Published in final edited form as:

Planta. 2012 September ; 236(3): 809–824. doi:10.1007/s00425-012-1669-z.

Lipidomic analysis of *N*-acylphosphatidylethanolamine molecular species in *Arabidopsis* suggests feedback regulation by *N*-acylethanolamines

Aruna Kilaru^{(1),(2),(6),7}, Pamela Tamura^{(3),(6)}, Giorgis Isaac^{(3),(5)}, Ruth Welti⁽³⁾, Barney J. Venables⁽²⁾, Edith Seier⁽⁴⁾, and Kent D. Chapman^{(2),8}

⁽¹⁾East Tennessee State University, Department of Biological Sciences, Johnson City, TN 37614, USA

⁽²⁾University of North Texas, Center for Plant Lipid Research, Department of Biological Sciences, Denton, TX 76203, USA

⁽³⁾Kansas State University, Kansas Lipidomics Research Center, Division of Biology, Manhattan, KS 66506, USA

⁽⁴⁾East Tennessee State University, Department of Mathematics and Statistics, Johnson City, TN 37614, USA

Abstract

N-Acylphosphatidylethanolamine (NAPE) and its hydrolysis product, *N*-acylethanolamine (NAE), are minor but ubiquitous lipids in multicellular eukaryotes. Various physiological processes are severely affected by altering the expression of fatty acid amide hydrolase (FAAH), an NAE-hydrolyzing enzyme. To determine the effect of altered FAAH activity on NAPE molecular species composition, NAE metabolism, and general membrane lipid metabolism, quantitative profiles of NAPEs, NAEs, galactolipids, and major and minor phospholipids for *FAAH* mutants of *Arabidopsis* were determined. The NAPE molecular species content was dramatically affected by reduced FAAH activity and elevated NAE content in *faah* knockouts, increasing by as much as 36-fold, far more than the NAE content, suggesting negative feedback regulation of phospholipase D-mediated NAPE hydrolysis by NAE. The *N*-acyl composition of NAPE remained similar to that of NAE, suggesting that the NAPE precursor pool largely determines NAE composition. Exogenous NAE 12:0 treatment elevated endogenous polyunsaturated NAE and NAPE levels in seedlings; NAE levels were increased more in *faah* knockouts than in wild-type or *FAAH* overexpressors. Treated seedlings with elevated NAE and NAPE levels showed impaired growth and reduced galactolipid synthesis by the “prokaryotic”, but not the “eukaryotic”, pathway. Overall, our data provide new insights into the regulation of NAPE-NAE metabolism and coordination of membrane lipid metabolism and seedling development.

⁽⁷⁾To whom correspondence may be addressed: Department of Biological Sciences, East Tennessee State University, PO Box 70703, Johnson City, TN 37614, USA. Tel.: 423-439-6931; Fax: 423-439-5958; kilaru@etsu.edu. ⁽⁸⁾To whom correspondence may be addressed: Department of Biological Sciences, University of North Texas, 1155 Union Circle, #305220, Denton, TX 76203-5017 USA. Tel.: 940-565-2969; Fax: 940-565-4136; chapman@unt.edu.

⁽⁵⁾Present address: Waters Corporation, 34 Maple Street, Milford, MA 01757

⁽⁶⁾Authors contributed equally to the work

Keywords

Mass spectrometry; Lipidomics; Lauroylethanolamide; Phospholipid

Introduction

N-Acylphosphatidylethanolamines (NAPEs) are a prevalent class of membrane phospholipids that occur in both plant and animal tissues (Chapman and Moore 1993b; Schmid 2000; Coulon et al. 2012). *N*-Acylethanolamines (NAEs), the hydrolysis products of NAPEs, have gained wide recognition as evolutionarily conserved lipid mediators that modulate various physiological processes in eukaryotic cells. A phospholipase D (PLD) capable of NAPE hydrolysis (Ueda et al. 2005a) and fatty acid amide hydrolases (FAAHs) that terminate NAE signaling (Thomas et al. 1997; Ueda et al. 2005b) were initially identified in mammalian systems. The metabolic pathway for NAE in plants is partly characterized based on molecular and *in vitro* and *in vivo* biochemical studies (Fig. 1). The hydrolysis of NAPE by an unidentified PLD generates NAE and phosphatidic acid (PA); *in vitro* studies have implicated PLD- β and/or - γ isoforms in NAPE hydrolysis (Pappan et al. 1998). NAE is catabolized to free fatty acid (FFA) and ethanolamine (EA) by FAAH-mediated hydrolysis (Shrestha et al. 2003). In addition, a lipoxygenase (LOX)-mediated oxidative pathway has been shown as an alternate, competitive metabolic pathway for metabolizing polyunsaturated (PU)-NAEs in cotton (Shrestha et al. 2002) and *Arabidopsis* (Kilaru et al. 2011a). Interestingly, *in vitro* studies have demonstrated that nano- to low micro-molar concentrations of NAE 12:0 inhibit PLD- α (Austin-Brown and Chapman 2002) and LOX activities (Keereetaweep et al. 2010) in a competitive manner.

In animals, the synthesis of NAPE involves the transfer of an acyl chain to phosphatidylethanolamine (PE) from the *sn*-1 position of phospholipids, including PE, phosphatidylcholine (PC), and their lysophospholipid derivatives, but not from FFAs or acyl-CoA (Natarajan et al. 1982). The enzyme *N*-acyltransferase, which catalyzes the transacylation, does not demonstrate any specificity with regard to chain length or degree of unsaturation (Sugiura et al. 1996). Thus, the composition of NAPE mostly reflects the *sn*-1 composition of its phospholipid donors, as observed in rodents (Kilaru et al. 2010; Kilaru et al. 2011b). In plant cells, although *in vitro* studies demonstrated that FFA may act as the acyl donor for NAPE synthesis (Chapman and Moore 1993a; McAndrew and Chapman 1998), a recently characterized *Arabidopsis* NAPE synthase (At1g78690) was shown to transfer an acyl chain from acyl-CoA to the head group of PE (Fig. 1; (Faure et al. 2009; Coulon et al. 2012)). A more recent study reported that this NAPE synthase enzyme may also have lysoglycerophospholipid *O*-acyltransferase activity (Bulat and Garrett 2011); however, only NAPE formation was demonstrated for this enzyme *in planta* (Faure et al. 2009), so the physiological significance of this *O*-acyltransferase activity reported *in vitro* remains unclear.

Our understanding of NAE metabolism and function in plants has expanded considerably with the availability of *FAAH* (At5g64440) overexpressor (OE) and T-DNA insertional knock-out (KO) mutants (reviewed in Kilaru et al. (2007) and Kim et al. (2010)). The

growth phenotypes observed in NAE metabolite mutants and their sensitivity to exogenous NAE 12:0 (Wang et al. 2006) and other abiotic (Teaster et al. 2007; Kim et al. 2009; Cotter et al. 2011) or biotic (Kang et al. 2008) stressors have been, in part, attributed to changes in NAE content and composition. The metabolism of NAEs in seedlings interacts with ABA signaling to modulate seedling growth (Teaster et al. 2007; Cotter et al. 2011), and this interaction may also influence responses to abiotic stress, especially for those responses that involve ABA. In terms of biotic stress, mature *Arabidopsis* plants over-expressing *FAAH1* showed compromised innate immunity to bacterial pathogens, and this was reported to be via altered salicylic acid (SA)-mediated regulation of host defense gene expression (Kang et al. 2008). The interaction of hormone signaling pathways with NAE metabolism is complex in that seedling growth regulation appears to depend on the modulation of NAE levels directly, whereas stress responses appear to be influenced by *FAAH* protein itself, independent of its catalytic activity (Kim et al. 2009). A hypothetical model for the regulation and action of the NAE regulatory pathway has been put forward (Chapman and Blancaflor, 2011), but further experimental evidence is required to more completely understand the mechanistic and functional details of this lipid mediator pathway. Here we have examined more broadly the lipid metabolites directly and indirectly related to the NAE regulatory pathway, specifically in the context of seeds and seedlings, a developmental period where considerable changes in NAE levels and metabolism have been associated with changes in physiology and growth. Further, because NAE 12:0 treatment can dramatically alter endogenous NAE profiles by inhibition of LOX-mediated oxidation (Keereetaweep et al. 2010), we used this as means to study the regulation of NAPE and NAE metabolite levels, in addition to utilizing *FAAH* mutants with altered NAE metabolism.

