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Lipoxygenase-mediated Oxidation of Polyunsaturated *N*-Acylethanolamines in *Arabidopsis*[*] [S]

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***N*-Acylethanolamines (NAEs) are bioactive fatty acid derivatives that occur in all eukaryotes. In plants, NAEs have potent negative growth-regulating properties, and fatty acid amide hydrolase (FAAH)-mediated hydrolysis is a primary catabolic pathway that operates during seedling establishment to deplete these compounds. Alternatively, polyunsaturated (PU)-NAEs may serve as substrates for lipid oxidation. In *Arabidopsis*, PU-NAEs (NAE 18:2 and NAE 18:3) were the most abundant NAE species in seeds, and their levels were depleted during seedling growth even in FAAH tDNA knock-out plants. Therefore, we hypothesized that lipoxygenase (LOX) participated in the metabolism of PU-NAEs through the formation of NAE-oxylipins. Comprehensive chromatographic and mass spectrometric methods were developed to identify NAE hydroperoxides and -hydroxides. Recombinant *Arabidopsis* LOX enzymes expressed in *Escherichia coli* utilized NAE 18:2 and NAE 18:3 as substrates with AtLOX1 and AtLOX5 exhibiting 9-LOX activity and AtLOX2, AtLOX3, AtLOX4, and AtLOX6 showing predominantly 13-LOX activity. Feeding experiments with exogenous PU-NAEs showed they were converted to hydroxide metabolites indicating that indeed *Arabidopsis* seedlings had the capacity for LOX-mediated metabolism of PU-NAEs *in planta*. Detectable levels of endogenous NAE-oxylipin metabolites were identified in FAAH fatty acid amide hydrolase seedlings but not in wild-type or FAAH overexpressors, suggesting that NAE hydroxide pools normally do not accumulate unless flux through hydrolysis is substantially reduced. These data suggest that *Arabidopsis* LOXs indeed compete with FAAH to metabolize PU-NAEs during seedling establishment. Identification of endogenous amide-conjugated oxylipins suggests potential significance of these metabolites *in vivo*, and FAAH mutants may offer opportunities to address this in the future.**

N-Acylethanolamines (NAEs)[3] are a class of fatty acid derivatives that is widely distributed in both plant and animal kingdoms. Their metabolic formation and catabolism are highly conserved (1, 2). In eukaryotes, NAEs are generated by the hydrolysis of a minor membrane lipid, *N*-acylphosphatidylethanolamine, by a phospholipase D (3, 4). The types of NAEs generated vary in their acyl chain length and degree of saturation and are dependent on the precursor pool. NAEs may be hydrolyzed to ethanolamine and free fatty acid by a fatty acid amide hydrolase (FAAH), an enzyme with broad substrate specificity (5–9).

Many NAE types are characterized as lipid mediators, and they regulate a wide range of physiological processes. For example, in mammals, *N*-arachidonylethanolamine (NAE 20:4; anandamide) is known to function as an endogenous ligand for cannabinoid receptors and is considered as a neurotransmitter (10). But more broadly, NAE 20:4 along with other NAE types now have been reported to play many important roles in mammalian physiology and behavior, including neuroprotection, pain perception, mental depression, appetite suppression, potentiation of memory, addiction, apoptosis, and reproduction (11).

Within the plant kingdom, NAEs are ubiquitous and most abundant in desiccated seeds; their levels are depleted substantially with germination and seedling establishment (12, 13). Several functional roles for NAEs in plants are now emerging. Pharmacological studies revealed the potent nature of lauroylethanolamide (NAE 12:0), which affects endomembrane trafficking, cell wall and cell shape formation, and cytoskeletal organization (14, 15), and inhibits plant lipoxygenase activity (16). More recently, with the availability of *Arabidopsis* mutants with altered NAE metabolism (tDNA insertional knock-out (KO) mutants and overexpressors (OE) of FAAH, At5g64440), NAEs have been implicated as negative regulators

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[3] The abbreviations used are: NAE, *N*-acylethanolamine; AOC, allene oxide cyclase; AOS, allene oxide synthase; FAAH, fatty acid amide hydrolase; FFA, free fatty acid; JA, jasmonic acid; KO, knock-out; LOX, lipoxygenase; NAE 20:4, *N*-arachidonylethanolamide; NAE 12:0, *N*-laurylethanolamide; NAE 18:2, *N*-linoleoylethanolamide; NAE 18:3, α/γ- *N*-linolenoylethanolamide; NP, normal phase; OE, overexpressor; PU, polyunsaturated; RP, reverse phase; TMS, trimethylsilyl; 13NAE-HPOD, hydroperoxy-9,11-octadecadienoylethanolamide; 9NAE-HPOD, (9*S*,12*Z*,10*E*)-9-hydroperoxy-10,12-octadecadienoylethanolamide; 13NAE-HPOT, (13*S*,9*Z*,11*E*,15*Z*)-13-hydroperoxy-9,11,15-octadecatrienoylethanolamide; 9NAE-HPOT, (9*S*,12*Z*,10*E*,15*Z*)-9-hydroperoxy-10,12,15-octadecatrienoylethanolamide; HOD, hydroxylinoleic acid; HOT, hydroxylinolenic acid.

of seedling growth and modulators of stress responses and interact with abscisic acid- and salicylic acid (SA)-mediated signaling pathways (1, 17–20).

The activity of saturated and unsaturated NAEs is typically terminated by FAAH-mediated hydrolysis (5–8). Alternatively, polyunsaturated (PU)-NAE species are also subjected to oxidative metabolism by the members of cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 families (21–30). In mammalian systems, the product of COX-2-mediated oxidation of anandamide, a prostaglandin analog, prostaglandin E_2-ethanolamide, and other eicosanoid ethanolamides were reported to be important signaling compounds (22, 31, 32). LOX-mediated NAE oxygenation is less understood, but anandamide (NAE 20:4), *N*-linoleoylethanolamide (NAE 18:2), and α/γ-*N*-linolenoylethanolamide (NAE 18:3) were reported to be substrates for several plant-derived LOXs such as barley, tomato, and soybean (27–29). Furthermore, *in vitro* studies demonstrated that the hydroperoxides of NAE 18:2 and NAE 18:3 were converted by alfalfa hydroperoxide lyase and/or flax allene oxide synthase (AOS) to yield NAE-oxylipins (28); however, their occurrence and relevance *in planta* are not yet understood.

