Enhanced seedling growth by 3-n-pentadecylphenolethanolamide is mediated by fatty acid amide hydrolases in upland cotton (*Gossypium hirsutum* L.)

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Abstract

Fatty acid amide hydrolase (FAAH) is a conserved amidase that is known to modulate the levels of endogenous N-acylenoethanolamines (NAEs) in both plants and animals. The activity of FAAH is enhanced in vitro by synthetic phenoxycylethanolamides resulting in greater hydrolysis of NAEs. Previously, 3-n-pentadecylphenolethanolamide (PDP-EA) was shown to exert positive effects on the development of Arabidopsis seedlings by enhancing Arabidopsis FAAH (AtFAAH) activity. However, there is little information regarding FAAH activity and the impact of PDP-EA in the development of seedlings of other plant species. Here, we examined the effects of PDP-EA on growth of upland cotton (*Gossypium hirsutum* L. cv. Coker 312) seedlings including two lines of transgenic seedlings overexpressing AtFAAH. Independent transgenic events showed accelerated true-leaf emergence compared with non-transgenic controls. Exogenous applications of PDP-EA led to increases in overall seedling growth in AtFAAH transgenic lines. These enhanced-growth phenotypes coincided with elevated FAAH activities toward NAEs and NAE oxylipins. Conversely, the endogenous contents of NAEs and NAE-oxylipin species, especially linoleoylethanolamide and 9-hydroxy linoleoylethanolamide, were lower in PDP-EA treated seedlings than in controls. Further, transcripts for endogenous cotton FAAH genes were increased following PDP-EA exposure. Collectively, our data corroborate that the enhancement of FAAH enzyme activity by PDP-EA stimulates NAE-hydrolysis and that this results in enhanced growth in seedlings of a perennial crop species, extending the role of NAE metabolism in seedling development beyond the model annual plant species, *Arabidopsis thaliana*.

Keywords

3-n-pentadecylphenolethanolamide, cotton, fatty acid amide hydrolase, N-acylenoethanolamines, oxylipins, seedling growth
Fatty acid amide hydrolase (FAAH) is a promiscuous amidase in plants, capable of hydrolyzing N-acylthanolamines (NAEs), NAE oxylipins, and microbial derived NAE-like quorum sensing compounds (Keereetaweep et al., 2013, 2015; Kilaru et al., 2011; Palmer et al., 2014; Shrestha et al., 2003; Wang et al., 2006). Identified originally in mammals, the rat and human FAAH enzymes hydrolyze the endocannabinoid NAE20:4 (anandamide) into arachidonic acid and ethanolamine to terminate this signaling pathway (Fowler et al., 2000; Giang & Cravatt, 1997). In plants, FAAH similarly mediates hydrolysis of NAEs. For example, the Arabidopsis FAAH (AtFAAH) converted NAE12:0, NAE14:0, NAE16:0, and NAE18:2 into their corresponding free fatty acids and ethanolamine (Shrestha et al., 2003, 2006). Further, AtFAAH has also been shown to cleave the amide bond of NAE-derived oxylipin, 9-hydroxy octadecadienoyl ethanolamidade (9-NAE-HOD), and multiple N-acyl-L-homoserine lactones (e.g., 3-oxo-C12-HSL) (Aziz et al., 2019; Keereetaweep et al., 2015; Palmer et al., 2014). In plants, therefore, FAAH exhibits an expanded substrate profile toward a wide range of acyl amide molecules including NAEs and NAE-like molecules.

NAE content and composition in plants vary depending on the type of plant species, tissue, and stage of development (Chapman et al., 1998; Gachet et al., 2017; Venables et al., 2005). Reports have shown that NAE content is the highest in desiccated seeds and that these levels dramatically decrease upon seed germination and seedling establishment, suggesting a potential role of FAAH hydrolysis during seedling growth (Venables et al., 2005). In Arabidopsis, the NAE profiles of both seeds and vegetative tissues revealed that 18C polyunsaturated NAEs (e.g., NAE18:2 and NAE18:3) are the most predominant (around 75% of total NAE content) types, whereas saturated NAEs (e.g., NAE12:0 and NAE18:0) are present in far lower amounts (Wang et al., 2006). More recently, it was shown that oxylipin metabolites of NAEs are produced from NAE 18:2 and NAE18:3 (Keereetaweep et al., 2013, 2015), and the hydroxylated derivative compounds were hydrolyzed by FAAH with equal efficiency to unsubstituted parent NAEs (Aziz et al., 2019).

Several genetic approaches have provided insights regarding FAAH-mediated NAE hydrolysis in Arabidopsis. Overexpression of Arabidopsis FAAH (AtFAAH) resulted in enhanced seedling growth and early flowering phenotypes when compared to either faah knockouts or wild-type controls (Teaster et al., 2007, 2012; Wang et al., 2006). Quantification of endogenous NAEs in these plants revealed that reduction of NAE content was associated with both increased in seedling growth and early flowering. When exogenous applications of unsaturated (e.g., NAE18:2) or saturated NAEs (e.g., NAE12:0) were made to the same genotypes, it was evident that Arabidopsis seedlings overexpressing AtFAAH were more resistant to the negative growth effects of NAEs than wild-type controls. By contrast, faah knockouts were more sensitive to added NAEs when compared to wild-type or AtFAAH overexpressing plants (Cotter et al., 2011; Teaster et al., 2007; Wang et al., 2006). Therefore, FAAH has the capacity to hydrolyze NAEs in planta which alleviates the inhibitory effects by these lipid mediators on plant development. The growth modulating role of AtFAAH resembles that of another well-characterized amidase superfamily member, namely, AMIDASE1 (AM1). AM1 modulates endogenous indole-3-acetamide (IAM) levels, which, like NAEs, modulates plant growth (Moya-Cuevas et al., 2021).

Lipoxygenase-generated, NAE-oxylipin metabolites are characterized by the presence of hydro (per)oxides at the C9 or C13 position in their acyl chains (Kilaru et al., 2011). Synthesis of these molecules from NAE18:2 and NAE18:3 were partly responsible, in addition to FAAH, for the depletion of NAEs upon seed germination. Experiments demonstrated that exposure to these NAE hydroxides differentially inhibited seedling development (Keereetaweep et al., 2013, 2015). In fact, the impact of the NAE oxylipins was shown to be far greater than that of their NAE-parent molecules. For instance, Arabidopsis wild-type seedlings exposed to 9-NAE-HOD had shorter roots compared to seedlings exposed to NAE18:2 or solvent controls (Keereetaweep et al., 2015). Follow-up experiments showed that the growth reduction was alleviated by overexpressing AtFAAH. Quantification of endogenous NAE-oxylipins in the same tissues supported the notion that reduced NAE oxylipin content was associated with enhanced seedling growth. Further, 9-NAE-HOD, but not 13-NAE-HOD (another NAE18:2-derived oxylipin), was reported to interact with, and require, an ABA signaling pathway to modulate seedling growth (Keereetaweep et al., 2015).

Consequently, a model has emerged whereby FAAH can hydrolyze either unsubstituted, polyunsaturated NAEs or their oxylipin derivatives to terminate their bioactivity. In the case of NAE18:2, this involves ABA signaling to modulate root elongation (Keereetaweep et al., 2015), and in the case of NAE18:3, this requires G-protein signaling to modulate chloroplast development and cotyledon expansion (Cannon et al., 2020; Cannon & Chapman, 2021; Yan et al., 2020).