We recently showed that perturbation of *FAAH* activity affected the NAPE and NAE profiles of brain tissues of mice (Kilaru et al. 2010) and also ischemic brain tissues of rats (Kilaru et al. 2011b). Here we have adapted a comprehensive, mass spectrometry (MS)-based approach, similar to that reported for mammalian tissues (Astarita and Piomelli 2009; Kilaru et al. 2010; Kilaru et al. 2011b), to identify and quantify *Arabidopsis* NAPE molecular species along with other major and minor phospholipids and galactolipids. With the availability of lipidomic tools and NAE metabolic mutants, we have demonstrated accumulation of NAPE under conditions that might otherwise lead to excessive accumulation of NAE, suggesting potential negative feedback regulation of NAE production. Moreover, these data may raise the possibility that NAPE could be responsible, in part, for some of the pleiotropic effects in NAE mutants (Motes et al. 2005; Wang et al. 2006; Teaster et al. 2007; Cotter et al. 2011).

Materials and methods

Chemicals

Lauroylethanolamide (NAE 12:0) was synthesized from lauroylchloride and ethanolamine and purified by organic extraction as described previously (Austin-Brown and Chapman 2002). Solvents (optima grade) were purchased from Thermo-Fisher.

Plant material and growth conditions

For all experiments, surface-sterilized *Arabidopsis* seeds of wild-type (WT), *faah* KO and *FAAH* OE were stratified for 3 days at 4 °C in the dark prior to germination. Growth was maintained for 8 days in liquid MS medium with or without 35 µM NAE 12:0 and a 16/8 light/dark cycle at 22 °C.

Total lipid extraction

Total lipids from seeds and seedlings of *Arabidopsis* were extracted with hot (70 °C) isopropyl alcohol/chloroform/water (2/1/0.45 by volume), as described previously (Venables et al. 2005). Lipid yield was determined gravimetrically, and extracts were stored under nitrogen at –80 °C.

NAE quantification

NAEs from the total lipid extracts were separated by normal phase HPLC, and their trimethylsilyl-ether derivatives were quantified by GC–MS against a mixture of deuterated internal standards as described previously (Venables et al. 2005).

Purification of lipid extracts

Arabidopsis seed and seedling lipid extracts were purified using cyanopropyl solid phase extraction (SPE) columns (500 mg, Alltech Associates, Deerfield, IL) to eliminate interfering substances such as abundant neutral lipids that may cause ion suppression and mask low-quantity species during MS analysis. Lipid extracts were dissolved in 1 mL chloroform/methanol (9:1). An aliquot equivalent to 3 mg lipid weight for seeds or 0.3 to 3 mg lipid weight for seedlings was combined with 1.1 nmol of *N*-17:0 di16:0 PE, which was synthesized as previously described (Kilaru et al. 2010) and employed as an internal NAPE standard. The lipid-plus-standard sample was evaporated under nitrogen, dissolved in 50 µL chloroform, and applied to an SPE column pre-conditioned with 10 mL of a hexane/ethyl ether/acetic acid mixture (4:1:0.05). After sample application, the neutral lipids were eluted from the column with 10 mL of the hexane/ethyl ether/acetic acid solvent. Polar lipids were eluted with 10 mL of chloroform/methanol/water (4:1:0.1). The solvent in the polar lipid fraction was evaporated under nitrogen, and the lipid was re-dissolved in 1 mL chloroform. SPE column purification was also performed on six replicates of the NAPE standard alone, 1.1 nmol per column. Cyanopropyl SPE columns were tested, using multiple NAPE standards, to assess if NAPE species were effectively retained on the columns during sample loading and neutral lipid elution. All NAPE standards were eluted in the polar lipid fraction without any loss in the neutral lipid fraction. Purified polar lipids were also used for phospholipid and galactolipid analyses.

NAPE analysis and quantification

Aliquots of 150–900 µL of purified polar lipid solution, equivalent to 1.2 mg lipid weight for seeds or approximately 260–470 µg lipid weight for seedlings, were taken for MS analysis and evaporated to dryness under nitrogen. The aliquots were re-dissolved in chloroform, and solvent was added so that the final ratio of chloroform/methanol/300 mM ammonium acetate in water was 300:665:35; the final volume was 1.4 mL.

Mass spectra were acquired on a triple quadrupole MS system (API 4000 QTrap, Applied Biosystems, Foster City, CA) equipped with an autosampler (LC Mini PAL, CTC Analytics AG, Zwingen, Switzerland). Samples were introduced by continuous infusion into the Turbo V electrospray ionization source at 30 $\mu\text{L}/\text{min}$. Sequential neutral loss (NL) scans produced a series of spectra, with each spectrum revealing a set of lipid species containing a common ammoniated *N*-fatty amide head group fragment corresponding to each common fatty acid. Lipids were detected in positive ion mode as $[\text{M} + \text{NH}_4]^+$ ions with the following scans: *N*-12:0 species with NL of m/z 340.2, *N*-14:0 species with NL of m/z 368.3, *N*-16:1 species with NL of m/z 394.3, *N*-16:0 species with NL of m/z 396.3, *N*-18:3 species with NL of m/z 418.3, *N*-18:2 species with NL of m/z 420.3, *N*-18:1 species with NL of m/z 422.3, *N*-18:0 species with NL of m/z 424.3, and *N*-20:1 species with NL of m/z 450.3. A scan for *N*-17:0 species (NL of m/z 410.3) was included to detect the internal standard. The declustering potential was set at +60 V, the entrance potential at +8 V, and the exit potential at +15 V. The ion spray voltage was set at +5.5 kV. The collision gas (nitrogen) was set at 2 (arbitrary units), and the collision energy was +45 V. The source temperature was 100 $^{\circ}\text{C}$, curtain gas was 20 (arbitrary units), ion source gases were 45 (arbitrary units), interface heater was on, and mass analyzers were adjusted to a resolution of 0.7 u full width at half height. For each spectrum, 100 cumulative scans were collected in multiple channel analyzer mode at a scan speed of 50 u/s.

The data were smoothed, the background of each spectrum was subtracted, and the peaks were centroided and integrated using a custom script and Applied Biosystems Analyst software. Peaks corresponding to the target lipids in each *N*-acyl class (each spectrum) were identified, the data were corrected for isotopic overlap within each spectrum (i.e., due to the diacylglycerol portion of NAPE), and molar amounts were quantified relative to the *N*-17:0 di16:0 PE internal standard. Six replicate samples from SPE column elution of the *N*-17:0 di16:0 PE standard alone were run through the same series of scans; two standard-only replicates were followed by 12 *Arabidopsis* samples on the instrument. The molar amounts of each lipid metabolite detected in the two averaged “NAPE standard-only” spectra were subtracted from the molar amounts of each metabolite detected in each experimental *Arabidopsis* spectrum to correct for chemical or instrumental noise. Finally, the data were corrected for isotopic overlap between head groups (NL fragments) and normalized to the sample lipid weight. Data were reported as mass spectral signal, normalized to *N*-17:0 di16:0 PE, per mg lipid weight, where the amount of signal produced by 1 nmol of *N*-17:0 di16:0 PE is 1.