Plant LOXs are classified based on location of oxygenation on the carbon atom (C-9 for 9-LOX or C-13 for 13-LOX) of the hydrocarbon backbone of linoleic or linolenic acids (18:2 or 18:3) generating the (9*S*)-hydroperoxy- and the (13*S*)-hydroperoxy derivatives of these fatty acids, respectively (33, 34). The various oxylipins derived from PU-free fatty acids (FFAs), including the hormone jasmonic acid (JA), play important roles in plant development and responses to biotic and abiotic stress (35–38). However, the extent or significance of NAE-derived oxylipin formation in plants remains largely unexplored. Several lines of evidence suggest that hydrolysis and oxidation pathways may compete in the metabolism of NAEs, particularly in seed germination and seedling growth. In all plants examined, PU-NAE types (NAE 18:2 and/or NAE18:3) are most prevalent in desiccated seeds (13). Metabolic feeding experiments with radiolabeled NAE 18:2 in cottonseed cell-free fractions and in intact seeds indicated that both hydrolysis and oxidation pathways metabolized NAE 18:2 *in vitro* and *in vivo* (23). Oxidation was inhibited by classical LOX inhibitors implicating 13-LOX and AOS in the formation of an interesting oxylipin, 12-oxo-13-hydroxy-*N*-(9*Z*)-octadecenoylethanolamide, from NAE 18:2. A time course of NAE 18:2 metabolism in cottonseed subcellular fractions showed that NAE oxidation and hydrolysis pathways were most active during early post-germinative growth (23), suggesting that NAE metabolism may be important for germination and seedling growth. Indeed, studies with *Arabidopsis* plants overexpressing FAAH (increased capacity of NAE hydrolysis) showed markedly enhanced seedling growth (20). On the other hand, the tDNA insertional knock-out in FAAH did not show a marked reduction in seedling growth, and the depletion of PU-NAEs in FAAH KO seedlings proceeded to a similar extent as in wild-type; an alternative metabolic pathway, possibly oxidation may be responsible, in part, for depletion of PU-NAEs in FAAH KOs. Although these studies point to a LOX-mediated pathway for the oxidation of PU-NAEs in plants, the specific enzymes that mediate NAE oxidation and their corresponding products *in planta* have not been characterized due to the lack of molecular tools and sensitive methods for identification.

Here, we utilized commercial 13- and 9-LOX enzymes (*Glycine max* (Gm) and *Solanum tuberosum* (St), respectively) and recombinant LOX pathway enzymes to generate an array of NAE-oxylipin standards *in vitro* and developed comprehensive profiling procedures to detect, separate, and characterize oxidative metabolites of NAEs. In *Arabidopsis*, enzymatic activities of six LOXs toward free fatty acid substrates were characterized recently. AtLOX1 and AtLOX5 were reported to be 9-LOXs, and AtLOX2, AtLOX3, AtLOX4, and AtLOX6 were shown to be 13-LOXs (39). We compared the potential of these *Arabidopsis* 13- and 9-LOXs to oxidize NAEs *versus* FFAs. In addition, we identified NAE-oxylipins produced by *Arabidopsis* seedlings with altered NAE metabolism with or without exogenous substrates. Together, our results continue to suggest that hydrolytic and oxidative pathways cooperate in the metabolism of NAEs in plants. We identified new oxylipin metabolites in *Arabidopsis* seedlings and established new tools for future studies of their physiological significance.

EXPERIMENTAL PROCEDURES

Materials—Linoleoylethanolamide (NAE 18:2) and α-linolenoylethanolamide (NAE 18:3) substrates and 13-GmLOX and 9-StLOX enzymes were purchased from Cayman Chemicals (Ann Arbor, MI). Vector pET28a was from Novagen (Schwalbach, Germany), and BL21 Star™ *Escherichia coli* cells were from Invitrogen. Solvents were purchased from Fisher and were all Optima grade. [1-^{14}C]Linoleic acid (53 mCi·mmol^{-1} in ethanol; 1 Ci = 37 GBq) was purchased from PerkinElmer Life Sciences. NAE[1-^{14}C]18:2 was synthesized from [1-^{14}C]linoleic acid and purified, as described previously (23).

NAE Quantification—Seeds and seedlings of *Arabidopsis* were homogenized in hot isopropyl alcohol (70 °C), and total lipids were extracted into chloroform. Lipid content was estimated gravimetrically, and samples were fractionated by normal phase (NP)-HPLC (HP1100 series; Agilent, Wilmington, DE) using an Alltech (Deerfield, IL) semi-preparative silica column (10 × 250 mm, 10 μm particle size). NAEs were identified via selective ion monitoring and quantified as TMS-ether derivatives by gas chromatography and mass spectrometry (GC-MS; model 6890 GC with a 5973 mass selective detector; Agilent) by isotope dilution mass spectrometry method, as described previously (13).

Analysis of Oxidation and Hydrolysis Activity in Seedlings—Enzyme activity was measured with 400 μg of crude protein extract isolated from 4- and 8-day-old seedlings, using NAE[1-^{14}C]18:2 as substrate. Reactions were carried out for 30 min, and lipid products were extracted and separated by thin layer chromatography as described previously (23). NAE catabolic activity was quantified by radiometric scanning (Bioscan, Washington, D. C., System 200 imaging scanner) of lipid-soluble enzyme reaction products (23).

Synthesis of NAE-Oxylipins—LOX reactions were carried out with commercially available 13-GmLOX and 9-StLOX (Cayman Chemicals) and NAE 18:2 and NAE 18:3 substrates, with and without reduction with sodium tetraborohydride to gener-

ate hydroxides and hydroperoxides, respectively. To generate free and NAE-oPDA, the reactions proceeded without reduction and by adding the enzymes AtAOC2 (kindly provided by C. Wasternack) and AtAOS (kindly provided by M. Stumpe). Lipids extracted from enzyme reactions were analyzed by HPLC and GC-MS as described previously (40, 41) with the modifications described below.

Separation of NAE-Oxylipins—Lipids extracted from LOX reactions were separated by reverse phase (RP)-HPLC using an ET250/2 Nucleosil 120-5 C18 column (250 × 2.1 mm, 5-μm particle size; Macherey & Nagel, Düren, Germany) on Agilent 1100 HPLC coupled to a UV diode array detector. A binary gradient system with solvent A, methanol/water/acetic acid (80:20:0.1, v/v), and solvent B, methanol/acetic acid (100:0.1, v/v), with a gradient program of 100% solvent A for 3 min, followed by a linear increase of solvent B up to 25% over 18 min, a linear increase of solvent B up to 100% over 2 min, and 100% solvent B for 20 min at a flow rate of 0.18 ml/min achieved separation of NAE-LOX products from that of FFA-LOX products. The RP fractions of NAE-LOX products were further separated by NP-HPLC using a Zorbax Rx-SIL column (150 × 2.1 mm, 5-μm particle size; Agilent, Waldbronn, Germany) and a solvent system of *n*-hexane/2-propanol/trifluoroacetic acid (100:12.5:0.02, v/v) and a flow rate of 0.15 ml/min. For detection of hydroxy fatty acid ethanolamides, the absorbance of the conjugated diene system at 234 nm was monitored. Enzyme-generated NAE-oxidative products were distinguished from autooxidative products on chiral OD-H column (150 × 2.1 mm, 5-μm particle size; Diacel, Osaka, Japan) and solvent system of *n*-hexane/2-propanol/acetic acid (100:12.5:0.05, v/v), and a flow rate of 0.1 ml/min was used.

Identification of NAE-LOX Products—Oxidative products of NAEs were further identified as TMS-derivatives by GC-MS using an Agilent 5973 network mass selective detector connected to Agilent 6890 gas chromatograph equipped with a capillary DB-5 column (30 m × 0.25 mm; 0.25-μm coating thickness; J&W Scientific, Agilent, Waldbronn, Germany). Helium was used as carrier gas (1 ml/min). The temperature gradient was 150 °C for 1 min, 150–200 °C at 4 °C/min, 200–250 °C at 5 °C/min, and 250 °C for 6 min. Electron energy of 70 eV, an ion source temperature of 230 °C, and a temperature of 260 °C for the transfer line were used.