Several small molecule inhibitors also have been utilized or developed to probe FAAH activity in vitro and in vivo, including general serine protease inhibitors like phenylmethylsulfonyl fluoride (PMSF) or substrate-mimic molecules like cyclohexyl carbamic acid 3-carboxamidobiphenyl-3-yl ester (URB597) and methoxy arachidonyl fluorophosphonate (MAFP). These and other small molecules have been used in vivo primarily in animal systems to modulate endogenous NAE levels (Bosier et al., 2013; Hamtiaux et al., 2012; Muccioli et al., 2007). On the other hand, few compounds have been identified that actually increase FAAH enzyme activity. One such class of compounds identified by Faure et al. (2014) was the phenoxyacetyl ethanololamides that were synthesized from natural products found in cashew nut shells (Faure et al., 2014). These molecules were capable of increasing FAAH activity toward NAEs in both animal and plant systems. Kinetic characterization of recombinant FAAHs showed the phenoxyacetyl ethanololamides, 3-npentadecylphenoletanolamide (PDP-EA) or cardanol-EA, both enhanced FAAH activity by relieving product inhibition from ethanolamine (Faure et al., 2014). As might be anticipated, both PDP-EA and cardanol-EA had growth-promoting properties in Arabidopsis seedlings that were associated with enhanced FAAH activities (Faure et al., 2014). Interestingly, these compounds also showed in vivo activity in animal cell cultures as FAAH-dependent enhancers of apoptosis, so the targeted effects of
these phenoxyacyl-ethanolamides were evolutionarily conserved across FAAH enzymes from plants and animals, both in vitro and in vivo (Faure et al., 2014).

Overexpression of AtFAAH in a heterologous plant system has not yet been explored. Similarly, the ability of phenoxyacyl-ethanolamides to increase FAAH activity alongside its effect(s) in seedling growth has not been expanded beyond the model annual species, Arabidopsis thaliana. Here, we generated upland cotton (G. hirsutum L.) transgenic lines overexpressing AtFAAH to assess its effect(s) in seedling growth and establishment. In addition, we followed up with applications with PDP-EA to cotton seedlings to evaluate its effect as a FAAH enhancer in seedling development. Collectively, our results indicate that the expression of AtFAAH in cotton seedlings is associated with an acceleration of leaf emergence. In addition, application of PDP-EA to cotton seedlings led to enhanced growth in young and older FAAH transgenic seedlings, whereas in older non-transgenic cotton seedlings, PDP-EA alone resulted in enhanced true-leaf expansion and increase leaf size. The increased cotton seedling growth appeared to be a result of the enhancement of FAAH activity and reduction in endogenous N-acylethanolamines. These results expand the concept that FAAH and phenoxyacyl-ethanolamides can act as positive modulators of seedling development in a perennial crop species.

2 | MATERIALS AND METHODS

2.1 | Binary vector and cotton transformation for AtFAAH transgenic production

The coding sequence of AtFAAH (AT5G64440) was cloned into the binary vector pCambia-1390 under the control of CaMV 35S.
promoter (Wang et al., 2006). AFAAH in the pCambia-1390 vector was electroporated in A. tumefaciens strain LBA4404; clones were selected on LB (Luria-Bertani) media with kanamycin (50 μg/ml). The callus from seedling explant tissues (5- to 7-day-old) was used for somatic embryogenic cell suspension culture (SEC) initiation, as reported elsewhere (Salimath et al., 2021). The SECs were propagated in 3 ml of modified Murashige and Skoog Medium (MSM) at 30°C, 16-h light/8-h dark cycle and placed on gyratory shaker at 110 RPM. SECs were subcultured in fresh liquid media every 18 to 22 days. Freshly grown SECs (2 ml) were co-cultured with Agrobacterium cells (OD600 1.5 to 2) harboring the binary vector AtFAAH: pCambia1390 for Agrobacterium-mediated transformation. Co-cultivated SECs were transferred to MSM plates supplemented with carbencillin for screening and selection of transgenic embryos. Hygromycin-selected embryos were developed and transferred to fresh MSM media with carbencillin and hygromycin. Plantlets with three to seven leaves were transferred into magenta boxes with MSM media for further growth, before transfer to soil and acclimation in a temperature-controlled growth room at 28°C, with a 16-h light/8-h dark cycle. T0 plants were transferred to a greenhouse at 28°C for maturation, seed production, and acclimation to T3 generation. Seedlings of the non-transgenic background, G. hirsutum Lcv Coker312, were used as the “wild-type” control for these studies. The germination rate (n = 18) of WT or transgenic seeds/seedlings was evaluated during an 8-day course, and the combined hypocotyl and primary root length was measured in 8-day-old seedlings (n = 18) (Figure S3). Image J software (Schneider et al., 2012) was used to measure the cotyledon area of WT or transgenic lines (n = 9) (Figure 1a-b). The stem length and average number of leaves were evaluated in 32-day-old cotton seedlings (n = 13) (Figure 1c–f). Two FAAH overexpressing lines were selected for our studies with ABA, NAES, or PDP-EA based on their substantial levels of AtFAAH transcripts (Figures 1f, 9, and S4).

2.2 | Exogenous applications of ABA and NAES

WT and AtFAAH-OE cotton seeds were soaked in 20 ml of 50 μM of abscisic acid (ABA) (Cayman Chemical), 9-NAE-HOD (9-hydroxy linoleoylthanolamide) (synthesis described later in materials and methods), or NAE12:0 (lauroylethanolamide) solution in 0.1% DMSO (final) for 6 h in the dark at room temperature, as reported elsewhere (Chakma et al., 2021). Afterwards, seeds were washed with distilled water and wrapped in paper rolls, and then, they were placed in 30 ml of ultrapure water for germination in a growth room at 28°C. Phenotypes were evaluated in 8-day-old seedlings (n = 9 per treatment).

2.3 | PDP-EA and plant material for treatment assays

Like the above described method, transgenic (AtFAAH-OE2 or AtFAAH-OE3) and non-transgenic (WT) cotton seeds were soaked in 20 ml of 30 μM PDP-EA (3-n-pentadecylphenolthanolamide) solution in 0.1% DMSO (final). PDP-EA was synthesized and characterized previously (Faure et al., 2014). Germination conditions were kept the same for this experiment. Germination was assessed by recording radicle emergence every 2 days during a 10-day time course (n = 18 per treatment). The length of hypocotyl and primary root was measured in 10-day-old seedlings and reported as a combined value in the data sets (n = 18 per treatment).

Cotton seedlings were grown in a growth room at 28°C, set to a 16-h light/8-h dark cycle. Soil grown 10-day-old seedlings were irrigated with 10 ml of 30 μM PDP-EA solution in 0.05% DMSO (final) every 4 days for 16 days. A solution of 0.05% DMSO was included in all experiments as solvent control. Stem length, leaf emergence, and leaf length and width were measured between 4 to 16 days (n = 9). At 16 days following exposure, roots were excised from whole plants, and their length was measured (n = 18).