Phospholipid/galactolipid analysis and quantification

As above, an automated electrospray ionization-triple quadrupole MS approach was used; data were acquired as described previously (Devaiah et al. 2006) with modifications. An aliquot of 15 to 90 μL of SPE-purified polar lipid, equivalent to 120 μg lipid weight for seeds or approximately 26-47 μg lipid weight for seedlings, was used. Precise amounts of phospholipid and galactolipid internal standards, obtained and quantified as previously described (Welti et al. 2002), were added in the following quantities: 0.6 nmol di12:0 PC, 0.6 nmol di24:1 PC, 0.6 nmol 13:0 lysoPC, 0.6 nmol 19:0 lysoPC, 0.3 nmol di12:0 PE, 0.3 nmol di23:0 PE, 0.3 nmol 14:0 lysoPE, 0.3 nmol 18:0 lysoPE, 0.3 nmol di14:0 PG, 0.3 nmol

di20:0 (diphytanoyl) PG, 0.3 nmol 14:0 lysoPG, 0.3 nmol 18:0 lysoPG, 0.3 nmol di14:0 PA, 0.3 nmol di20:0 (diphytanoyl) PA, 0.2 nmol di14:0 PS, 0.2 nmol di20:0 (diphytanoyl) PS, 0.23 nmol 16:0-18:0 PI, 0.16 nmol di18:0 PI, 2.01 nmol 16:0-18:0 MGDG, 0.39 nmol di18:0 MGDG, 0.49 nmol 16:0-18:0 DGDG, and 0.71 nmol di18:0 DGDG. Solvent was added to the sample-plus-standard mixture so that the final ratio of chloroform/methanol/300 mM ammonium acetate in water was approximately 300:665:35, and the final volume was 1.4 mL.

Mass spectra were acquired on the triple quadrupole MS and autosampler system described in the previous section. Sequential precursor (Pre) and NL scans of the extracts produced a series of spectra with each spectrum revealing a set of lipid species containing a common head group fragment. Lipid species were detected with the following scans: PC and lysoPC, $[M + H]^+$ ions in positive ion mode with Pre of m/z 184.1; PE and lysoPE, $[M + H]^+$ ions in positive ion mode with NL of m/z 141.0; PG, $[M + NH_4]^+$ ions in positive ion mode with NL of m/z 189.0; lysoPG, $[M - H]^-$ ions in negative ion mode with Pre of m/z 152.9; PI, $[M + NH_4]^+$ ions in positive ion mode with NL of m/z 277.0; PS, $[M + H]^+$ ions in positive ion mode with NL of m/z 185.0; PA, $[M + NH_4]^+$ ions in positive ion mode with NL of m/z 115.0; MGDG, $[M + NH_4]^+$ ions in positive ion mode with NL of m/z 179.1; and DGDG, $[M + NH_4]^+$ ions in positive ion mode with NL of m/z 341.1. The ion spray voltage was set at +5.5 kV or -4.5 kV. Declustering potentials were +100 V for PE, PC, PA, PG, PI, and PS, +90 V for MGDG and DGDG, and -100 V for lysoPG. Entrance potentials were +15 V for PE, +14 V for PC, PA, PG, PI and PS, +10 V for MGDG and DGDG, and -10 V for lysoPG. Exit potentials were +11 V for PE and +14 V for PC, PA, PG, PI, and PS, +23 V for MGDG and DGDG, and -5 V for lysoPG. The collision gas (nitrogen) was set at 2 (arbitrary units). The collision energies were +28 V for PE, +40 V for PC, +25 V for PA and PI, +20 V for PG, +26 V for PS, +21 V for MGDG, +24 V for DGDG, and -53 V for lysoPG. The source temperature, curtain gas, ion source gases, interface heater, and mass analyzers were adjusted as for NAPE analysis. For each spectrum, 9 to 65 continuum scans were collected in multiple channel analyzer (MCA) mode at a scan speed of 45-100 u per s.

The data were smoothed, the background of each spectrum was subtracted, and the peaks were centroided and integrated using a custom script and Applied Biosystems Analyst software. Peaks corresponding to the target lipids were identified, the data were corrected for isotopic overlap, and molar amounts were calculated in comparison to the internal standards in the same lipid class. The same spectra were acquired on samples containing phospholipid/galactolipid internal standard mixture only and were used to correct for chemical or instrumental noise by subtracting the molar amounts of each lipid metabolite detected in the “internal standards-only” spectra from the molar amounts of each metabolite calculated from the experimental *Arabidopsis* spectra. Each “internal standards-only” spectrum was used to correct the data from the following ten samples run on the instrument. Finally, the data were adjusted to account for the fraction of sample analyzed and normalized to the sample lipid weight.

Data Analysis

All metabolite data obtained per mg lipid content were normalized to the sample fresh weight (FW) and reported as nmol/g FW or normalized MS signal/g FW; the amount of signal produced by 1 nmol of *N*-17:0 di16:0 PE is 1. Data are averages of four replicates and expressed as mean $\pm$ SD (Supplemental Table 1). A two-way analysis of variance was conducted for each one of the eleven metabolites quantified, as response variable and using genotype (WT, KO, and OE) and condition (seed and treated and untreated 8-day-old seedlings) as factors. For most of the metabolites, the interaction between genotype and condition was significant ($P < 0.05$). A two-tailed, unpaired Student's *t*-test was used to determine the significant difference among the datasets ($P < 0.05$). A two-way analysis of variance was also conducted for each NAE and NAPE species with their proportion of the total metabolite as a response variable and using genotype and condition as factors, to determine the significance of their interaction on composition. Analysis of phospholipid/galactolipid class totals from one WT 8-day-old seedling replicate showed that values fell statistically outside the mean by a Q-test (Shoemaker et al. 1974); only these values were removed from calculations.

Results

Identification and quantification of NAPE, NAE, and PE, the major metabolites of the NAE metabolic pathway

While characterization of the NAE pathway remains far from complete (Fig. 1), our study focused on the consequences of altered NAE metabolism on the metabolite levels of NAPE and other phospholipids. In our previous work with mice, we developed a method for direct infusion electrospray ionization mass spectrometry analysis of NAPEs (Kilaru et al. 2010). Here, we extended this procedure to comprehensively analyze the NAPEs in seeds and seedlings of *Arabidopsis*. To reduce ion suppression during direct infusion electrospray ionization mass spectrometry, polar lipids were separated from neutral lipids by SPE before analysis. When dissolved in solvent containing ammonium acetate, NAPEs within the polar lipid fraction form positively charged ammonium adducts ($[M + NH_4]^+$), which fragment into neutral head group and charged diacylglycerol (DAG) fragments in the mass spectrometer. For example, fragmentation of *N*-18:2 di18:2 PE generates a NL of *m/z* 420.3 (Fig. 2a). Sequential scanning for NL of various ammoniated *N*-acylethanolamine phosphate head groups provides a diagnostic method to detect the molecular species of NAPE in all *N*-acyl classes. NAEs, which are generated upon PLD hydrolysis (Fig. 2b), were analyzed by GC-MS. PE was analyzed as previously described by monitoring positive ions that can generate a NL of *m/z* 141.0 (Brugger et al. 1997); Fig. 2c). The total lipids extracted from seeds and seedlings of *Arabidopsis* were thus analyzed for content and molecular composition of NAPE, NAE, PE, and other phospho- and galactolipids (Supplemental Table 1).

Altered FAAH expression affects NAE metabolite content but not composition in seeds

Total NAPE, NAE, and PE levels in WT, *faah* KO and *FAAH* OE seeds were quantified (Fig. 3). The total NAPE content in seeds of all three genotypes was ~10-fold higher than their NAE and ~10-fold lower than their PE content (Fig. 3a). The total NAPE content in

OE seeds was ~2-fold lower than in WT and KO seeds. Although the absence of FAAH activity resulted in elevated levels of NAE in *faah* KO seeds, their NAPE and PE levels were not significantly different from those in WT (Fig. 3a). In contrast, the NAE and PE levels were significantly lower in *FAAH* OE seeds relative to WT (Fig. 3a). These data suggest that overexpression of *AtFAAH* had a stronger effect than *faah* KO on the NAPE, NAE, and PE metabolite pools; we have suggested elsewhere that additional mechanisms exist for the metabolism of NAEs, specifically PU-NAEs (Kilaru et al. 2011a).