The compounds also were characterized by LC-MS/MS using an MS Surveyor HPLC system equipped with a photodiode array detector and coupled with an LCQ Advantage electrospray ionization ion trap mass spectrometer (Thermo Finnigan, Austin, TX). NAE-LOX products were separated by RP-HPLC (EC 250/2 Nucleodure 100-5 C18ec column, 250 mm × 2.1 mm, 5-μm particle size; Macherey-Nagel, Düren, Germany). The solvent system for gradient separation in A was methanol/water/acetic acid (50:50:0.1, v/v) and in B was methanol/acetic acid (100:0.1, v/v). The gradient elution profile was as follows. first, a flow rate of 0.18 ml/min, 0–3 min from 50% A and 50% B to 45% A and 55% B; and second, 3–20 min from 45% A and 55% B to 100% B and a flow rate increase to 0.36 ml/min The mass spectrometer was operated in negative ion mode with the source voltage set to 4 kV and a capillary voltage of 27 V at a temperature of 300 °C. For identification, scans were collected in full scan mode between *m/z* values of 50 and 600. For MS/MS experiments, the collision energy was 2 V.

Characterization of Recombinant Arabidopsis LOX Activity against PU-FFAs and NAEs—The cDNAs of AtLOX1 through six were cloned into pET28a vectors (Novagen, Schwalbach, Germany) using PCR to generate appropriate restriction sites. Recombinant proteins with N-terminal His tag were expressed in BL21 Star™ cells (Invitrogen). Cells were harvested and lysed via sonication in lysis buffer (0.1 M Tris/HCl, pH 7.5, 300 mM NaCl, 10% (v/v) glycerol, 0.1% Tween 20), and cell lysates were obtained after centrifugation at 20,000 × *g* to remove insoluble proteins and cell debris. Lysates were incubated with 50 μg of NAE and FFA each at room temperature for 30 min and reduced with $SnCl_2$. Lipids were extracted and analyzed via RP- followed by NP-HPLC, as described earlier.

Identification of NAE-LOX Products in Planta—Seeds and 4-day-old seedlings that were provided with or without 100 μM NAE 18:2 or NAE 18:3 for 24 h were analyzed for endogenous products of NAE oxidation. About 50–100 mg of seed or 1 g of plant tissue was homogenized in 20 ml of hexane/isopropyl alcohol solution (3:2 with 0.0025% 2-butyl-6-hydroxytoluene) and capped under nitrogen. The extract was shaken at 4 °C for 10 min and then centrifuged at 3200 × *g* at 4 °C for 10 min. To the clear supernatant, 12 ml of 6.6% potassium sulfate solution was added, and vigorous shaking at 4 °C was repeated for 10 min, followed by centrifugation as indicated above. The upper hexane-rich layer was transferred and evaporated to dryness under a stream of nitrogen gas and resuspended in hexane/isopropyl alcohol (100:5). Samples were vortexed vigorously, and the supernatant was transferred and dried again under nitrogen. Lipids were then resuspended in 80 μl of methanol/water/acetic acid (75:25:0.1), transferred to HPLC-glass inserts, and analyzed by RP-HPLC followed by NP-HPLC and GC- and/or LC-MS as described above for NAE-oxylipins.

RESULTS

Evidence for Oxidation of PU-NAEs in Arabidopsis—Cloning and characterization of *Arabidopsis* FAAH (AtFAAH) have advanced our understanding of NAE metabolism in seeds and seedlings (20). Quantification of total NAEs in desiccated seeds of wild-type (WT) and FAAH-altered plants (OEs and KOs) showed that FAAH OEs, as expected, had lower NAE content (~1100 ng/g fresh weight), whereas the KO seeds had higher NAE content (~2500 ng/g fresh weight) when compared with WT (~1800 ng/g fresh weight; Fig. 1*A*). However, irrespective of the FAAH expression, the NAE content in all the three genotypes declined to similar levels with the progression of seedling development (4-day-old and 8-day-old; Fig. 1*A*). Furthermore, quantification of individual NAE species revealed that the proportion of each NAE species among the three genotypes remained very similar within the developmental stage with PU-NAEs (NAE 18:2 and NAE 18:3) being the most abundant type in desiccated seeds (Fig. 1*A*). For all genotypes, the NAE composition was altered with growth; for example, when compared with seeds, 8-day-old seedlings showed 50% reduction in the proportion of PU-NAEs and a corresponding increase in the proportion of saturated NAE species (NAE 12:0 and NAE 14:0).

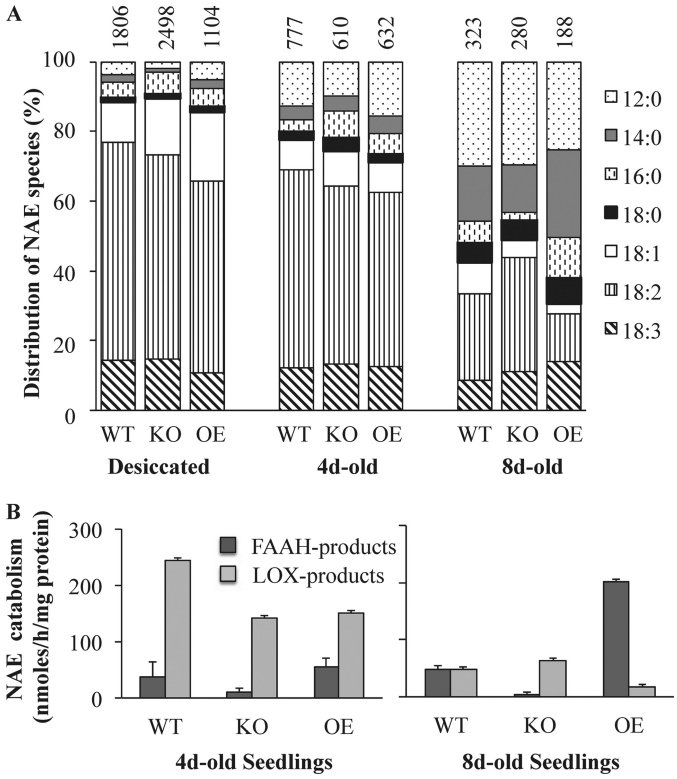

FIGURE 1. **Metabolism of NAEs in *Arabidopsis*.** *A,* total NAE content and distribution of NAE species in desiccated seeds and 4- and 8-day (*d*)-old seedlings of wild type (*WT*), FAAH tDNA knock-outs, and FAAH overexpressors. Total NAE content is shown *above* each bar, in ng/g fresh weight. Crude enzyme extracts from 4- and 8-day-old seedlings were provided with radiolabeled NAE 18:2, and their product separated on TLC were quantified to determine the hydrolysis or oxidation activity (*B*). *Error bars* show the standard deviation of three independent experiments.