2.4 | Identification and sequence retrieval of FAAH genes from cotton

Sequences of the Arabidopsis fatty acid amide hydrolase (AtFAAH) gene locus (AT5G64440) were retrieved from TAIR website (https://www.arabidopsis.org/). AtFAAH was used as template to search for cotton homologs in NCBI (https://www.ncbi.nlm.nih.gov/), CottonGen (http://www.cottongen.org) and ccNET (http://structuralbiology.cau.edu.cn/gossypium) databases by using the BLAST option with default settings (You et al., 2017; Yu et al., 2014) (Table S3). All the putative FAAHs were examined for the presence of the amidase signature domain using the ScanProsite tool (Figure S12a) (Gattiker et al., 2002). MEME suite was employed for identification of other conserved motifs in their amino acid sequences (Figure S12b) (Bailey et al., 2006). Phylogenetic analysis was conducted with Phylogeny.fr online server with default settings (Figure 8) (Dereeper et al., 2008). GalaxyWEB was used to construct cotton FAAH homology models, using Arabidopsis FAAH (6DHV) as the template (Figure S13) (Ko et al., 2012).

2.5 | RNA isolation, cDNA preparation, and quantitative real-time PCR (RT-qPCR)

For Figure 1f, four biological replicates (four true leaves from four different seedlings) of non-transgenics or AtFAAH transgenics were utilized for RNA extraction, cDNA synthesis, and RT-qPCR experiments. For Figure S4, 10 leaves from 10 different AtFAAH transgenics were utilized for RNA extraction, cDNA synthesis, and RT-PCR experiments. For Figure 8c, three whole 10-day-old seedlings, tissues (stems, leaves, and roots) dissected from three different 15 day-old seedlings, and petals dissected from three different seven-week-old plants were used for tissue expression analysis. For Figure 9, three biological replicates (three true leaves from three different seedlings) of non-transgenics or AtFAAH transgenic cotton seedlings from each...
treatment (0.05% DMSO or 30 μM PDP-EA) were used for RNA extraction, cDNA synthesis, and RT-qPCR analysis.

Hot borate extraction method was used for RNA extraction with few modifications (Wan & Wilkins, 1994). Briefly, 200 mg of leaves was flash frozen in liquid N₂ and then crushed into a fine powder with mortar and pestle, 800 μl of preheated (80°C) borate buffer (200 mM sodium tetraborate decahydrate, Sigma-Aldrich; 30 mM EGTA, Sigma-Aldrich; 1.5% deoxycholic acid, Sigma-Aldrich; 1.5% SDS, Fisher Scientific; 10 mM of dithiothreitol [DTT], Sigma-Aldrich; and 3% polyvinylpyrrolidone [PVP], Sigma-Aldrich), and 10 μl of Proteinase K (Thermo Fisher) (20 mg/ml) were added. The mixture was loaded onto shredder spin columns (Qiagen RNasey Plant Mini Kit); purification steps were followed as described by the manufacture. Then, 50 ng of RNA was used for cDNA synthesis using the Applied Biosystems High Capacity cDNA Reverse Transcription Thermo Fisher kit.

cDNA from all samples was normalized to 100 ng/μl and used for RT-qPCR analysis. Applied Biosystems PowerUp™ SYBR™ Green Master Mix- Fisher Scientific kit was used for the assays using a reaction volume of 10 μl. Primers were designed to evaluate endogenous (GhFAAH) and transgenic (AtFAAH) transcript abundance (Table S5). The delta-CT method was used to evaluate relative transcript abundance (Livak & Schmittgen, 2001), with Ubiquitin 1 (UBQ1) as the “housekeeping” gene for normalization and loading control for RT-qPCR and RT-PCR, respectively.

2.6 | Lipid extraction and quantification of NAEs

In these experiments, a pool of three true leaves from one seedling represents one biological replicate (600 mg dry weight), and in total, three biological replicates of non-transgenics or AtFAAH transgenic cotton seedlings from each treatment (0.05% DMSO or 30 μM PDP-EA) were used independently for lipid extraction and NAE profiling (Figure 4). Lyophilized tissues were used for lipid extraction, using the previously developed methods (Keereetaweep & Chapman, 2016; Wang et al., 2006). Briefly, dry-tissues were powdered in a bead beater and homogenized in preheated (70°C) isopropanol, and then, water and chloroform were added to create a monophasic solvent for lipid extraction to a ratio 2:1:0.45 (IPA:CHCl₃:H₂O). A total of 250 ng of internal standard d₄-NAE16:0 (Cayman Chemical) was added to the extracts for later normalization and quantification purposes. Lipid extracts were partitioned into two phases, and the chloroform phase containing the lipids was washed and recovered for further fractionation (Venables et al., 2005). Samples were separated in normal phase (NP)-HPLC on a VP 250 × 10 mm Nucleodur 100–10 column, using linear gradients of 60% hexane and 40% isopropanol during 20 min, followed by 20% hexane and 80% isopropanol during 5 min, and then 100% hexane over 5 min (Chapman et al., 1999; Venables et al., 2005). NAE-enriched fractions were monitored at 214 nm (UV), and collection was made between 11 and 15 min. Next, these fractions were evaporated and suspended in 50 μl of BSTFA (N,O-Bis [trimethylsilyl] trifluoroacetamide) for derivatization at 50°C for 30 min. The derivatized fractions were finally re suspended in 20 μl of hexane for GCMS separation. GCMS analysis was conducted with the following conditions: temperature of the injector at 260°C, oven at 40°C to 300°C (with 10°C/min ratios), purge flow of 48 (kPa), and 50 (ml/min), using Helium as the carrier gas. GCMS was equipped with a capillary column J&W HP-5 ms GC. The MS analyzer was adjusted for electron-impact ionization (EI-MS), and data were collected using the single ion monitoring (SIM) mode. NAEs were also confirmed separately by their MS spectra in total ion scans. NAEs appeared as trimethylsilyl (TMS)-derivatives (Figures S8 and S9). Fragmented molecular ions were used for diagnostic and quantification purposes (Table S1), as reported elsewhere (Venables et al., 2005; Wang et al., 2006).

