure 6C). The lack of bleaching in FAAH 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 7) (Blancaflor et al., 2003; Teaster et al., 2007).

Effects of NAE 18:3 on seedling development were stage specific (see Supplemental Figure 2 online). Sensitivity to NAE 18:3 was observed up to about 6 d 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 (see Supplemental Figure 3 online), 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 8). These effects of chlorophyll degradation were specific for NAE 18:3 (Figure 8B). 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 d of exposure (Figures 8C and 8D). 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 (pxa1 and kat2; Kunz et al., 2009), indicating that a functional β-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 et al. (2009), attributable to accumulation of FFAs, was likely not responsible for the NAE 18:3–specific bleaching observed here in cotyledons of seedlings.

Figure 7. 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. [See online article for color version of this figure.]

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Figure 8. 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.
Seedling bleaching by NAE 18:3 was reversible (Figure 9). When seedlings were transferred to growth medium without NAE 18:3, cotyledons regreened, and chlorophyll content and autofluorescence were restored over a period of 4 d (Figure 9). This indicates that NAE 18:3 did not induce a toxic, irreversible effect. Even faah1 knockout seedlings, which showed a more rapid and severe response to NAE 18:3 relative to the wild type, were able to recover at the cellular- and whole-seedling levels when NAE 18:3 was withdrawn (Figure 9). 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 (see Supplemental Figure 4 online). 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 regreening.

Seedling Phenotypes are Due to a LOX-Derived Ethanolamide Oxylipin

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 (Keereetaweep 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 10 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 11) 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 11C). Quantification of ethanolamide and FFA oxylipins confirmed the inhibition of LOX in vivo by NAE 12:0.
under these conditions (Figure 12). 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 13). 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 hydroperoxides. Because of 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 attributable to its greater accessibility (it is less polar than the oxylipins) to internal plant tissues.

Endogenous Ethanolamide Oxylipins Are 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 extractable oxylipins could be observed. Chua and coworkers (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 (see Supplemental Figure 2 online), 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 four additional days of exposure (Figure 14). 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 signaling- 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 CYCLO-OXYGENASE-2 (COX-2) (Kozak et al., 2002, 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, 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 (CYP) 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 2). 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 15 summarizes the results taken together from steady state metabolite profiling experiments for both ethanolamide and FFA oxylipins (Figures 2 to 5 and 12) and illustrates the relative partitioning and metabolic directions in the various mutant backgrounds. Whereas these pathway metabolites were identified during normal seedling growth (Figures 2 and 4), their steady-state levels could be exaggerated by the addition of exogenous NAE 18:3 (Figures 3 and 5), helping to clarify these conclusions. Pharmacological inhibition of LOX activity by the addition of the competitive inhibitor, NAE 12:0, demonstrated

![Figure 11. NAE 12:0 Reversed the Effects of NAE 18:3 in a Dose-Dependent Manner.](image)

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.

[See online article for color version of this figure.]

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 11). 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

[See online article for color version of this figure.]
further the metabolism in planta of NAE 18:3 by LOX pathway enzymes (Figure 12; 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) because of its stability, but this NAE is among the least-abundant NAE types in desiccated Arabidopsis seeds, especially compared with 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 d after sowing bleached and lost chlorophyll. This effect was specific for NAE 18:3 and was organ (cotyledon) and stage specific (see Supplemental Figure 3 online). FAAH1 OE did not show the bleaching phenotype (Figure 6A), 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 5 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 and 6]) or with a general LOX inhibitor [Figures 10 to 12]) or by removing NAE 18:3 and depleting these ethanolamide oxylipins during a recovery period (Figures 3 and 9).

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 2 and 3). 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 NAE 18:3, the metabolism of NAE 18:3 in planta with LOX pathway enzymes appears to be essential for normal seedling development.

Figure 13. 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)xoy-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 so of three replicate samples. chl, chlorophyll; FW, fresh weight.