To ascertain the metabolic fate of PU-NAE species in developing *Arabidopsis* seedlings, radiolabeled NAE 18:2 was presented as a substrate to the crude homogenates of 4- and 8-day-old WT, FAAH OE, and KO seedlings, and the corresponding lipid soluble, radiolabeled products were analyzed by thin layer chromatography. The enzyme extracts of 4-day-old seedlings of all the three genotypes readily oxidized NAE 18:2; in WT, this activity was at least 5-fold higher than the amidase activity (Fig. 1*B*). In contrast, 8-day-old seedlings showed drastically reduced oxidative activity; in the case of WT, it was equivalent to hydrolysis activity. However, 8-day-old OE seedlings displayed a 10-fold higher FAAH activity than the oxidative activity. In KOs, as expected, hydrolase activity was negligible in both 4- and 8-day-old seedlings (Fig. 1*B*). These results were consistent with previous results in cotton seedlings, which showed a capacity for both hydrolysis and oxidation of NAEs (23). The ability of homogenates of *Arabidopsis* FAAH KOs to oxidize but not hydrolyze NAE 18:2 *in vitro* suggests that NAE oxidation can serve as a major route for the depletion of PU-NAEs in these developing seedlings.

LOX-mediated Oxidation of PU-NAEs—Typically, 13- and 9-LOX enzymes oxidize PU-FFAs to their corresponding 13/9-hydroperoxides, which are subsequently reduced to hydroxides (37). Previous studies have shown the ability of plant-derived 13-LOX to oxidize PU-NAEs as well (27–29). Shrestha *et al.* (23) demonstrated the ability of imbibed cottonseed extracts to convert NAE 18:2 hydroperoxides to 12-oxo-13-hydroxy-*N*-(9*Z*)-octadecanoylethanolamine. In accordance with PU-FFA oxidation and extending on the previously available *in vitro* data, we developed a schematic model for LOX-mediated oxidation of NAE 18:2 and NAE 18:3 (Fig. 2). We hypothesized that NAE 18:2 is likely to undergo 13- and 9-LOX-mediated oxidation to generate (13*S*,9*Z*,11*E*)-13-hydroperoxy-9,11-octadecadienoylethanolamide (13NAE-HPOD) and (9*S*,12*Z*,10*E*)-9-hydroperoxy-10,12-octadecadienoylethanolamide (9NAE-HPOD; Fig. 2), respectively. Similarly, NAE 18:3 peroxidation will produce (13*S*,9*Z*,11*E*,15*Z*)-13-hydroperoxy-9,11,15-octadecatrienoylethanolamide (13NAE-HPOT) and (9*S*,12*Z*,10*E*,15*Z*)-9-hydroperoxy-10,12,15-octadecatrienoylethanolamide (9NAE-HPOT; Fig. 2), respectively. Typically, 13/9-hydroperoxides are unstable and are further reduced to their corresponding hydroxides (13/9NAE-HOD or -NAE-HOT). It is possible that 13NAE-HPOD and 13NAE-HPOT might act as substrates for 13-AOS to generate 12-oxo-13-hydroxy-NAE 18:2 (23) and (12,13*S*)-epoxy-NAE 18:3 (28), respectively. The 13-AOS product, (12,13*S*)-epoxy-NAE 18:3, may undergo cyclization by 13-AOC to form 13NAE-oPDA, similar to the free fatty acid oPDA except conjugated to ethanolamine. These collective oxidative products of NAE 18:2 and NAE 18:3, collectively referred to as NAE-oxylipins, were synthesized enzymatically and used as standards to develop methods for their separation and characterization.

Synthesis and Separation of NAE-Oxylipins—LOX reactions were carried out individually with each substrate (NAE 18:2, NAE 18:3, FFA 18:2, and FFA 18:3) and enzyme combination (13-GmLox and 9-StLox), with or without reduction to generate corresponding hydroxides and hydroperoxides, respectively. Additionally, the hydroperoxide products of NAE 18:3 and FFA 18:3 were incubated with 13-AOS and 13-AOC to generate corresponding oPDA. It was important to include the FFA products in this separation procedure because we expected these metabolites to be co-extracted with NAE-oxylipins and to be more prevalent than the NAE products in plant tissue samples. An initial retention time (R_t) was determined for each product on RP- and NP-HPLC. Subsequently, the binary solvent gradient system on RP-HPLC was adjusted to achieve the separation of oxidative products by their degree of saturation and polarity, which allowed the elution of NAE products earlier than that of the FFA products (Fig. 3*A*). However, on RP-HPLC, 9/13-hydroxides and hydroperoxides of each substrate were not separable. Free oPDA was eluted with NAE 18:2 products (Fig. 3*A*). The eluates of NAE products were pooled and further separated by NP-HPLC (Fig. 3*B*). NAE-oPDA was separated on RP (Fig. 3*A*) and was further analyzed on GC/LC-MS. Because NAE 20:4 has not been reported in plants, we included its 13-LOX derivative (15/12NAE-HET) in our method development here to serve as an internal standard for subsequent quantification (Fig. 3, *A* and *B*). An aliquot of each fraction corresponding to an individual compound from NP-HPLC was separated by chiral phase HPLC to confirm enzymatic NAE oxidation. Further identification and characterization of NP-HPLC fractions were carried out by GC-MS and/or LC-MS/MS.