NAE 18:2 and NAPE with 18:2 at the *N*-acyl position were the most abundant species in WT, KO, and OE seeds (Fig. 3b). The NAPEs with saturated species at the *N*-acyl position contributed to ~25% or less of the total NAPE (Fig. 3c). Although NAPE 20:1 and NAPE 16:1 were detected, corresponding NAE species were not, perhaps because their concentration was below the limits of detection in these analyses. NAE 12:0 and 14:0 were previously reported and were excluded from this study since exogenous treatment with NAE 12:0 would interfere with quantification of the same endogenous species and NAE14:0 was near the limits of detection in seeds. The lower amounts of NAPE and NAE noted in OE seeds, relative to WT, was similar for most molecular species groups based on their *N*-acyl composition (Fig. 3b). Regardless of altered FAAH metabolism and altered levels of NAPE and NAE, the overall composition of *N*-acyl species contributing to total NAPE and NAE content in seeds were significantly different across the genotypes (Fig. 3c).

Exogenous NAE treatment results in seedlings with altered NAPE, NAE, and PE profiles

Previous studies have shown that exogenous NAE 12:0 treatment affects germination and seedling growth. The physiological ramifications of NAE treatment on *FAAH* mutants were genotype-dependent and included altered endogenous NAE levels, specifically those of PU-NAEs (Wang et al. 2006; Teaster et al. 2007; Keereetawee et al. 2010; Cotter et al. 2011). Here, we have determined the effects of exogenous NAE treatment on the content and composition of NAPE, NAE, and PE in *Arabidopsis* seedlings (Fig. 4).

When *Arabidopsis* seeds were germinated and grown in the presence of 35 μ M exogenous NAE 12:0, growth of *FAAH* OE seedlings, which actively hydrolyze NAEs, remained unaffected relative to the OE seedlings that were grown in the absence of NAE 12:0. However, growth of WT and *faah* KO seedlings was visibly stunted when grown in the presence of exogenous NAE 12:0 (Fig. 4a). The NAE and NAPE content of 8-day-old untreated WT seedlings was 7- and 10-fold lower than that of the seeds, respectively, whereas the PE content was lower by about 2-fold (compare Fig. 4b with 3a). Unlike in seeds, the NAPE and PE content of 8-day-old seedlings (control) did not vary significantly among the genotypes (Fig. 4b).

Exogenous NAE treatment of seedlings resulted in a significant increase in the absolute amounts of NAPE, NAE, and PE for all genotypes (Fig. 4b). The most dramatic changes were noted with NAPE levels. In WT, KO, and OE seedlings, the NAPE levels increased by 22-, 36-, and 12-fold, respectively, relative to untreated 8-day-old seedlings (Fig. 4b). A less pronounced but significant increase in absolute levels of NAE (4-8-fold) and PE (2-fold) was also observed in seedlings exposed to NAE 12:0 when compared with the untreated seedlings (Fig. 4b). The NAE and PE content of WT, KO, and OE seedlings, when grown in

the presence of exogenous NAE 12:0, were similar to the levels observed in seeds. However, the increase in NAPE content in treated seedlings resulted in 2- to 3-fold higher levels than observed in seeds (compare Fig. 4b with 3a).

In addition to the decrease in levels of NAPE in 8-day-old control seedlings relative to seeds, their *N*-acyl composition was also significantly different from that of seeds, specifically *N*-18:2 species were less dominant in 8-day-old control seedlings relative to seeds (Fig. 5a compared to Fig. 3b). The seedlings, although showed an abundance of PU species there was no significant difference in NAPE content and composition among the genotypes (Fig. 5a). The increases in NAPE content observed in the seedlings when grown in the presence of exogenous NAE 12:0 is attributed mostly to increases in the unsaturated *N*-18:2, *N*-18:3, and *N*-18:1 NAPE species but not the saturated species (Fig. 5b). In seeds and untreated 8-day old seedlings, the unsaturated *N*-acyl species contributed < 80 % of the total NAPE (Figs. 3c, 5a), in contrast to 95 % in NAE-treated seedlings (Fig. 5b). With exogenous NAE 12:0 treatment, the differences in composition of NAPE molecular species among genotypes were minor, yet significantly different, along with the amounts of *N*-acyl species that differed dramatically among genotypes (Fig. 5b). This suggests that there is likely no induction of a new metabolic process in the mutant genotypes, but rather an exaggerated regulation of the normal NAPE/NAE synthesis and turnover. One interesting observation is that the proportional amount of 18:2 NAPE was much higher in the pool of seed NAPEs compared to that in the NAPE pool in seedlings (untreated) (compare Fig 3b to Fig 5a). This may reflect a preference by the unknown PLD for *N*-18:2 containing species during normal seedling establishment.

The specific effects of NAE 12:0 treatment on NAE content and composition were very similar to those on NAPE (Fig. 6a and b). The unsaturated NAE levels increased 4- to 6-fold in treated 8-day-old seedlings relative to untreated seedlings. As expected, the *faah* KO seedlings treated with NAE 12:0 showed the highest increase in NAE content among the three genotypes (with NAE 18:2 being the most abundant species), due to their reduced capacity for NAE hydrolysis. The composition of NAE-treated 8-day-old seedlings, although shared a similar profile across the genotypes and closely resembled that of the seeds, it was significantly different from seeds and between genotypes (compare Fig. 6b and 3c).

Lipidomic profiling enables identification of PE components of NAPE molecular species

NAPEs are formed by *N*-acylation of the primary amine group of PE. Detailed analysis of the individual NAPE molecular species revealed that the predominant glycerol backbone fatty acids of the PE component were similar regardless of the *N*-acyl group (e.g., *N*-16:0, *N*-18:3, *N*-18:2, or *N*-18:1) (Fig. 7). For example, 34:2 and 36:4 were the most prevalent glycerol backbone fatty acids (numbers designate totals of two fatty acids) within most of the NAPE *N*-acyl groups (Fig. 7), particularly in seeds. In untreated 8-day-old seedlings, 34:3 and 36:5 were also prominent in various *N*-acyl groups. In seeds, ~ 28, 65, and 7% of the total NAPE molecular species contained PE components with 34-, 36-, and 38-carbon fatty acids, respectively. The composition did not vary among the genotypes but was altered during seedling development (Fig. 7). In 8-day-old seedlings, PE components with 34- and

36-carbon fatty acids constituted 48 and 47% of the total NAPEs, respectively, and 38-carbon fatty acids constituted only 5%. This is similar to the PE composition in leaves, which typically contain substantial amounts of PE with 34 and 36 acyl carbons (Devaiah et al. 2006). There were substantial increases in longer chain molecular species in treated seedlings of all genotypes, with 38:3 and 38:4 diacylglycerol components making up 12% of the NAE composition (Fig. 7 and Supplemental Table 1). Prevalence of longer chain species, as observed in seeds, but not in untreated seedlings, suggests that NAE 12:0 treatment of the seedlings renders the NAE composition more “seed-like”. Similar observations were made with profiles of the PE precursor pool available for *N*-acylation to form NAPEs. The most abundant PE species in WT, *faah* KO, and *FAAH* OE seeds was 36:4 PE (Fig. 8). PE levels, which were not different among the genotypes in untreated 8-day-old seedlings, were increased by exogenous NAE treatment in WT and *faah* KO but not *FAAH* OE seedlings (Fig. 8).