2.7 | Standards, extraction and quantification for NAE- and FFA-oxylipins

NAE-oxylipin standards were synthesized as described previously with some modifications (Aziz et al., 2019; Keereetaweep et al., 2013, 2015). Briefly, 500 μg of NAE18:2 was incubated with a commercially purified 13-LOX from Glycine max (Cayman Chemical) or recombinant 9-LOX from Solanum tuberosum (9-LOX was cloned into pYES2 yeast expression vector, Thermo Fisher Scientific) to yield 9-NAE-HPOD, or 13-NAE-HPOD. Then, 200 μg of NaBH₄ was used to reduce the hydroperoxides into NAE-hydroxides, namely, 9-NAE-HOD and 13-NAE-HOD. Free fatty acids (FFA) oxylipins, namely, 9-HOD and 13-HOD, were purchased from Cayman Chemical. NAE and FFA-oxylipins were separated by reverse phase (RP)-HPLC on an EC 250/2 Nucleosil 120-5 C18 column; fractions were collected between 20 and 30 min and then analyzed in GCMS (Figures S10 and S11 and Table S2). NAE- and FFA- oxylipins were extracted as described previously (Christensen et al., 2013; Keereetaweep et al., 2013, 2015; Keereetaweep & Chapman, 2016; Kilaru et al., 2012), with some modifications. For these experiments, the pool of three true leaves from one seedling represents one biological replicate (900 mg dry weight), and in total, three biological replicates of non-transgenics or AtFAAH transgenic cotton seedlings from each treatment (0.05% DMSO, or 30 μM PDP-EA) were used independently for lipid extraction and NAE- and FFA- oxylipin profiling (Figure 5). Lyophilized leaves were ground and homogenized in 3 ml of HIP buffer containing hexane:isopropl alcohol (1.5:1), and butylated hydroxytoluene (BHT) (0.0025%). A total of 250 ng of internal standard d₄₂-9HOD (Cayman Chemical) was added in each sample. Extracts were capped under N₂ stream and kept on ice at all times, and then, samples were shaken for 1 h at 4°C and then centrifuged at 2,000 RPM for 10 min. Supernatants were incubated with 200 mg of NaBH₄ and shaken for 30 min at 4°C. Upon centrifugation (10 min at 2,000 RPM), hexane-rich upper phase was recovered dried under N₂ stream and re-suspended in methanol:water (80:20). Samples were separated in RP-HPLC on an EC 250/2 Nucleosil 120-5 C18 column under UV monitoring (234 nm), and a gradient system of two solvents at a flow rate of 0.18 ml/min. Solvents A and B were made of methanol:water:acetic acid (80:20:0.1,
v/v/v) and methanol:acetic acid (100:0.1, v/v), respectively. NAE- and FFA-oxylipins enriched fractions were collected between 20 and 30 min and then analyzed by GCMS, using the full-scan and SIM modes. Oxylipins were identified as their TMS-derivatives by retention times, and full mass scans were used to confirm their identity again known standards. Diagnostic ions were used to confirm identification in SIM mode and for quantification against the deuterated internal standard d$_4$-9HOD (Cayman Chemical) added at the time of extraction (Figures S10, S11, and Table S2).

## 2.8 | FAAH activity assay

Four biological replicates (four true leaves from four different 32-day-old seedlings) from each genotype (wild-type (WT), AtFAAH-OE2, and AtFAAH-OE3) were excised and ground with mortar and pestle under liquid N$_2$. Two milliliters of homogenization buffer (10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM MgCl$_2$, 400 mM sucrose, and 100 mM potassium phosphate, 0.2 mM DDM, p = 7.2) was added to the fine powder, mixed, and incubated for 30 min on ice. Samples were centrifuged at 600g for 20 min, and the clarified supernatant was used as the enzyme source, as reported previously (Kim et al., 2013). Pierce Bovine Serum Albumin (BCA) Protein Assay Kit (Thermo Scientific) was used to construct BSA standard curves to quantify total protein concentration in the samples. Homogenates (100 μg protein) were incubated with 100 μM of NAE16:0, NAE18:2 or 9-NAE-HOD substrates in the presence of 30 μM PDP-EA or 0.05% DMSO (solvent control) at 30°C for 10 min. Reactions were stopped with the FAAH inhibitor, p-nitromethylsulfonyl fluoride (PSMF). An aliquot of 15 μl from the reaction was taken and mixed with 45 μl of fluorescamine (3.6 mM in acetone) and 97.5 μl of milli-q water for detection of ethanolamine products using a microplate reader (Agilent BioTek Synergy H4) adjusted to 390 nm excitation and 475 nm emission, as described elsewhere (Palmer et al., 2014). A standard curve with free ethanolamine standard (Sigma Aldrich) was used to convert fluorescent values into μmol of ethanolamine produced in the reaction per unit of time (min) per amount of protein (mg).

## 2.9 | Statistical and multivariate analysis

Comparisons made for phenotype, metabolite, and RT-qPCR data sets among treatment groups were performed by one-way ANOVA and Tukey’s test (P < 0.05) or Student’s t test (P < 0.05) using MetaboAnalyst online version 5.0 (https://www.metaboanalyst.ca/) (Xia et al., 2009). Partial least-squares discriminant analysis (PLS-DA) and variable importance in projection (VIP) scores were conducted with MetaboAnalyst 5.0 as well. Settings included, data normalization (log transformation), and data scaling (mean centering). PLS-DA plot displayed a 95% confidence region surrounding the replicates from each treatment. PLS-DA was built with components 1 and 2 from the pairwise score plots. VIP scores plot employed component 1 for metabolic ranking of NAE, NAE- and FFA-oxylipin metabolites.

## 3 | RESULTS

### 3.1 | Phenotypic characterization of cotton seedlings expressing Arabidopsis FAAH (AtFAAH)

The effects of cisgenic overexpression of the FAAH coding sequence in A. thaliana have been well documented (Teaster et al., 2007, 2012; Wang et al., 2006). However, overexpression of AtFAAH in a heterologous host has yet to be evaluated extensively in other plant systems. Here, we decided to investigate the seedling growth effects associated with overexpression of AtFAAH in upland cotton (G. hirsutum L.). Cotton transformation remains largely genotype dependent and time consuming. Embryogenic cell lines in culture were used as an explant source for A. tumefaciens-mediated transformation. Regenerated plantlets (T0) were selected on antibiotic-containing media, matured and transferred to soil for growth and seed production (Figures S1 and S2). We developed several independent transgenic events expressing AtFAAH, and two T3-advanced events (AtFAAH-OE2 and AtFAAH-OE3) were selected for further characterization.

Several seedling growth parameters were compared in plants expressing AtFAAH (Figures S3 and 1). There were no significant differences between the germination rates, of wild-type and AtFAAH transgenic cotton seedlings (Figure S3a,b; P > 0.05, n = 18). The combined length of the hypocotyl and primary root was similar in both 8-day-old wild-type and AtFAAH cotton lines (Figure S3a,c; P > 0.05, n = 9). There were no significant differences between the average cotyledon areas of 10-day-old wild-type and AtFAAH transgenic cotton seedlings (Figure 1a,b; P > 0.05, n = 9). However, in older cotton seedlings (32-day-old) either AtFAAH-OE2 or AtFAAH-OE3 had greater number of leaves compared to non-transgenic plants (Figure 1c,e; P < 0.05, n = 13), whereas the length of the stem was similar in all genotypes (Figure 1c,d; P > 0.05, n = 13). Hence, while FAAH did not enhance the size of organs in young cotton seedlings like it did when overexpressed in Arabidopsis, it still appeared to accelerate plant development as observed by accelerated leaf emergence. RT-qPCR and RT-PCR were used to confirm expression of AtFAAH in the transgenic lines AtFAAH-OE2 and AtFAAH-OE3. Non-transgenic cotton seedlings were included as negative control for expression of AtFAAH (Figure 1f, n = 4). RT-PCR was performed in 10 sibling plants of each transgenic line. Data showed the presence of AtFAAH transcript in all transgenic plants tested; non-transgenic (wild-type) was included as negative control. UBQ1 was used as loading control (Figure S4).