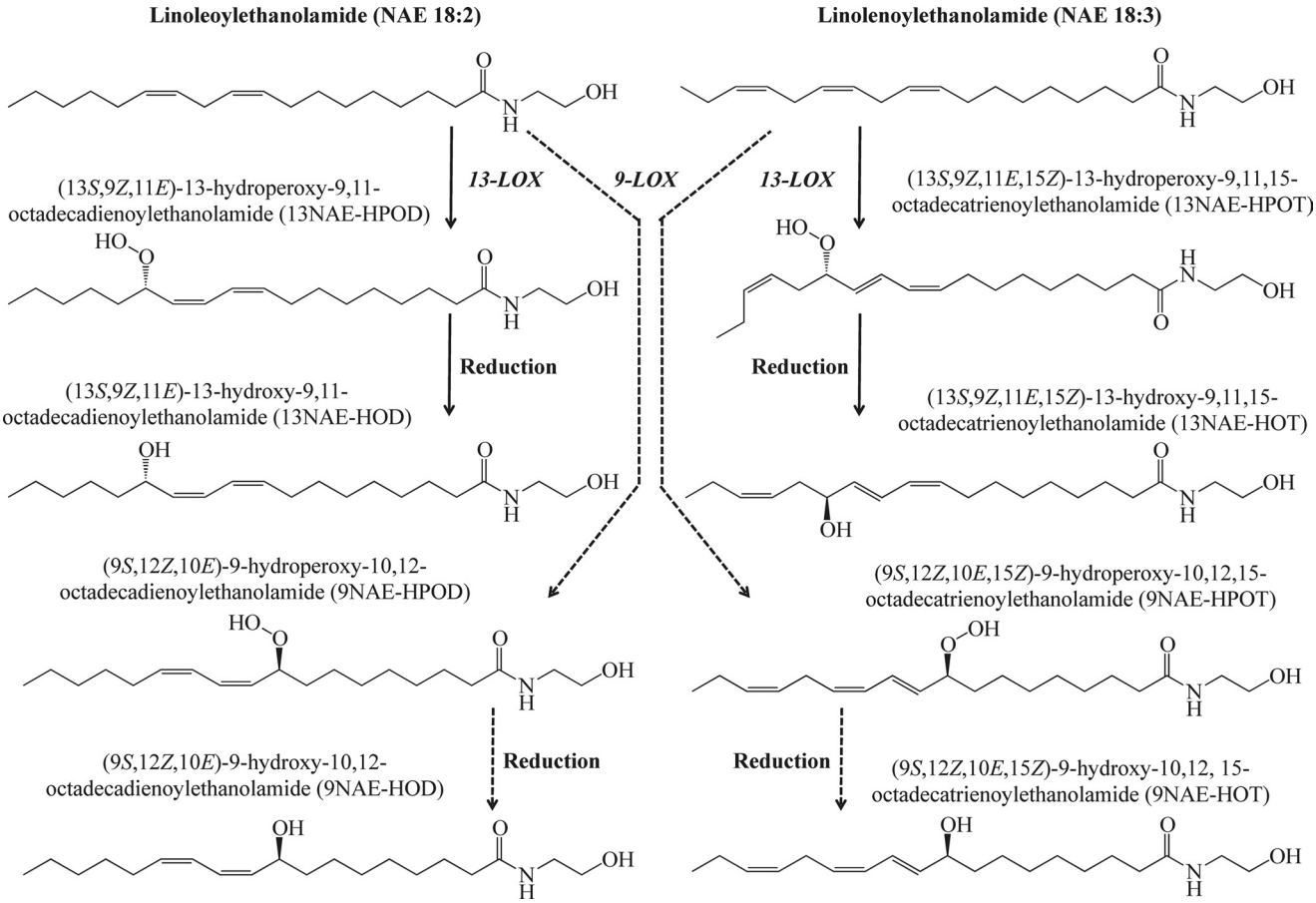

FIGURE 2. **Schematic model of LOX-mediated oxidation of NAE 18:2 and NAE 18:3.** The PU-NAE species, NAE 18:2 and NAE 18:3, are likely to undergo 13- and/or 9-LOX-mediated oxidation to generate corresponding hydroperoxides, which can be further reduced to hydroxides. The structures of NAE-oxidative products were predicted based on LOX-derived products of PU-FFAs.

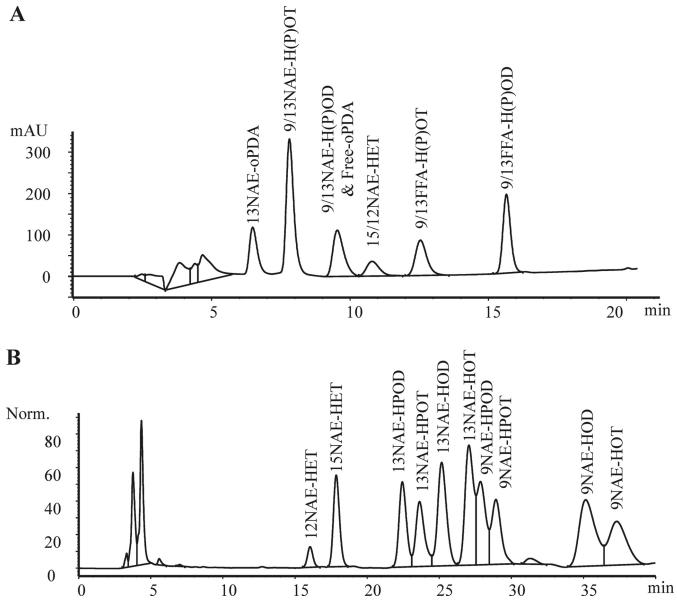

FIGURE 3. **Separation of NAE- and free-oxylipins.** LOX products generated *in vitro*, with and without reduction, from PU-NAEs and FFAs, using 13-GmLOX and 9-StLOX enzymes were separated on RP-HPLC (*A*). mAU, milli-absorbance units. Fractions eluted from RP that were generated from PU-NAEs were pooled together and further separated on NP (*B*). All the fractions eluted on RP- and NP-HPLC were identified by GC/LC-MS. *Norm*, normalized.

Identification of NAE-Oxylipins—The NP fractions were derivatized with *N,O*-bis(trimethylsilyl) trifluoroacetamide prior to GC-MS analysis. We generated characteristic mass spectra by GC-MS for 9/13NAE hydroxides (Fig. 4; Table 1). The retention times for 13- and 9-hydroxides of NAE 18:2 and NAE 18:3 by GC were very similar (Table 1). The molecular ions for TMS-derivatized NAE-HODs and NAE-HOTs were *m/z* 483 and *m/z* 481 respectively. Fragment ion *m/z* 116 is diagnostic of ethanolamide compounds. Based on the fragmentation pattern, additional diagnostic ions for NAE hydroxides were identified (Table 1). The 13- and 9NAE-HOD/Ts were distinguished by the characteristic ions at *m/z* 412 and *m/z* 360, respectively (Fig. 4).

Because the NAE hydroperoxides and NAE-oPDA were sensitive to the derivatization process, we relied on LC-MS/MS for further characterization of those compounds (supplemental Figs. 1–3; Table 2). LC-MS/MS also confirmed identification and characterization of NAE hydroxides providing further corroboration for the compounds identified by GC-MS. The 13- and 9NAE-HPOT as well as 13- and 9NAE-HOT elute on LC at 7 min followed by 13-and 9NAE-HPOD and 13- and 9NAE-HOD at 8.7 min (Table 2). Irrespective of the position of oxygenation (9 or 13), the products on LC-MS shared the same molecular ion (same *m/z*), the $[M - H]^-$ ion and the acetate adduct $[M + OAc]^-$. However, the MS/MS of $[M\text{-}H]^-$ ion gen-