Similarities and differences in galactolipid and phospholipid composition in *FAAH* mutants

Quantification of digalactosyldiacylglycerols (DGDG) and monogalactosyldiacylglycerols (MGDG) indicated that their levels were significantly lower in *FAAH* OE seeds than in WT seeds (Fig. 9a). Regardless of the genotype, the levels of MGDG were increased by more than 4-fold in 8-day-old seedlings relative to seeds, while the DGDG levels did not change significantly with seedling development (Fig. 9b). Increases in MGDG levels were strongest in OE seedlings (18-fold increase relative to seeds). Although exogenous application of NAE 12:0 resulted in a two-fold reduction in galactolipid content relative to untreated seedlings (Fig. 9c), the levels remained higher than those observed in seeds.

The most abundant phospholipid in the seeds was PC, followed by PI and PE. All of the major phospholipids showed significantly lower levels in OE seeds than in WT and KO seeds, but this difference did not persist in 8-day-old seedlings. Phospholipid content was in general higher in seeds, relative to seedlings, by ~ 2-fold. The levels of major and minor phospholipids were moderately increased in seedlings, specifically WT and *faah* KO but not *FAAH* OE, when grown in the presence of NAE 12:0, relative to untreated seedlings (Fig. 9). Major and minor phospholipids (phosphatidylserine (PS), lysoPC, and lysoPE) were detected at lower levels in *FAAH* OE seeds than in WT and KO seeds, but no difference among the genotypes was observed in untreated seedlings (Fig. 9). These data suggest that the seedlings adjust their PE supply to meet the exaggerated synthesis of NAE (and NAE) that is occurring in the presence of exogenous NAE 12:0.

Impaired prokaryotic galactolipid synthesis in seedlings treated with NAE 12:0

Comprehensive profiling of the precursor diacylglycerol species for glycerolipid synthesis led to the identification of a wide range of effects associated with altered NAE and NAE levels (Supplemental Table 1). In 8-day-old WT seedlings, MGDG and DGDG together made up ~ 43 % of the total glycerolipids. Levels of molecular species derived *via* the “prokaryotic” pathway, or synthesized in plastids (Ohlrogge and Browse 1995; Benning 2009), such as MGDG 34:6, were severely reduced in seedlings exposed to NAE 12:0 (Fig. 10a). On the contrary, levels of molecular species such as MGDG 36:6, derived *via* the

“eukaryotic” pathway, or synthesized and contributed from the endoplasmic reticulum, increased with NAE 12:0 treatment (Fig. 10b). This perturbation of the prokaryotic pathway was somewhat alleviated in *FAAH* OE seedlings (Fig. 10a), which also had lower levels of NAE and NAPE compared to WT and *faah* KO genotypes (Figs. 4-6). In other words, it appears that the content of NAE and NAPE in plant tissues may influence the proportion of prokaryotic lipid synthesis selectively. This may be a consequence of a dramatic demand for extraplasmidial PE for the synthesis and turnover of NAPE.

Discussion

The NAE regulatory pathway is part of the endocannabinoid signaling system in vertebrates, and as such it has received considerable experimental attention. Targeted lipidomics provided new opportunities to gain a clearer understanding of the effects of perturbations of the intricate network of the NAE metabolic pathway in vertebrate systems (Astarita and Piomelli 2009; Astarita et al. 2009; Kilaru et al. 2010; Kilaru et al. 2011b). Results with mammalian systems suggested that the physiological effects of NAE metabolism might involve changes in other metabolites in the pathway, including NAPE. For example, selective lipidomic analysis of *FAAH* (–/–) mice revealed differences in content and composition of NAPE, NAE, and other major and minor phospholipids in brain and heart tissues, indicating differential regulation of the NAE metabolic pathway (Kilaru et al. 2010). Despite the physiological implications that NAE metabolites have on plant growth and development, an in depth understanding of the pathway and its regulation in plants is essentially lacking. Here, we have extended methods developed for mammalian systems to plants and profiled the NAPE content and composition, along with NAE, PE, and other phospholipids, in *Arabidopsis* seeds and seedlings (Figs. 3-10).

In *Arabidopsis*, the metabolites NAPE, NAE, and PE were most abundant in seeds, and the levels declined by ~ 10-, 7-, and 2-fold, respectively, as seeds germinated and seedlings developed (Figs. 3, 4). Modulation of NAE levels is considered important for proper seedling establishment (Wang et al. 2006; Teaster et al. 2007; Cotter et al. 2011). However, metabolite analyses revealed that normal seedling establishment might require a coordinated decline in NAE metabolites, especially NAPE, which showed the greatest decline during normal seedling growth. Perhaps the lower NAPE content in *FAAH* OE seeds, relative to WT seeds, in addition to reduced NAE content (Fig. 3; Wang et al. 2006), together help to explain their enhanced growth phenotype, as observed in 8-day-old *FAAH* OE seedlings grown in the presence or absence of NAE 12:0 treatment (Fig. 4a; Wang et al. 2006; Teaster et al. 2007; Cotter et al. 2011). Interestingly, 50 to 80 % of the total NAPE and NAE content in seeds was composed of PU-*N*-acyl species (18:2 and 18:3; Fig. 3), suggesting that it is specifically their decline in seedlings (Figs. 5,6) that substantially contributes to seedling development. This concept is supported by the fact that *faah* KO seeds, despite their slightly higher levels of NAE (but not NAPE), showed normal germination and growth as long as they retained the capacity to metabolize the large amount of PU-NAEs *via* oxidation (Kilaru et al. 2011a). However, if oxidation also was blocked by LOX inhibitors such as NAE 12:0 (Figs. 4-6, (Keereetaweep et al. 2010)) or nordihydroguaiaretic acid (Keereetaweep et al. 2010), seedling development was substantially retarded, especially in *faah* KO. Additionally, the NAPE and NAE metabolite profiles were markedly elevated and more

similar to those in seeds than in seedlings. Taken together, this suggests that activation of PU-NAPE and -NAE metabolism is associated with normal seedling establishment.

Perhaps the most significant observation here was the dramatic elevation in NAPE levels in seedlings after application of exogenous NAE 12:0 (36-fold in *faah* KO), which was much more than the increase in NAE content itself (8-fold; Fig. 4). In addition to being a competitive inhibitor of LOXs (Keereetaweep et al. 2010) and PLD- α (Austin-Brown and Chapman 2002), NAE 12:0 is known to affect membrane trafficking and cytoskeletal organization (Blancaflor et al. 2003; Motes et al. 2005). It is possible that the physiological actions of NAE 12:0 may be, in part, attributed to increases in levels of NAPE, which has an unusual structural influence on membrane organization and function (Sandoval et al. 1995). NAPE levels also were elevated during elicitor-induced signaling in tobacco cells (Chapman et al. 1995) and chilling stress (Chapman and Sprinkle 1996). Furthermore, a 13-fold increase in NAPE levels was observed in anoxia-stressed potato cells (Rawlyer and Braendle 2001). Together, these data suggest that, while more experimental evidence needs to be obtained, it may be important to take a closer look at the role of NAPE in cellular responses to stress, as this lipid class may have unrecognized roles in plant cell function.