### 3.2 | Effect(s) of ABA and NAEs in cotton seedling growth

The effects of ABA and NAEs on Arabidopsis have been described elsewhere in the literature (Cotter et al., 2011; Keeeretaweep et al., 2015; Teaster et al., 2007). ABA cooperatively acts with NAEs to negatively reduce seedling growth in Arabidopsis (Teaster...
et al., 2007). We predicted that like in Arabidopsis, cotton AtFAAH transgenic seedlings might be hypersensitive to ABA and more tolerant to NAEs applied exogenously. We pre-treated transgenic and non-transgenic seeds with 50 μM of ABA, 9-NAE-HOD, or NAE12:0, using a method reported elsewhere (Chakma et al., 2021), and measured seedling growth (combined length of radicle, hypocotyl and root) in 8-day-old-seedlings (Figure 2). Compared to wild-type, both AtFAAH-OE2 and AtFAAH-OE3 were tolerant to ABA-mediated growth reduction. Similarly, both cotton AtFAAH lines were more tolerant to NAE12:0 or 9-NAE-HOD than non-transgenic seedlings (Figure 2a,b; P < 0.05; n = 9). Altogether, these data suggest that cotton AtFAAH lines are resistant to both ABA- and NAE-mediated growth reduction. Ectopic expression of AtFAAH in cotton results in seedlings that are capable of processing exogenously applied NAEs. The considerable ABA tolerance of AtFAAH-cotton lines is opposite to what was observed for Arabidopsis, suggesting that there may be differences in the ABA-NAE interaction in these two species.

3.3 | Effect(s) of PDP-EA in cotton seedling growth

PDP-EA was shown to enhance FAAH activity and early seedling growth in Arabidopsis (Faure et al., 2014). To assess whether PDP-EA could exert similar physiological responses in cotton, (1) we evaluated PDP-EA effects on cotton seedlings at early stages of development (Figure 3a,b), and (2) effects of PDP-EA were assessed in older, soil grown seedlings (Figure 3c–f). At 2- and 4- days post-germination (dpg), the germination rates of PDP-EA-treated AtFAAH-OE2 or AtFAAH-OE3 were significantly elevated compared to their respective DMSO-controls or WT (PDP-EA or DMSO) (Figure S5; P < 0.05; n = 18). At 6 to 10 dpg, the germination rates plateaued for all treatments. At 10 dpg, the combined length of the hypocotyl and primary root was longer in both PDP-EA-treated AtFAAH transgenic lines than in their respective DMSO-controls or WT (PDP-EA or DMSO) (Figure 3a,b; P < 0.05; n = 18). These combined data suggest that
Figure 3  Exogenous application of 3-n-pentadecylphenoylethanolamide (PDP-EA) in cotton seedlings. (a) Representative images of 10-day-old non-transgenic wild-type (WT) and AtFAAH transgenics pretreated with 0.05% DMSO (solvent control) or 30 μM PDP-EA. (b) Combined length of hypocotyl and primary root length in the same treatments and genotypes. Bars represent the means and standard deviation (±SD) from 18 plants per group studied. (c) Representative images of non-transgenic wild-type (WT) and AtFAAH transgenics fed with 0.05% DMSO (solvent control) or 30 μM PDP-EA at 16-days of exposure. (d) Stem length was evaluated in seedlings irrigated with DMSO or PDP-EA at 4, 8, 12, and 16 days after exposure with DMSO or PDP-EA. Bars represent the means and standard deviation (±SD) from nine plants per group studied. (e) Representative images of roots collected from wild-type (WT) and AtFAAH transgenics irrigated with DMSO or PDP-EA at 16-days of exposure. (f) The length of the root was measured for DMSO- or PDP-EA-treated cotton seedlings (WT or AtFAAH-OE). Bars represent the means and standard deviation (±SD) from 18 plants per group studied. One-way ANOVA with Tukey post hoc test were used for statistical analyses of all data sets. Different letters represent significant differences (P < 0.05) within the group analyzed.
PDP-EA enhances early seedling development in cotton seedlings where the AtFAAH is ectopically expressed.

At 16 days post irrigation with DMSO or PDP-EA, we assessed seedling changes in stems, roots, leaves, and leaf emergence (Figures 3c–f, S6, and S7). The stem length of PDP-EA-irrigated transgenic seedlings was longer compared to their respective DMSO-treated controls (Figure 3c,d; P < 0.05; n = 9). Although appearing slightly taller, no significant differences were found between the stem length of DMSO- or PDP-EA-treated non-transgenic seedlings (Figure 3c,d; P > 0.05; n = 9). At 16 days post irrigation, the root length of PDP-EA-irrigated cotton transgenics was significantly longer than DMSO-treated transgenics or WT (DMSO or PDP-EA) seedlings (Figure 3e,f; P < 0.05; n = 18). These combined data demonstrate that PDP-EA enhances growth in older seedlings expressing the transgene AtFAAH.

Following addition of PDP-EA, the width and length of the first true leaves in AtFAAH-OE2, AtFAAH-OE3, and wild type cotton seedlings were greater than those of the corresponding DMSO-treated controls (Figure S6; P < 0.05; n = 9). A similar outcome also was observed for the second true leaves (Figure S6; P < 0.05; n = 9). This growth parameter suggests that addition of PDP-EA enhances the leaf size of both non-transgenic (WT) and transgenic lines (AtFAAH-OE). Leaf size was the only parameter that we measured where PDP-EA had an enhanced growth effect that was independent of the AtFAAH transgene. By contrast, AtFAAH-OE2 or AtFAAH-OE3 lines had greater number of leaves than non-transgenics (WT) with or without the PDP-EA (Figure S7; P < 0.05; n = 9), suggesting that AtFAAH expression alone can enhance leaf emergence independent of PDP-EA. Altogether, while there are a few growth parameters influenced individually by either AtFAAH expression or PDP-EA treatment, combining AtFAAH expression and PDP-EA treatment showed the most dramatic enhancement of growth in cotton seedlings.

### 3.4 NAE profiles in PDP-EA-treated and AtFAAH transgenic plants

Given that FAAH activity was enhanced by PDP-EA in vitro (Faure et al., 2014), we predicted that cotton seedlings treated with PDP-EA might have modified NAE profiles. To test this possibility, NAE content and composition were measured in PDP-treated cotton seedlings. Ten-day-old cotton seedlings were irrigated with 30 μM PDP-EA over a time course of 16 days, leaves were excised, lyophilized, and subjected to lipid extraction, and analysis of NAE content and composition (Figure 4a,b). The total NAE content was highest in untreated, non-transgenic plants, and lowest in AtFAAH transgenics treated with PDP-EA. As might be anticipated from enhanced FAAH activity, PDP-treated and AtFAAH overexpressing plants had lower levels of NAEs (Figure 4a). Inspection of NAE profiles demonstrated differences for individual NAE types (Figure 4b). Leaves of wild-type plants irrigated with PDP-EA exhibited between ≈onefold to threefold lower levels of saturated NAE (NAE12:0), and polyunsaturated NAEs (NAE18:2, NAE18:3, and NAE18:1) (Figure 4b; P < 0.05, n = 3). NAE reductions were also observed for NAE14:0 and NAE16:0. Notably, NAE16:0 was not detectable in AtFAAH transgenics without or with PDP-EA treatment. PDP-EA treatment of AtFAAH transgenics resulted in significantly lower levels of NAE18:2 and NAE14:0 (P < 0.05), and undetectable levels of NAE12:0. Somewhat surprisingly, the amount of NAE18:0 remained unchanged regardless of the treatments made to wild-type or AtFAAH lines (Figure 4b; P > 0.05, n = 3). Altogether, these results demonstrate that treatment of cotton seedlings with PDP-EA is sufficient to reduce the content of most NAE types to levels similar to that by ectopic expression of AtFAAH, and that treatment of PDP-EA further enhances NAE content decline in planta.