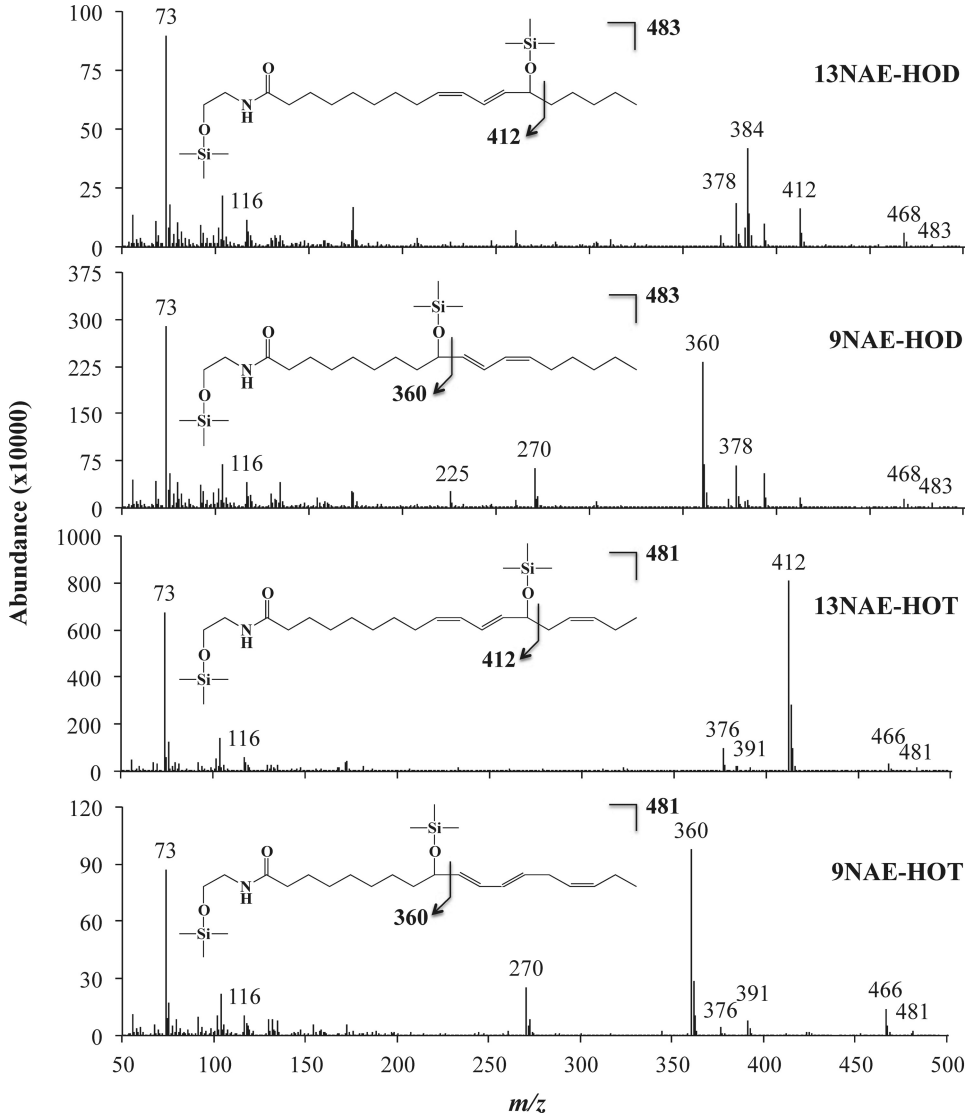

FIGURE 4. **Characterization of NAE-oxylipins.** NAE-oxylipin standards synthesized and separated by HPLC were analyzed on GC-MS as their TMS-derivatives. Mass fingerprints indicating fragmentation patterns for TMS-derivatives of 13NAE-HOD, 9NAE-HOD, 13NAE-HOT, and 9NAE-HOT are shown.

TABLE 1

Retention time (min) and diagnostic ions (*m/z*) of NAE-oxylipins identified by GC-MS

OTMS is trimethylsilyl ester.

Product	R_t	M^+	$M^+ - CH_3$	$M^+ - CH_3$-90	C_2H_3-OTMS	Other ions
	min					
13NAE-HOD	18.2	483	468	378	116	384, 412
9NAE-HOD	17.9	483	468	378	116	225, 270, 360
13NAE-HOT	18.1	481	466	376	116	412
9NAE-HOT	17.9	481	466	376	116	270, 360

TABLE 2

Retention time (min) and diagnostic ions (*m/z*) of NAE-oxylipins identified by LC-MS

Product	R_t	$[M - H]^-$	$[M + Ac\text{-}H]^-$	MS/MS of $[M - H]^-$
	min			
13NAE-HPOD	8.7	354.3	414.1	336.6
9NAE-HPOD	8.7	354.3	414.1	336.6, 228.1
13NAE-HOD	8.7	338.6	398.2	320.1, 238.1, 179.2
9NAE-HOD	8.7	338.6	398.2	320.1, 214.1
13NAE-HPOT	7.0	352.3	412.1	ND[a]
9NAE-HPOT	7.0	352.3	412.1	ND
13NAE-HOT	7.0	336.5	396.4	ND
9NAE-HOT	7.0	336.5	396.4	ND

[a] ND, not determined.

erated several diagnostic fragments that distinguished 9-products from the 13-products (supplemental Figs. 1 and 2; Table 2). Because of low compound amount of the NAE 18:3-oxylipins, the MS/MS spectra are missing. As suspected, we were able to generate NAE-oPDA by dehydration of 13NAE-HPOT with AtAOS and cyclization with AtAOC2 (supplemental Fig. 3; Table 2). However, we did not detect an AtAOS-generated product of 13NAE-HPOD, unlike the situation in cotton seedling microsomes (23).

Evidence for AtLOX-mediated Oxidation of PU-NAEs—Six *LOX* genes have been identified in *Arabidopsis* of which

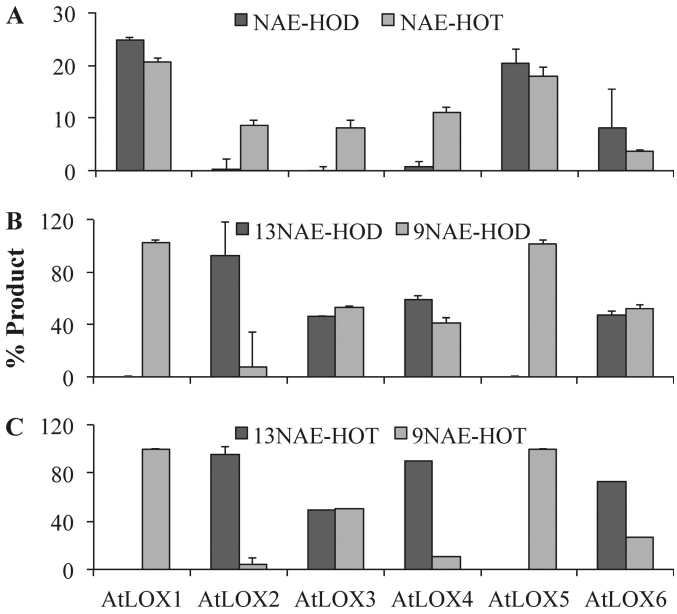

FIGURE 5. **LOX-mediated oxidation of PU-NAEs.** Oxidation of PU-NAEs relative to PU-FFA by heterologously expressed *AtLOX1–6*, to their corresponding hydroperoxides (and chemical reduction to hydroxides), is shown in *A*. The products NAE-HOD and NAE-HOT were further separated on NP-HPLC to identify the preferred position of oxygenation (*B* and *C*, respectively). *Error bars* show the standard deviation of three independent experiments.

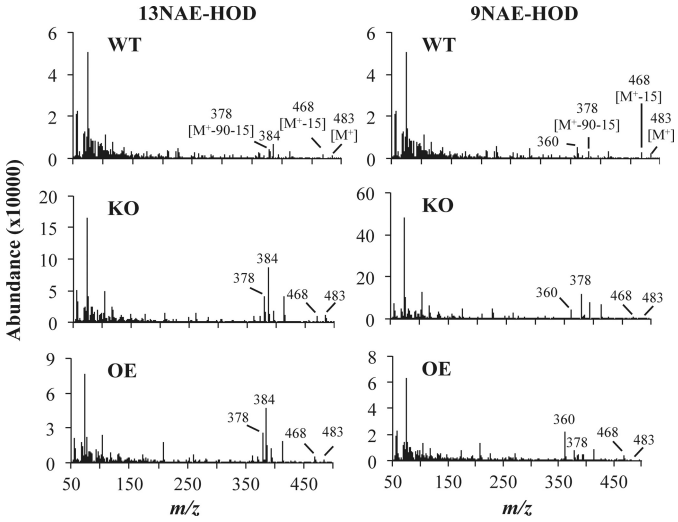

FIGURE 6. **Identification of oxidative products of NAE 18:2.** Lipid extractions of 4-day-old WT, FAAH KO, and FAAH OE seedlings exposed to 100 μM NAE 18:2 for 24 h showed evidence for endogenous 13- and 9NAE hydroxides.