Interestingly, feeding of exogenous NAE 12:0 affected mostly the levels of PU-*N*-acyl groups of the NAPE species (Fig. 5), similar to profiles of the NAEs (Fig. 6). The *N*-acyl groups of NAPE (and NAE) were dominated by *N*-18:2, *N*-18:3, and *N*-18:1, regardless of the genotype, whereas the glycerol backbone *O*-acyl patterns of NAPE remained similar among samples (Fig. 7), indicating that existing PE pools were utilized for NAPE synthesis. On the other hand, the results suggest that perhaps there was a preference for unsaturated 18C acyl-CoA/FFA for NAPE formation under NAE 12:0-stimulated conditions. Further, there was only a moderately higher amount of PE in NAE 12:0-treated seedlings compared to untreated seedlings. In fact, the ratio of PE to NAPE was greater than 6 to 1 in seeds (Fig. 3), but in treated seedlings this ratio was closer to 2 to 1 (Fig. 4). Overall amounts of the normally minor NAPE were as high in treated seedling tissues as the common and abundant PE was in untreated seedling tissues. The marked elevation of NAPE (much more than NAE on a percentage basis) suggests that NAE 12:0 treatment results in negative feedback regulation of the PLD-mediated hydrolysis of NAPE. Whether this is due mostly to a pharmacological inhibition of the PLD enzyme in situ, or an exaggeration of a normal biochemical regulatory process will require further examination. NAPE formation, on the other hand, seems to be under less tight control, allowing NAPE to accumulate in NAE-treated tissues, especially when FAAH activity was compromised (*faah* KO). Increased capacity for NAE turnover (*FAAH* OE) appeared to reduce the severity of the accumulation of NAE pathway metabolites (including NAPE), the re-direction of lipid metabolism toward NAPE formation, and the severity of inhibition of seedling growth caused by exogenous NAE treatment (Figs. 3-10). Overall, the regulation of NAPE levels and composition in plants appears to require a constitutive metabolism of pathway intermediates, but this is perturbed by the inhibition of NAE catabolism. These data also suggest that the PLD-mediated formation of NAE is subject to substantial regulation and may require further attention as a control point of the NAE regulatory pathway in plants.

Comprehensive lipidomic analyses revealed significant reductions in the levels of galactolipids and phospholipids in *FAAH* OE seeds, relative to WT seeds (Fig. 9). The reasons are unclear for the reductions in major membrane lipid classes in the *FAAH* OE seeds on a seed mass basis. These differences may be due to differences in water content or the mass of something else other than lipid. We measured seed oil percentage of these genotypes by time-domain, ^1H -NMR, and the oil content did not vary significantly between genotypes. This suggests that the bulk storage lipid metabolism and compartmentation is not substantially influenced by changes in *FAAH* expression. Other studies have demonstrated that ABA signaling pathways and NAE metabolism interact during seedling establishment (Teaster et al. 2007; Cotter et al. 2011). It is possible that altered *FAAH* expression also influences embryo/seed development pathways through its interaction with ABA signaling, but this stage of development remains to be explored.

During seedling development, an increase in NAE content was associated with a marked reduction in galactolipid biosynthesis *via* the prokaryotic pathway (Fig. 10). For example, in NAE-treated seedlings, the levels of MGDG 34:6 (synthesized by the prokaryotic pathway) were severely reduced while MGDG 36:6 (synthesized by the eukaryotic pathway) levels were increased. This was especially obvious for wild type and *faah* knockout seedlings, whereas this trend was somewhat reversed, though not completely, in *FAAH* OE seedlings, suggesting that indeed it was related to alterations in the capacity for NAE turnover. The change in flux between chloroplast and endoplasmic reticulum membrane lipid synthesis pathways may be a reflection of the significant demand for PE and NAPE formation and their extraplastidial synthesis, such that overall membrane glycerolipid synthesis by the eukaryotic pathway was upregulated, resulting in a general increased availability of ER-derived glycerolipid to support chloroplast MGDG synthesis. On the other hand, DGDG species derived from the eukaryotic pathway were not altered in a similar manner to MGDG, as would be expected. Further, PC and PG metabolites seemed relatively unchanged (glycerolipid classes formed by eukaryotic and prokaryotic pathways, respectively, almost entirely). It is possible that the formation of DGDG from MGDG is unable to keep up with additional MGDG from the eukaryotic pathway, but this seems unlikely. Alternatively, the selective alteration in MGDG species might be a result of reduced seedling growth and stunted development of chloroplasts in NAE-treated seedlings (a reduced requirement for chloroplast lipids), but it is difficult to reconcile how this would result in a selective change within MGDG species and be exhibited not more broadly in chloroplast lipids. Perhaps exogenous NAE acts to inhibit a later step in plastidial MGDG formation, resulting in markedly reduced MGDG formation via the prokaryotic pathway, and increased MGDG formation via the eukaryotic pathway. Nonetheless, the evaluation of this alteration in flux between the prokaryotic and eukaryotic pathways may provide new insight into the functional significance of NAE metabolism in seedlings. In addition to gross morphological changes in seedlings grown in NAE 12:0, there may be a regulation of cellular lipid metabolism in general by the NAE regulatory pathway that remains to be explored in the future. In conclusion, comprehensive profiling of the metabolites of the NAE pathway in *Arabidopsis* has provided new insights into potential regulatory points of this pathway and how NAE-NAPE metabolism might influence seedling growth.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by a grant from the United States Department of Energy, Office of Basic Energy Sciences (BES, grant number DE-FG02-05ER15647) and a seed grant from the University of North Texas to KDC. Method development and equipment acquisition at the Kansas Lipidomics Research Center was funded by National Science Foundation (DBI 0521587; MCB 0455318 and 0920663), Kansas IDeA Networks of Biomedical Research Excellence (INBRE) of National Institute of Health (P20RR16475), and Kansas State University.

Abbreviations

DGDG	Digalactosyldiacylglycerol
EA	Ethanolamine
FAAH	Fatty acid amide hydrolase
FFA	Free fatty acid
FW	Fresh weight
KO	Knock-out
LOX	Lipoxygenase
MGDG	Monogalactosyldiacylglycerol
MS	Mass spectrometry
NAE	<i>N</i> -acylethanolamine
NAPE	<i>N</i> -acyl PE
NL	Neutral loss
OE	Overexpressor
PA	Phosphatidic acid
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PLD	Phospholipase D
Pre	Precursor
PS	Phosphatidylserine
PU	Polyunsaturated
SPE	Solid phase extraction
X:Y	Designates total fatty acid or acyl carbons:carbon-carbon double bonds