### 3.5 NAE and free fatty acid oxylipin profiles for wild-type and AtFAAH transgenic cotton seedlings

The 9-LOX- derived NAE18:2 oxylipin, namely, 9-hydroxy-octadecadienoyl-ethanolamide (9-NAE-HOD) has been reported to be part of an ABA-mediated mechanism to modulate overall seedling growth in...
Arabidopsis (Keereetaweep et al., 2015). 9-NAE-HOD can be hydrolyzed by AtFAAH, which points to a direct participation of FAAH in 9-NAE-HOD metabolism (Aziz et al., 2019). Here, we tested whether the LOX metabolites of NAE and their corresponding free fatty acids (FFA) oxylipins were altered in cotton seedlings in a FAAH dependent manner, and if the levels were further altered in PDP-EA treated plants (Figure 5). The content of 13-NAE-HOD was unaltered when wild-type or AtFAAH cotton lines were irrigated without or with PDP-EA (Figure 5, upper left panel, P > 0.05, n = 3). The 9-NAE-HOD contents in wild-type and AtFAAH OEs were not significantly different, although average amounts in both AtFAAH transgenic lines were lower than those in non-transgenics. On the other hand, 9-NAE-HOD levels were dramatically lower in all genotypes treated with PDP-EA. In fact, 9-NAE-HOD levels were below detection in both AtFAAH transgenic lines treated with PDP-EA (Figure 5, upper right panel). The amount of FFA oxylipin 13-HOD was similar in wild-type and AtFAAH transgenics without PDP-EA (Figure 5, lower left panel, P > 0.05, n = 3), but levels declined upon irrigation with PDP-EA (P < 0.05). The levels of 9-HOD were significantly lower in AtFAAH transgenics compared with wild type; however, unlike non-transgenic plants where 9-HOD levels were significantly reduced upon PDP-EA treatment, levels of the free 9-HOD were actually higher in AtFAAH transgenics when plants were irrigated with PDP-EA (Figure 5, lower right panel, P < 0.05). Taken together these data in cotton seedlings suggest that FAAH is especially capable of depleting the endogenous levels of 9-NAE-HOD in both non-transgenics and transgenic plants, and that this FAAH-mediated reduction in 9-NAE-HOD is inversely associated with enhancement of plant growth and development. In other words, plants with lowest levels of 9-NAE-HOD (and its metabolic precursor NAE 18:2) were most enhanced in growth and development (compare Figure 5, upper right panel; Figure 4b; and Figure 3). Also notable, these data suggest that endogenous cotton FAAH activities may also especially modulate endogenous NAE18:2 and 9-NAE-HOD content, since levels of these metabolites were lower in non-transgenics following PDP-EA-treatment (Figures 4b and 5).

### 3.6 Lipophilic factors associated with AtFAAH expression and PDP-EA treatment in cotton seedlings

Four major groupings were revealed from partial least squares discriminant analysis (PLS-DA) of NAEs, NAE hydroxides and FFA-hydroxides data sets for non-transgenic and AtFAAH transgenic

![Figure 5](image-url)  
Comparison of endogenous N-acylethanolamines (NAE) oxylipins and free fatty acid (FFA) oxylipin profiles in non-transgenic wild-type (WT) and AtFAAH cotton transgenics fed with 0.05% DMSO (control) and 30 μM PDP-EA. Tissues were collected at 16-day-post treatment and subjected to oxylipin extraction. Bars represent the means and standard deviation (±SD) from three biological samples. One-way ANOVA with Tukey post hoc test was used for statistical analyses of the oxylipin profiles. Different letters indicate statistical differences (P < 0.05). Abbreviation “n.d” represents no detectable levels of the metabolite.
FIGURE 6  Partial Least Squares Discriminant Analysis (PLS-DA) and variable importance in projection (VIP) scores for the combined NAE-oxylipins, FFA-oxylipins, and NAE data sets. (a) Shaded colored clusters in PLS-DA graph represent 95% confidence intervals, rectangles with dashed lines were drawn to enclose and name the data set clusters, namely, clusters 1 to 4 (C1 to C4). (b) VIP scores plot depict relative abundance and ranks each metabolite (from top to bottom) in terms of its importance in the data spatial distribution. Red represents the high values, whereas blue represents the low values. Abbreviations: WT_D, wild-type fed with 0.05% DMSO; WT_30μM_P, wild-type fed with 30 μM PDP-EA; AF_O2_D, AtFAAH cotton line 2 fed with 0.05% DMSO; AF_O3_D, AtFAAH cotton line 3 fed with 0.05% DMSO; AF_O2_30μM_P, AtFAAH cotton line 2 fed with 30 μM PDP-EA; AF_O3_30μM_P, AtFAAH cotton line 3 fed with 30 μM PDP-EA.
cotton seedlings without or with PDP-EA treatment (Figure 6a). Cluster 1 represents non-transgenic seedlings with no treatment. Cluster 2 (30 μM PDP-EA, non-transgenic) and Cluster 3 (AtFAAH-OE2 and AtFAAH-OE3, solvent controls) were separated from Cluster 4 (30 μM PDP-EA-treated AtFAAH-OE2 and AtFAAH-OE3), supporting distinct differences in these lipid metabolites among these genotypes and treatments. In order to assess the impact of the metabolites as factors responsible for the spatial positioning of these data sets, we utilized the variable importance in projection (VIP) to rank the metabolites that govern the PLS-DA clustering patterns (Figure 6b). The VIP plot indicated that NAE16:0, NAE12:0, NAE18:2, NAE14:0, and 9-NAE-HOD have the top five highest scores, whereas NAE18:3, 9-HOD, 13-NAE-HOD, 13-HOD, NAE18:1, and NAE18:0 seem to be minor contributors of the clustering patterns observed in the PLS-DA plot (Figure 6b). Altogether, these statistical analyses support the conclusion that NAE and NAE-HOD metabolites may be associated with the growth phenotypes observed in PDP-EA-treated wild-type or AtFAAH cotton seedlings.

3.7 | FAAH activity assays in wild-type and AtFAAH cotton lines

To confirm that FAAH activity in cotton seedlings was higher in the transgenic FAAH overexpressors, and that this activity was responsive to PDP-EA, fluorescent-based assays were used to quantify the amount of ethanolamine produced from three different types of acylethanolamines in cell-free homogenate fractions extracted from leaves of 32-day-old plants (Figure 7). The rate of FAAH activity is presented as the amount of ethanolamine produced (μmol) per unit of time (min) per total amount of protein (mg).

The FAAH activities in AtFAAH-OE2 or AtFAAH-OE3 transgenics tested toward NAE16:0, NAE18:2, and 9-NAE-HOD were elevated between 3- and 11-fold above FAAH activities measured in homogenates from non-transgenic, wild-type controls (Figure 7; \( P < 0.05 \), \( n = 4 \)). In the enzyme assays where PDP-EA was added directly, the FAAH activities for wild-type, AtFAAH-OE-2, or AtFAAH-OE3 were greater than in the absence of PDP-EA (between twofold and fivefold; \( P < 0.05 \), \( n = 4 \)). Overall, the highest FAAH activities were consistently detected in AtFAAH overexpressing leaf extracts measured with added PDP-EA regardless of the NAE substrate tested. (Figure 7; \( P < 0.05 \), \( n = 4 \)). These combined data indicate that expression of AtFAAH in cotton increases the capacity of the hydrolysis of NAE and NAE-hydroxides. Furthermore, these data also suggest that PDP-EA is capable of enhancing the activity of endogenous cotton FAAHs as well as increasing activity of FAAH in both transgenic lines. Due to the inherently variability in assays where plant homogenates (enzyme source) are used, it is important to interpret these data with some caution. Future experiments to test the in vitro hydrolysis of NAEs by recombinant endogenous cotton FAAHs in the presence of PDP-EA will help to support these results, as was done for AtFAAH (Faure et al., 2014).