AtLOX1 and *AtLOX5* were identified as having 9-LOX activities and the remaining having 13-LOX activities (33, 34). We tested the ability of heterologously expressed AtLOXs to oxidize PU-NAEs relative to PU-FFAs and also determined their preferential position for oxygenation (Fig. 5). When equivalent amounts of PU-FFA and PU-NAE were provided to various AtLOXs, 25% or less of the product was generated from PU-NAE, whereas the predominant proportion was from PU-FFA substrate (Fig. 5*A*). Among the AtLOXs, AtLOX1 and -5 showed highest activity toward PU-NAEs, generating ~25% of the oxylipin product from PU-NAEs; additionally, the activity was similar toward either NAE 18:2 or NAE 18:3. AtLOX2–4, although less active toward PU-NAE substrates than the FFA substrates (generating< 10% of the oxylipin product), appeared to prefer NAE 18:3 as a substrate than NAE 18:2 (Fig. 5*A*).

Further analysis of NAE-HOD and NAE-HOT products on NP-HPLC revealed that 100% of the product generated by AtLOX1 and -5 were 9-LOX products (Fig. 5, *B* and *C*). The NAE-HODs generated by AtLOX2–4 were <1% of the total oxidation products. For AtLOX6, NAE-HODs were <10% of the total oxidative products generated from PU-NAE/FFA substrates (Fig. 5*A*). Among these enzymes, only AtLOX2 showed predominantly 13-LOX activity, and the remaining showed both 9- and 13-LOX activity toward NAE 18:2 substrate (Fig. 5*B*). With NAE 18:3 as a substrate, both AtLOX2 and -4 showed predominantly 13-LOX activity, and AtLOX3 and -6 showed 9- and 13-LOX activity (Fig. 5*C*). In addition it should be stressed that all recombinant enzymes showed high activity at least against PU-FFA, because in all cases these substrates were converted to their products in substantial amounts (data not shown). The ability of recombinant AtLOXs to oxidize PU-NAE substrates, even in the presence of PU-FFA substrates, supports the possibility of LOX-mediated NAE-oxidation *in planta* and perhaps with a preference for 9-LOX activity.

Capacity for NAE Oxidation in Arabidopsis Seedlings—Identifying endogenous NAE-oxylipins in *Arabidopsis* was complicated by not knowing the precise metabolites of the pathways. Also of concern, the low levels of endogenous NAEs in tissues (about 100 times less than FFA) and the likelihood of even lower and perhaps trace levels of transient or unstable oxidative metabolites makes the detectable amounts of any NAE oxidative products very low. Consequently, we utilized a metabolic feeding approach to assess the capacity for *Arabidopsis*, in general, to oxidize most abundant PU-NAEs *in planta* and to identify LOX metabolites of NAEs. Evidence for NAE oxidation was observed in 4-day-old WT, KO, and OE seedlings in the presence of 100 μM exogenous substrate of either NAE 18:2 or NAE 18:3 (Fig. 6). Four-day-old seedlings were allowed to grow in the presence of substrate for 24 h after which the lipid extracts from tissue homogenates were analyzed for NAE-oxylipins. Because NAE hydroperoxides were unstable, all lipid extracts were reduced with $NaBH_4$ prior to separation on HPLC and therefore were not part of our analysis for endogenous NAE-oxylipins. The oxidative products of NAE 18:2, 9-, and 13NAE-HOD were identifiable in the extracts of all the three genotypes (Fig. 6). Seedlings supplied with NAE 18:3 also generated 9- and 13NAE-HOTs (Fig. 7); however, we did not detect any 13-AOS or 13-AOC product for NAE 18:3. In the absence of 4-day-old seedlings, NAE-oxylipins were not detectable in MS media-only preparations treated with NAE 18:2 or NAE 18:3 for 24 h indicating that there was no detectable autoxidation of the substrates.

Identification of Endogenous NAE-Oxylipins—PU-NAEs are most abundant in seeds and seedlings and are likely to undergo oxidation (Fig. 1). Therefore, we analyzed seeds and 4- and 8-day-old seedlings of WT, KO, and OE *Arabidopsis* for the presence of NAE-oxylipins. LOX-derived oxidative products, particularly the hydroxides of NAE 18:2, were readily detectable in 4-day-old KO seedlings but not in desiccated seeds and 8-day-old seedlings (Fig. 8). Absence of any oxidative products

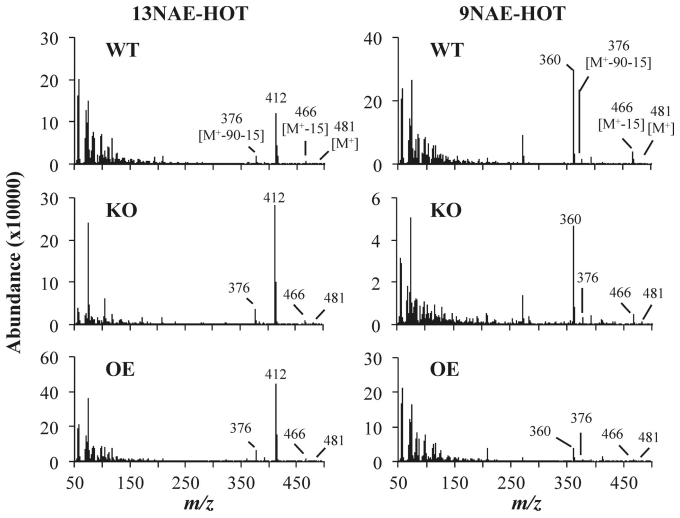

FIGURE 7. **Identification of oxidative products of NAE 18:3.** Lipid extractions of 4-day-old WT, FAAH KO, and FAAH OE seedlings exposed to 100 μM NAE 18:3 for 24 h showed evidence for endogenous 13- and 9NAE hydroxides.

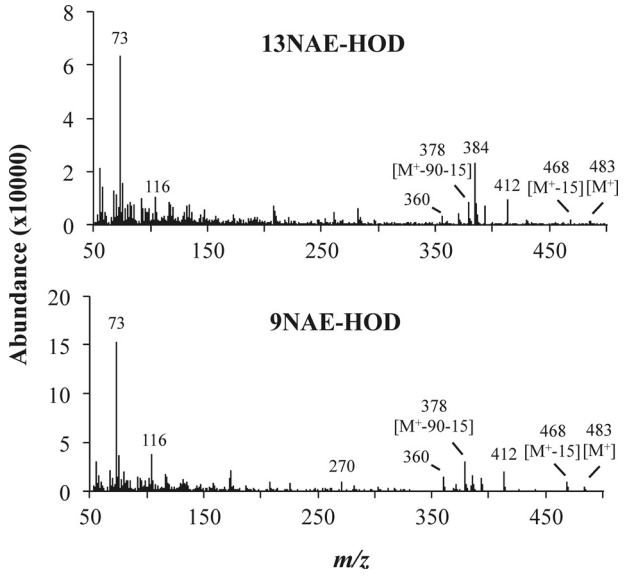

FIGURE 8. **Identification of endogenous NAE-oxylipins.** Lipid extractions of 4-day-old FAAH KO seedlings showed evidence for endogenous 13NAE-HOD and 9NAE-HOD. Products were separated on reverse and normal phase HPLC and identified as TMS-derivatives by GC-MS. Characteristic mass fragmentation pattern for 13/9NAE-HOD is shown.