[See online article for color version of this figure.]

Figure 14. 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 so 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.
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 hydroperoxides to seedlings could induce bleaching (Figure 13), consistent with visible phenotypes (Figure 6) and metabolite profiles (Figure 3) of mutants treated with NAE 18:3. On the other hand, none of the FFA oxylipins induced chloroplast disruption in cotyledons (Figure 13), pointing to specific metabolism of NAE 18:3 by LOX enzymes as a means of disrupting thylakoid membrane organization (see Supplemental Figure 4 online). 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 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 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 15). The NAEs in desiccated seeds are depleted to low levels and NAE oxylipins do not accumulate substantially under normal conditions of seedling development (Figure 2). On the other hand, at early stages of establishment, seedlings retain the capacity to arrest growth and reactivate 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, 2002). We suggest that NAE metabolism is part of this backup 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; so under normal conditions, levels of NAE oxylipins would be low. This is analogous to the depletion of ABA levels around seedling establishment; so under normal conditions, levels of NAE oxylipins would be low. This is analogous to the depletion of ABA levels around seedling establishment. Here, we also show that when seedling growth is arrested by treatment with ABA, levels of endogenous 9- and 13-NAE-HOT metabolites increase in a dose-dependent fashion (Figure 14). 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 14). 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 ABA 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 attributable 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 ethanola-mide oxylipins have potent biological activities during seedling establishment.

METHODS

Chemicals

9S-hydroxy-10E,12Z-octadecadienoic-9,10,12,13-d4 acid, 9S-hydroxy-10E,12Z,15Z-octadecatrienoic acid, 13S-hydroxy-9Z,11E,15Z-octadecatrienoic acid, OPDA, NAE 18:3, 13-LOX from soybean (Glycine max; 13-GmLOX), and 9-LOX from potato (Solanum tuberosum; 9-StLOX) were purchased from Cayman Chemicals.

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 NAE OPDA. NAE oxylipins were separated on reverse-phase (RP) HPLC. The fraction collector was set to collect samples from 6 to 11 min (1 min/ fraction). The first NAE oxylipin to be eluted around 6.2 min was NAE OPDA, and 9- and 13-NAE HOT were eluted together around 7.5 min. Normal phase HPLC was used to further separate 13- and 9-NAE HOT, which were eluted around 26 and 34 min, respectively.

LOX-mediated oxidative reactions with 13-GmLOX and 9-StLOX were performed 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 tetrathionate. Hydroperoxide product from 13-GmLOX and NAE 18:3 were further incubated with Arabidopsis enzymes AOC2 (recombinant plasmids in Escherichia 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 min to generate NAE OPDA (Van Der Steelt 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 0.025% [v/v] 2-butyl-6-hydroxytoluene). One hundred nanograms of 9,11,9,13-hydroxy-octadecadienoic acid-d4 was added as internal standard in each sample prior to extraction. Screw-cap test tubes (Pyrex 16 × 125 mm) were immediately capped under nitrogen stream. The extract was shaken at 4°C for 30 min and then centrifuged at 450g for 10 min. The hexane-rich phase was collected and combined with 2 mL of 6.6% potassium sulfate in aqueous solution. To reduce all peroxides to hydroxides for analysis, ~10 mg of sodium tetrahydroborate was added and the extract was then centrifuged at 4500g for 10 min. The hexane-rich phase was recovered, dried under nitrogen, and resuspended in 200 μL of 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 of 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. RP-HPLC was performed on a EC 250/2 nucleosil 120-5 C18 column (250 × 25 mm, 5-μm particle size; ID 2 mm; Macherey and Nagel) 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 postrun of 100% solvent A for 10 min. For NAE oxylipin standards, NAE oxylipins collected from RP-HPLC were further separated by normal-phase HPLC performed on a Zorbax Rx-SIL column (150 × 2.1 mm, 5-μm particle size; Agilent) 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, 2.1 × 150 mm; Diacel Chemical Industries).