3.8 | Identification of groups I and II fatty acid amide hydrolase (FAAHs) in upland cotton

Recently, two major phylogenetic clusters of FAAHs, namely, groups I and II FAAH have been found in most angiosperm plants outside the Brassicaceae (Aziz & Chapman, 2020), and this prompted us to investigate in more detail the groups of FAAHs encoded in the cotton genome. Here, we identified six FAAH homologs in upland cotton (G. hirsutum L), and based on similarities of their predicted amino acid sequences, these homologs were also divided into two groups. Group I FAAH comprised GhFAAH-la, -lb, and -lc, whereas group II FAAH was represented by GhFAAH-IIa, -IIb, and -IIc (Figure 8a; Table S4).

All six cotton FAAHs contained the conserved catalytic residues (Ser-Ser-Lys) in their amidase signature domains, identical to those functionally determined for AtFAAH (Figure 8b; Figure S12a). MEME analysis showed the position of additional conserved motifs in the FAAH amino acid sequences (Figure S12b). Further, homology modeling of these proteins showed that like AtFAAH and GhFAAHs in both groups are predicted to have two subunits (Figure S13). Overall, it appears that in terms of FAAH isoforms, the situation in cotton is quite complex compared with the single FAAH isoform in Arabidopsis, with cotton having six FAAH isoforms divided into two groups.

To assess expression levels of cotton FAAHs in different plant organs and stages, we performed RT-qPCR analysis in seedlings, stems, leaves, roots, and petals tissues. Comparison between groups I
and II FAAH transcripts showed that FAAH I genes were expressed at higher levels than FAAH II overall in plant tissues (Figure 8c). Both groups of FAAH transcripts were more abundant in seedlings and leaves compared to stems, roots, and petals. Overall, these data indicated that groups I and II FAAHs can be expressed in multiple tissues with somewhat of a preference in seedlings and leaves at least among the tissues and stages tested.

We used RT-qPCR to quantify the amounts of endogenous GhFAAH transcripts and AtFAAH transcripts (in transgenics) in leaves of seedlings after a 16-day-post treatment with 30 μM PDP-EA. The GhFAAH-Ia, -Ib, and -Ic transcripts of PDP-EA treated wild-type or AtFAAH transgenics were elevated between ≈twofold to threefold above controls without PDP-EA (Figure 9; \( P < 0.05, n = 3 \)). GhFAAH-Ic transcripts in PDP-EA treated genotypes were unchanged relative to controls in the absence of PDP-EA (Figure 9; \( P > 0.05 \), except for AtFAAH-OE3, where a slightly higher level of GhFAAH-Ic transcripts was noted in PDP-EA treated seedlings (\( P < 0.05, n = 3 \)). As anticipated, AtFAAH transcripts were not detected in non-transgenics, and levels in AtFAAH-OE2 and AtFAAH-OE3 remained unchanged following application of 30 μM PDP-EA. Altogether, these data suggest that PDP-EA may impact transcript levels for selective cotton FAAH isoforms, which may add to increases in FAAH activity that were observed in PDP-EA treated plants. However, the AtFAAH transcripts (under control of the CaMV35S promoter) were unresponsive to PDP-EA.
FIGURE 9  Reverse transcriptase-quantitative PCR (RT-qPCR) analysis of endogenous and transgenic fatty acid amide hydrolase (FAAH) transcripts in non-transgenic wild-type (WT) and AtFAAH cotton transgenics at 16-days-post treatment with 0.05% DMSO (control) or 30 μM PDP-EA. Relative RNA values were calculated with the deltaCT method, using Ubiquitin1 (UBQ1) as the housekeeping gene. Bars represent the means and standard deviation (±SD) from three biological replicates. One-way ANOVA with Tukey post hoc test was used for statistical analyses of the data sets. Different letters represent significant differences (P < 0.05) within the group analyzed. Abbreviation “n.d.” represents no detectable levels of the transcript.

4 | DISCUSSION

Fatty acid amide hydrolase (FAAH) terminates the signaling activity of lipid molecules, namely, N-acylthanolamines (NAEs) (Aziz et al., 2019; Teaster et al., 2012; Wang et al., 2006). In Arabidopsis, several lines of evidence have shown that modulation of NAE content by AtFAAH is associated with multiple physiological processes, including seedling establishment, primary root elongation, flowering, chloroplast development, and plant pathogen interactions (Cannon et al., 2020; Kang et al., 2008; Keereetawee et al., 2013, 2015; Palmer et al., 2014; Wang et al., 2006). Here, we demonstrate that like in the model plant, Arabidopsis, Arabidopsis transgenics overexpressing the AtFAAH gene (Faure et al., 2014), enhancing FAAH activity in a perennial crop plant (upland cotton) by overexpression and by pharmacological treatment with a FAAH enhancer (PDP-EA), can enhance seedling growth. We showed that applications of micromolar concentrations of PDP-EA can stimulate cotton seedling development in cotton lines overexpressing AtFAAH. The enhancement of growth coincided with both a decrease of NAE and NAE-oxylipin contents, and a stimulation of FAAH enzyme activities. An ability to modulate the interplay between NAE metabolism and plant responses could provide novel biotechnological and agricultural applications.

To functionally characterize the transgenics used in the study, AtFAAH cotton seeds were exposed to micromolar concentrations of NAE12:0, NAE18:2 hydroxide (9-NAE-HOD), or ABA. Compared to controls, transgenic seedlings were more tolerant to the inhibitory effects of NAE12:0, 9-NAE-HOD, or ABA. The tolerance toward NAEs in cotton transgenics was expected due to an increased capacity for FAAH activity, which in turn ameliorated the negative growth effects of NAE12:0 or 9-NAE-HOD. Such an outcome resembled that of reports where Arabidopsis AtFAAH lines showed tolerance to exogenous NAEs (Kim et al., 2009; Wang et al., 2006) due to enhanced FAAH activity. By contrast, the tolerance to ABA in these same cotton lines was surprising. Unlike the Arabidopsis AtFAAH lines where ABA-exposure led to hypersensitivity (Cotter et al., 2011; Keereetawee et al., 2015; Teaster et al., 2007), AtFAAH cotton lines exhibited insensitivity to ABA. Although the same transgene (AtFAAH) was used in both Arabidopsis and cotton transgenics, it is possible that the mechanisms linking NAE metabolism to ABA signaling may be different between the two plant species. For example, in Arabidopsis, ABA application increased NAE hydroxide levels (and vice versa) presumably through increased lipoxigenase (LOX) gene expression and activity. This interaction between NAE hydroxide levels and ABA accumulation was proposed as a mechanism to induce a “secondary dormancy” in Arabidopsis and arrest growth during a developmental window in very early seedling establishment (Blancaflor et al., 2014). Perhaps the activation of secondary dormancy differs somewhat between cotton and Arabidopsis. In cotton, it may be that the regulation of LOX is more complex or different (there are six 9-LOX isoforms in cotton compared with two 9-LOXes in Arabidopsis) (Shaban et al., 2018) or that the higher FAAH activity in cotton seedlings is able to more rapidly hydrolyze the NAE hydroxides and prevent their accumulation. Notably, others showed that ABA hypersensitivity was independent of catalytic activity (Kim et al., 2009). Thus, it may be that there is a FAAH protein interaction with another protein or macromolecule that influences ABA signaling in Arabidopsis that may be absent in cotton—either because the partner is not present or the sequence similarity is too different for it to occur (Kim et al., 2009). Alternatively, there may be interactions between NAE metabolism and other phytohormone pathways (e.g., salicylic acid) to modulate seedling growth. Previous research in Arabidopsis has pointed out the association of SA-mediated pathways in the context of AtFAAH overexpressed plants (Kang et al., 2008). In any case, additional research will be required to more fully examine the interaction between NAEs and ABA in cotton seedlings.