in seeds and seedlings of WT and OE is not surprising because of the active and competing hydrolysis pathway in these genotypes. However, the lack of FAAH activity and presence of high oxidative activity are likely to contribute to the generation, and thus detection, of oxidative products in 4-day-old KO seedlings, despite the probable rapid flux through this pathway. Although 4-day-old KO seedlings are likely to oxidize both NAE 18:2 and NAE 18:3, detection of oxidative products for only NAE 18:2 is most likely attributed to the 3-fold abundance of NAE 18:2 over NAE 18:3 in seedlings, likely indicating LOX metabolites of NAE18:3 are below detection even in FAAH knock-outs. It is possible that the ethanolamide moieties of NAE-oxylipins are cleaved by an endogenous amidase after which these metabolites would mix with FFA oxylipins and become undetectable in this larger pool, but this remains to be investigated in more detail. In any case, demonstration of the formation of new endogenous NAE-derived oxylipins in plant tissues points to the need to further examine this class of lipids. Further studies with the NAE metabolite mutants such as the FAAH knock-out plants have the potential to help understand the biological/metabolic significance of these compounds.

DISCUSSION

In mammalian systems, research on NAEs and their metabolism has been intensive in recent years because of their ubiquitous nature and widespread physiological and behavioral activities (42–45). Several novel NAE-derived lipids, their regulation mechanisms, and functions have been identified (24–26, 32, 46). Studies on NAEs in plants have followed this mammalian research, and the conserved hydrolysis of NAEs is perhaps the most understood regulatory step in NAE metabolism in eukaryotes in general (1, 2, 9). However, there has been increasing evidence, both *in vitro* and *in vivo*, in plants and animals suggesting that these lipids are also substrates for oxidative metabolism by a number of fatty acid oxygenases such as LOXs, COXs, and cytochrome P450s (21–23, 25, 28).

Active metabolism of NAEs in plant systems has been associated with seed germination and seedling growth. For example, in various plants, such as cotton, pea, peanut, and *Arabidopsis*, NAE content was most abundant in the desiccated seeds and rapidly declined upon imbibition and seedling establishment (20, 47). In *Arabidopsis*, when NAE metabolism was perturbed by overexpression of FAAH, the transgenic OE lines with increased FAAH activity had lower NAE content than the WT plants (Fig. 1*A*), displayed enhanced growth and early maturity (20), hypersensitivity to abscisic acid (19), and were susceptible to biotic stress (17). These studies suggest a pivotal role for NAEs in balancing growth and stress responses. However, FAAH KO plants were not substantially affected by the lack of FAAH activity, except that they had higher NAE content in seeds (Fig. 1*A*) and were sensitive to exogenous NAE 12:0 treatments (20). The ability to decrease NAE content during the progression of germination and growth (Fig. 1*A*) and the oxidative activity of crude enzyme extracts toward PU-NAE substrates (Fig. 1*B*) suggested that NAE levels *in planta* might be metabolized in part by a competing pathway, such as oxidation, thus helping to explain the lack of a strong growth phenotype in FAAH KO plants.

Based on the *in vitro* evidence for NAE oxidation and LOX-mediated oxidation of FFAs, we hypothesized the primary products of LOX-mediated oxidation of PU-NAEs (Fig. 2). However, developing a method to identify these plausible products was a challenge because of the abundance of FFA products relative to NAE metabolites in plant tissues and transient nature of some of the products such as hydroperoxides. Previous studies that reported NAE-oxylipins were mostly *in vitro* and were restricted to identification of one or two products in a controlled reaction (23, 28). The methods that were developed in this study, including extraction of trace compounds from plant tissues, allow for separation, detection, and quantification of multiple metabolites of PU-NAEs concomitantly with those of PU-FFA (Figs. 3 and 4).

Irrespective of FAAH expression, all the three genotypes WT, KO, and OE, showed higher oxidative activity toward PU-NAEs in 4-day-old seedlings, suggesting that oxidation is an important pathway for the catabolism of PU-NAEs in *Arabidopsis*, at least in the early stages of seedling development (4 days). Evidence that this oxidation is LOX-mediated comes from several independent observations. First, NAE-oxidative products can be generated *in vitro* using heterologously expressed LOXs derived from a variety of species such as cucumber, *Physcomitrella* (data not shown), and *Arabidopsis* (Fig. 5), in addition to the commercially available GmLOX and StLOX (Fig. 4). Second, we were able to generate NAE-oPDA from NAE 18:3, using heterologously expressed AtAOC and AtAOS (supplemental Fig. 3) suggesting that the entire LOX pathway that generates free oPDA from 18:3 can also utilize NAE 18:3 and metabolites that derive therefrom. Third, we showed previously that inhibition of LOX activity in *Arabidopsis* seedlings resulted in accumulation of PU-NAEs, and this effect was more severe in FAAH KO seedlings than the WT or OE seedlings supporting the importance of this alternative LOX-mediated activity in depleting NAE levels during seedling development (16). Fourth, *Arabidopsis* seedlings exhibited substantial NAE-oxidation activities *in vitro* toward NAE 18:2 (Fig. 1*B*) and *in vivo* toward exogenously fed NAE18:2 or NAE 18:3 (Figs. 6 and 7), indicating that *Arabidopsis* seedlings indeed had the capacity for LOX-mediated oxidation of NAEs. Finally, the identification of 9- and 13NAE 18:2 hydroxides in FAAH knock-out seedlings where NAE hydrolysis is substantially blocked indicates that LOX operates *in vivo* in *Arabidopsis* to oxidize NAEs. Taken together, our results indicate that hydrolysis by FAAH and oxidation by LOX can compete *in vivo* for the depletion of polyunsaturated NAEs in *Arabidopsis* seedlings, and these studies highlight the discovery of new ethanolamide-conjugated oxylipin metabolites in plants that warrant further biochemical and physiological investigations.

It will be of particular interest to identify which LOX isozymes are involved in the PU-NAE pathway. Comparative studies of recombinant *Arabidopsis* 13- and 9-LOX enzymes revealed that their affinity for PU-NAEs or FFA did not differ greatly, except where 9-LOX showed 10-fold higher affinity for NAE 18:3 when compared with FFA 18:3 (Fig. 5). In experiments with both NAE and FFA substrates, the products generated were predominantly from FFA substrates (Fig. 5), and the lower proportion of PU-NAE oxidative products generated in the LOX reaction assays may be due to the 2–4-fold higher V_{max} exhibited by LOX toward FFA substrates when compared with PU-NAEs (16). Nevertheless, the apparent "substrate promiscuity" of LOX enzymes that has been demonstrated for esterified acyl substrates recently (39, 48) can now be extended to ethanolamide-conjugated acyl substrates. This may have important physiological implications in plants because the amide-conjugated JA oxylipins (JA-Ile) are believed to be the major regulatory molecules *in situ*, as opposed to the free acid (49, 50). Hence, additional conjugated oxylipins with potential yet-to-be discovered activities should be investigated. The work described here helps to encourage the search for additional oxylipins in plants and should facilitate their analysis in plant tissues.

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