Identification of LOX-Mediated Products by GC-MS

Identity of oxylipins as TMS derivatives was confirmed by GC-MS performed with an Agilent GC 7890A/MSD 5975C system using a capillary
HP-5MS column (30 m × 0.250 mm, 0.25-mm coating thickness; Agilent Technologies) in full mass scan mode. The oven temperature gradient was 40°C for 1 min, 40 to 200°C at 50°C/min, 200 to 285°C at 5°C/min, followed by 300°C for 15 min. Ultrapure 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. Ethanolamine derivatives were quantified against corresponding FFA standard curves using the same deuterated standard, 9-HOD-d₄. Fragmentation patterns and diagnostic and quantitative ions (m/z) of oxylipins identified by GC-MS are presented in Figure 1, Supplemental Figure 1 online, and Supplemental Table 1 online. Full mass scans were performed to confirm identities of each compound.

Plant Materials and Tissue Collection

FAAH1 OE and faah1 T-DNA knockouts (SALK_095108) are described by Wang et al. (2006). The faah1 (At5g64440) mutant was crossed with a T-DNA insertion 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 oflox1 (SALK_038475) andlox5 (SALK_012188) were crossed to obtain the double mutantlox1lox5 and were genotyped to verify the disruption of the respective genes. The loss-of-functionlox2 (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 3 min each, rinsed with sterile deionized water three times, and subsequently stratified in sterile deionized water for 3 d at 4°C in the dark prior to sowing in liquid or solid Murashige and Skoog medium (0.5×) (Teaster et al., 2007). Seeds were germinated and grown in controlled conditions with 16-h-light/8-h-dark cycle (60 µmol m⁻² s⁻¹) at 20 to 22°C. For many experiments, Arabidopsis seedlings were grown for 4 d prior to treatment with 100 µM NAE 18:3 (or 0.07% DMSO as solvent control). Treated seedlings were collected at 24, 48, 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 and 96 h after the transfer. For experiments with ABA treatment, ABA (concentration based on cis-isomer) was added to seedlings at day 3, and seedlings were collected for analysis at day 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, five to 10 seedlings were collected, weighed, and homogenized in 1 mL of 80% acetone. The extracts were centrifuged at 3000g for 2 to 3 min. The supernatant was collected, and absorbance was measured at 647 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: chlorophyll b (mg/L) = 20.47 A₆64.5/A₆47 – 4.73 A₆47; Chlorophyll a = 12.63 A₆₄₄.5 to 2.52 A₆₄₄ (Inskoep 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 ×63 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) in 10 mM potassium phosphate buffer, pH 7.0, under vacuum for 5 min (three times) and subsequently maintained in fixative at 4°C overnight. Cotyledon pieces were washed six times (15 min each) with potassium phosphate buffer followed by postfixation 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) using flat embedding molds. Ultrathin sections (<100 nm) were cut in cross section from polymerized blocks 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 (At5g45140), LOX1 (At1g55020), and LOX5 (At5g22400).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Identification of Endogenous NAE 18:3 and FFA 18:3 Oxylipins.

Supplemental Figure 2. NAE 18:3 Negatively Regulates Growth and Induces Bleaching of Cotyledons within a Narrow Window of Developmental Sensitivity.

Supplemental Figure 3. Arabidopsis Seedlings Show Organ-Specific Sensitivity to Exogenous NAE 18:3.

Supplemental Figure 4. Representative Electron Micrographs of Chloroplasts in Cotyledons of Wild-Type Seedlings Treated with NAE 18:3.

Supplemental Table 1. Retention Times and Diagnostic/Quantitative Ions of NAE Oxylipins and Corresponding FFA as Detected by GC-MS.

Supplemental Table 2. Quantification of NAE 18:3 and LOX-Derived Metabolites from Different Parts of Arabidopsis Plants (Col-0).

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AUTHOR CONTRIBUTIONS


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