References

- Astarita G, Geaga J, Ahmed F, Piomelli D. Targeted lipidomics as a tool to investigate endocannabinoid function. *Int Rev Neurobiol.* 2009; 85:35–55. [PubMed: 19607960]
- Astarita G, Piomelli D. Lipidomic analysis of endocannabinoid metabolism in biological samples. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2009; 877:2755–2767.
- Austin-Brown SL, Chapman KD. Inhibition of phospholipase D alpha by N-acylethanolamines. *Plant Physiol.* 2002; 129:1892–1898. [PubMed: 12177503]
- Benning C. Mechanisms of lipid transport involved in organelle biogenesis in plant cells. *Annu Rev Cell Dev Biol.* 2009; 25:71–91. [PubMed: 19572810]
- Blancaflor EB, Hou G, Chapman KD. Elevated levels of N-lauroylethanolamine, an endogenous constituent of desiccated seeds, disrupt normal root development in *Arabidopsis thaliana* seedlings. *Planta.* 2003; 217:206–217. [PubMed: 12783328]
- Brugger B, Erben G, Sandhoff R, Wieland FT, Lehmann WD. Quantitative analysis of biological membrane lipids at the low picomole level by nano-electrospray ionization tandem mass spectrometry. *Proc Natl Acad Sci U S A.* 1997; 94:2339–2344. [PubMed: 9122196]
- Bulat E, Garrett TA. Putative N-acylphosphatidylethanolamine synthase from *Arabidopsis thaliana* is a lysoglycerophospholipid acyltransferase. *J Biol Chem.* 2011; 286:33819–33831. [PubMed: 21803774]
- Chapman KD, Lin I, DeSouza AD. Metabolism of cottonseed microsomal N-acylphosphatidylethanolamine. *Arch Biochem Biophys.* 1995; 318:401–407. [PubMed: 7733669]
- Chapman KD, Moore TS Jr. Catalytic properties of a newly discovered acyltransferase that synthesizes N-acylphosphatidylethanolamine in cottonseed (*Gossypium hirsutum* L.) microsomes. *Plant Physiol.* 1993a; 102:761–769. [PubMed: 12231864]
- Chapman KD, Moore TS Jr. N-acylphosphatidylethanolamine synthesis in plants: occurrence, molecular composition, and phospholipid origin. *Arch Biochem Biophys.* 1993b; 301:21–33. [PubMed: 8442663]
- Chapman KD, Sprinkle WB. Developmental, tissue-specific and environmental factors regulate the biosynthesis of N-acylphosphatidylethanolamine in cotton (*Gossypium hirsutum* L.). *J Plant Physiol.* 1996; 149:277–284.
- Cotter MQ, Teaster ND, Blancaflor EB, Chapman KD. N-acylethanolamine (NAE) inhibits growth in *Arabidopsis thaliana* seedlings via ABI3-dependent and -independent pathways. *Plant Signal Behav.* 2011; 6:671–679. [PubMed: 21633189]
- Coulon D, Faure L, Salmon M, Wattelet V, Bessoule JJ. Occurrence, biosynthesis and functions of N-acylphosphatidylethanolamines (NAPE): Not just precursors of N-acylethanolamines (NAE). *Biochimie.* 2012; 94:75–85. [PubMed: 21575672]
- Devaiah SP, Roth MR, Baughman E, Li M, Tamura P, Jeannotte R, Welti R, Wang X. Quantitative profiling of polar glycerolipid species from organs of wild-type *Arabidopsis* and a phospholipase Dalpha1 knockout mutant. *Phytochemistry.* 2006; 67:1907–1924. [PubMed: 16843506]
- Faure L, Coulon D, Laroche-Traineau J, Le Guedard M, Schmitter JM, Testet E, Lessire R, Bessoule JJ. Discovery and characterization of an *Arabidopsis thaliana* N-acylphosphatidylethanolamine synthase. *J Biol Chem.* 2009; 284:18734–18741. [PubMed: 19447891]
- Gillum MP, Zhang D, Zhang XM, Erion DM, Jamison RA, Choi C, Dong J, Shanabrough M, Duenas HR, Frederick DW, Hsiao JJ, Horvath TL, Lo CM, Tso P, Cline GW, Shulman GI. N-acylphosphatidylethanolamine, a gut-derived circulating factor induced by fat ingestion, inhibits food intake. *Cell.* 2008; 135:813–824. [PubMed: 19041747]
- Kang L, Wang YS, Uppalapati SR, Wang K, Tang Y, Vadapalli V, Venables BJ, Chapman KD, Blancaflor EB, Mysore KS. Overexpression of a fatty acid amide hydrolase compromises innate immunity in *Arabidopsis*. *Plant J.* 2008; 56:336–349. [PubMed: 18643971]
- Keereetaweep J, Kilaru A, Feussner I, Venables BJ, Chapman KD. Lauroylethanolamide is a potent competitive inhibitor of lipoxygenase activity. *FEBS Lett.* 2010; 584:3215–3222. [PubMed: 20541546]

- Kilaru A, Blancaflor EB, Venables BJ, Tripathy S, Mysore KS, Chapman KD. The N-acylethanolamine-mediated regulatory pathway in plants. *Chem Biodivers*. 2007; 4:1933–1955. [PubMed: 17712835]
- Kilaru A, Herrfurth C, Keereetaweep J, Hornung E, Venables BJ, Feussner I, Chapman KD. Lipoxygenase-mediated oxidation of polyunsaturated N-acylethanolamines in *Arabidopsis*. *J Biol Chem*. 2011a; 286:15205–15214. [PubMed: 21372125]
- Kilaru A, Isaac G, Tamura P, Baxter D, Duncan SR, Venables BJ, Welti R, Koulen P, Chapman KD. Lipid profiling reveals tissue-specific differences for ethanolamide lipids in mice lacking fatty acid amide hydrolase. *Lipids*. 2010; 45:863–875. [PubMed: 20714818]
- Kilaru A, Tamura P, Garg P, Isaac G, Baxter D, Duncan SR, Welti R, Koulen P, Chapman KD, Venables BJ. Changes in N-acylethanolamine pathway related metabolites in a rat model of cerebral ischemia/reperfusion. *J Glycom and Lipidom*. 2011b; 1:101.
- Kim S-C, Chapman KD, Blancaflor EB. Fatty acid amide lipid mediators in plants. *Plant Sci*. 2010; 178:411–419.
- Kim S-C, Kang L, Nagaraj S, Blancaflor EB, Mysore KS, Chapman KD. Mutations in *Arabidopsis* fatty acid amide hydrolase reveal that catalytic activity influences growth but not sensitivity to abscisic acid or pathogens. *J Biol Chem*. 2009; 284:34065–34074. [PubMed: 19801664]
- Li M, Qin C, Welti R, Wang X. Double knockouts of phospholipases Dzeta1 and Dzeta2 in *Arabidopsis* affect root elongation during phosphate-limited growth but do not affect root hair patterning. *Plant Physiol*. 2006; 140:761–770. [PubMed: 16384909]
- McAndrew RS, Chapman KD. Enzymology of cottonseed microsomal N-acylphosphatidylethanolamine synthase: kinetic properties and mechanism-based inactivation. *Biochim Biophys Acta*. 1998; 1390:21–36. [PubMed: 9487138]
- Motes CM, Pechter P, Yoo CM, Wang YS, Chapman KD, Blancaflor EB. Differential effects of two phospholipase D inhibitors, 1-butanol and N-acylethanolamine, on in vivo cytoskeletal organization and *Arabidopsis* seedling growth. *Protoplasma*. 2005; 226:109–123. [PubMed: 16333570]
- Natarajan V, Reddy PV, Schmid PC, Schmid HH. N-Acylation of ethanolamine phospholipids in canine myocardium. *Biochim Biophys Acta*. 1982; 712:342–355. [PubMed: 7126608]
- Ohlrogge J, Browse J. Lipid biosynthesis. *Plant Cell*. 1995; 7:957–970. [PubMed: 7640528]
- Pappan K, Austin-Brown S, Chapman KD, Wang X. Substrate selectivities and lipid modulation of plant phospholipase D alpha, -beta, and -gamma. *Arch Biochem Biophys*. 1998; 353:131–140. [PubMed: 9578608]
- Rawlyer AJ, Braendle RA. N-Acylphosphatidylethanolamine accumulation in potato cells upon energy shortage caused by anoxia or respiratory inhibitors. *Plant Physiol*. 2001; 127:240–251. [PubMed: 11553752]
- Sandoval JA, Huang ZH, Garrett DC, Gage DA, Chapman KD. N-acylphosphatidylethanolamine in dry and imbibing cottonseeds. Amounts, molecular species, and enzymatic synthesis. *Plant Physiol*. 1995; 109:269–275. [PubMed: 7480326]
- Schmid HH. Pathways and mechanisms of N-acylethanolamine biosynthesis: can anandamide be generated selectively? *Chem Phys Lipids*. 2000; 108:71–87. [PubMed: 11106783]
- Shoemaker, DP.; Garland, CW.; Steinfeld, JI. Experiments in physical chemistry. McGraw-Hill; New York: 1974.
- Shrestha R, Dixon RA, Chapman KD. Molecular identification of a functional homologue of the mammalian fatty acid amide hydrolase in *Arabidopsis thaliana*. *J Biol Chem*. 2003; 278:34990–34997. [PubMed: 12824167]
- Shrestha R, Noordermeer MA, Van der Stelt M, Veldink GA, Chapman KD. N-acylethanolamines are metabolized by lipoxygenase and amidohydrolase in competing pathways during cottonseed inhibition. *Plant Physiology*. 2002; 130:391–401. [PubMed: 12226518]
- Sugiura T, Kondo S, Sukagawa A, Tonegawa T, Nakane S, Yamashita A, Waku K. Enzymatic synthesis of anandamide, an endogenous cannabinoid receptor ligand, through N-acylphosphatidylethanolamine pathway in testis: involvement of Ca(2+)-dependent transacylase and phosphodiesterase activities. *Biochem Biophys Res Commun*. 1996; 218:113–117. [PubMed: 8573114]

- Teaster ND, Motes CM, Tang Y, Wiant WC, Cotter MQ, Wang YS, Kilaru A, Venables BJ, Hasenstein KH, Gonzalez G, Blancaflor EB, Chapman KD. N-Acylethanolamine metabolism interacts with abscisic acid signaling in *Arabidopsis thaliana* seedlings. *Plant Cell*. 2007; 19:2454–2469. [PubMed: 17766402]
- Thomas EA, Cravatt BF, Danielson PE, Gilula NB, Sutcliffe JG. Fatty acid amide hydrolase, the degradative enzyme for anandamide and oleamide, has selective distribution in neurons within the rat central nervous system. *J Neurosci Res*. 1997; 50:1047–1052. [PubMed: 9452020]
- Ueda N, Okamoto Y, Morishita J. N-acylphosphatidylethanolamine-hydrolyzing phospholipase D: a novel enzyme of the beta-lactamase fold family releasing anandamide and other N-acylethanolamines. *Life Sci*. 2005a; 77:1750–1758. [PubMed: 15949819]
- Ueda N, Tsuboi K, Lambert DM. A second N-acylethanolamine hydrolase in mammalian tissues. *Neuropharmacology*. 2005b; 48:1079–1085. [PubMed: 15910884]
- Venables BJ, Waggoner CA, Chapman KD. N-acylethanolamines in seeds of selected legumes. *Phytochemistry*. 2005; 66:1913–1918. [PubMed: 16054175]
- Wang YS, Shrestha R, Kilaru A, Wiant W, Venables BJ, Chapman KD, Blancaflor EB. Manipulation of *Arabidopsis* fatty acid amide hydrolase expression modifies plant growth and sensitivity to N-acylethanolamines. *Proc Natl Acad Sci U S A*. 2006; 103:12197–12202. [PubMed: 16880402]
- Welti R, Li W, Li M, Sang Y, Biesiada H, Zhou HE, Rajashekar CB, Williams TD, Wang X. Profiling membrane lipids in plant stress responses: Role of phospholipase D α in freezing-induced lipid changes in *Arabidopsis*. *J Biol Chem*. 2002; 277:31994–32002. [PubMed: 12077151]

Fig. 1.

Schematic representation of NAE metabolic pathway in plants. The NAE metabolic pathway shown here is based on both *in vivo* (solid lines) and *in vitro* (dashed lines) evidence. Saturated and unsaturated NAEs generated by the hydrolysis of NAPEs by PLD are hydrolyzed by FAAH to free fatty acids (FFAs) and ethanolamine (EA). In addition, polyunsaturated (PU)-NAEs can also be oxidized by LOX to form NAE-oxylipins. Both PLD and LOX are inhibited by NAE 12:0. *N*-acylation of PEs, with FFA and/or acyl-CoA as an acyl donor mediated by NAPE synthase, generates the NAPE

a. *N*-18:2 di18:2 PE **b. NAE 18:2** **c. di18:2 PE** **Fig. 2.**

Structures and fragmentation patterns of NAPE and related molecules. **a** Fragmentation pattern of *N*-18:2 di18:2 PE (shown as $[M + NH_4]^+$). **b** *N*-18:2 di18:2 PE is the precursor of NAE 18:2. **c** The precursor of *N*-18:2 di18:2 PE is di18:2 PE (shown as $[M + H]^+$). Neutral loss (NL) fragments used in lipidomic analysis are shown for *N*-18:2 di18:2 PE and di18:2 PE

Fig. 3.

Content and composition of NAE metabolites in *Arabidopsis* seeds with altered *FAAH* expression. **a** Total NAPE, NAE, and PE levels were quantified in *Arabidopsis* WT, *faah* KO, and *FAAH* OE seeds. **b, c** The NAPE and NAE content and composition, based on *N*-acyl moiety, were compared among the three genotypes. $N = 4 \pm SD$. Significance ($P < 0.05$) was determined by unpaired Student's *t*-test and is indicated by 'a' for KO or OE vs. WT

Fig. 4.

Effect of exogenous NAE 12:0 treatment on *Arabidopsis* 8-day-old seedlings. **a** WT and *faah* KO seeds germinated and grown in the presence of 35 μ M NAE 12:0 for 8 days showed reduced growth, compared to their controls and *FAAH* OE seeds with or without treatment. **b** Total NAPE, NAE, and PE levels were elevated in 8-day-old seedlings with NAE 12:0 treatment. $N = 4 \pm$ SD. Significance ($P < 0.05$) was determined by unpaired Student's t-test and is indicated by 'a' for KO or OE vs. WT and 'b' for NAE 12:0-treated vs. 8 day-control seedlings

Fig. 5.

Content and composition of NAPE in 8-day-old seedlings. **a** NAPE (grouped by common *N*-acyl moiety) content and composition was similar among 8-day-old WT, *faah* KO, and *FAAH* OE seedlings. **b** The levels were increased and the compositions altered with exogenous NAE 12:0 treatment. *N* = 4 ± SD. Significance (*P* < 0.05) was determined by unpaired Student's *t*-test and is indicated by 'a' for KO or OE vs. WT and 'b' for NAE 12:0-treated vs. 8 day-control seedlings

Fig. 6.

Content and composition of NAE in 8-day-old seedlings. NAE content and composition of 8-day-old seedlings of WT, *faah* KO, and *FAAH* OE genotypes (a) were altered with exogenous NAE 12:0 treatment (b). Differential effects were observed among the genotypes. $N = 4 \pm SD$. Significance ($P < 0.05$) was determined by unpaired Student's t-test and is indicated by 'a' for KO or OE vs. WT and 'b' for NAE 12:0-treated vs. 8 day-control seedlings

Fig. 7.

The profiles of NAE molecular species in *Arabidopsis* seeds and seedlings. The profiles of PE components of NAE molecular species, based on NAE *N*-acyl group, for WT, *faah* KO, and *FAAH* OE seeds (a) and 8-day-old seedlings (b) were compared with those of 8-day-old seedlings grown in the presence of 35 μ M NAE 12:0 (c)

Fig. 8.

Molecular species composition of PE in *Arabidopsis* is affected by NAE 12:0 treatment. The profiles of PE molecular species for WT, *faah* KO, and *FAAH* OE seeds (a), 8-day-old seedlings (b), and 8-day-old seedlings grown in the presence of 35 μ M NAE 12:0 (c) are shown. $N = 4 \pm$ SD. Significance ($P < 0.05$) was determined by unpaired Student's t-test and is indicated by 'a' for KO or OE vs. WT and 'b' for NAE 12:0-treated vs. 8 day-control seedlings

a.
Seeds

b.
**8 d-
Control**

c.
**8 d-
Treated**

Fig. 9.

Galactolipid and phospholipid content of *Arabidopsis* seeds and 8-day-old seedlings. The amount of galactolipids (DGDG and MGDG), major phospholipids (PG, PC, PE, PI, and PA), and minor phospholipids (PS, LysoPG, LysoPC, and LysoPE) were compared among seeds (**a**) and 8-day-old control (**b**) and 35 μ M NAE 12:0-treated (**c**) seedlings. $N = 4 \pm SD$. Significance ($P < 0.05$) was determined by unpaired Student's t-test and is indicated by 'a' for KO or OE vs. WT and 'b' for NAE 12:0-treated vs. 8 day-control seedlings

Fig. 10.

Exogenous NAE 12:0 affects prokaryotic pathway to lipid synthesis. **a** *Arabidopsis* 8-day-old seedlings grown in the presence of 35 μ M NAE 12:0 showed reduced levels of prokaryotic molecular species MGDG 34:6. **b** Levels of eukaryotic species MGDG 36:6 were not decreased in 8-day-old NAE-treated seedlings relative to 8-day-old control seedlings. $N = 4 \pm$ SD. Significance ($P < 0.05$) was determined by unpaired Student's t-test and is indicated by 'a' for KO or OE vs. WT and 'b' for NAE 12:0-treated vs. 8 day-control seedlings