PDP-EA enhanced overall seedling growth of Arabidopsis transgenics overexpressing the AtFAAH gene (Faure et al., 2014). Consistent with these results, the growth of PDP-EA-treated AtFAAH cotton transgenics was enhanced in both young and older seedlings. Seed germination was enhanced as was seedling root elongation in PDP-EA treated transgenic seedlings. Furthermore, older AtFAAH cotton seedlings irrigated with PDP-EA had longer stems, roots, and larger leaves compared to corresponding solvent controls. Somewhat different from Arabidopsis, PDP-EA alone had no obvious effects in non-transgenic cotton genotypes, either in seed germination or seedling development at early stages. However, PDP-EA treatment alone did show positive effects on leaf expansion in older, non-transgenic seedlings, suggesting that at least some enhancement of growth in cotton could be attributed to PDP-EA treatment alone. One mechanism by which these phenoxyacetyl-ethanolamides exert their effects in plants is through relief from product feedback inhibition on the FAAH enzyme, which results in an increase in FAAH activity in vitro and in vivo (Faure et al., 2014). Consistent with these previous results in
Arabidopsis plants and in mammalian cell cultures, we found that when PDP-EA was added to crude, cell-free extracts from wild-type or AtFAAH-overexpressing cotton seedlings, there was an elevated hydrolysis of saturated (NAE16:0), unsaturated (NAE18:2), and hydroxylated (9-NAE-HOD) NAE substrates. These findings support the notion that not only is PDP-EA capable of stimulating hydrolysis activity of AtFAAH but may also enhance the activity of endogenous cotton FAAHs, although this needs to be followed up with studies on purified FAAH enzymes. The lack of effects of PDP-EA on several growth parameters in non-transgenic (WT) cotton seedlings (above) may suggest that PDP-EA is not as effective on cotton FAAH enzymes as Arabidopsis. Because there are multiple endogenous FAAH isoforms expressed in cotton seedlings, more work is required to determine whether any enhancement in activity is a feature of one or multiple FAAH isoforms in cotton seedlings.

Non transgenic wild-type and AtFAAH transgenic cotton seedlings showed significant decreases in the content of NAE and NAE oxylipins—both of which have been shown to negatively modulate seedling growth in Arabidopsis. Total NAEs, several individual NAE types, and NAE hydroxides were reduced in a PDP-EA- and FAAH-dependent manner. Reductions were pronounced especially for 9-NAE-HOD and its metabolic precursor NAE 18:2. The misregulation of 9-NAE-HOD content and its potent negative effects in seedling growth were reported in Arabidopsis, where 9-NAE-HOD was shown to interact with ABA signaling to arrest seedling growth (Keeretawee et al., 2015). Although the connection between NAE and ABA signaling remains to be investigated in cotton (above), overall our data support associations between PDP-EA stimulation of FAAH activity, the reduction of NAE content, especially the potent 9-NAE-HOD, and the modulation of seedling growth.

It is worth noting that although some endogenous NAEs (e.g., NAE12:0) or NAE-hydroxides (e.g., 9-NAE-HOD) were completely depleted in PDP-EA treated AtFAAH cotton lines, there were certain NAE species that remained unchanged in the same tissues (e.g., NAE18:0 and 13-NAE-HOD), thus suggesting a limited hydrolytic activity by FAAH. One explanation could be differences in substrate preferences, although this seems less likely due to the wide range of substrates hydrolyzed by AtFAAH. A more likely explanation is a limitation in accessibility of FAAH to some NAEs. Although AtFAAH is strongly expressed in cotton transgenics, the substrate pool of some lipophilic species could be limited to certain cellular compartments that might not accessible to the enzyme (AtFAAH) or to endogenous cotton FAAHs, even in cases where their amidohydrolase activities were elevated by PDP-EA.

Analysis of FAAH amino acid sequences in cotton revealed two phylogenetic groups, namely, group I FAAHs (GhFAAH-Ia, GhFAAH-Ib, and GhFAAH-Ic), and group II FAAHs (GhFAAH-IIa, GhFAAH-IIb, and GhFAAH-IIc). FAAH enzymes in these two major groups were categorized for other angiosperms (Aziz & Chapman, 2020). RT-qPCR data showed that these genes are all expressed in most cotton organs, with seedlings and leaves having the highest relative transcript levels in general. Three important pieces of information were obtained when AtFAAH and endogenous cotton FAAHs where compared in the presence or absence of PDP-EA. First, the relative amount of AtFAAH transcripts in transgenic lines was unchanged by PDP-EA treatment. Hence, it is reasonable to speculate that AtFAAH may be exerting its effect at the protein level rather than at the transcript level. Second, AtFAAH transcript levels are far more abundant than endogenous cotton FAAHs (GhFAAHs). This likely is explained by the fact that AtFAAH expression in the transgenic cotton lines is regulated by the strong CaMV 35S promoter (Amack & Antunes, 2020), whereas the expression of cotton FAAHs (GhFAAHs) are regulated by their endogenous promoters. Third, to our surprise, we noted that the relative transcript abundance for certain GhFAAHs (e.g., GhFAAH-Ia and GhFAAH-Ib) were increased in the presence of PDP-EA. These data suggest that in addition to the direct effects of PDP-EA on the FAAH enzyme itself, PDP-EA may also influence FAAH gene expression in cotton and modulate upstream or downstream signaling pathways associated with NAE metabolism and growth. It may be that PDP-EA activates FAAH transcription directly by some as-yet undefined mechanism. Alternatively, it may be that through binding directly to FAAH that PDP-EA influences the stability of the FAAH enzyme, and altered protein turnover rates may indirectly induce cotton FAAH expression. This unexpected finding will require further examination including, but not restricted to, an investigation of cotton FAAH promoter activities.

Here, our findings extend the scope of FAAH modulation of seedling growth beyond a model species to a perennial crop species. Indeed, we showed that PDP-EA can enhance the hydrolysis of multiple NAEs by FAAH in cotton seedlings and that this behavior seems to be associated with the positive effects observed at different stages of cotton seedling development. Further, targeted lipidomics in seedling tissues made it possible to identify certain NAEs that are most likely associated with these enhanced growth phenotypes. Fine tuning of the metabolism of these endogenous lipophilic signaling molecules may lead to novel strategies for the enhancement of seedling establishment in other crops.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

OAG and KDC designed the experiments. BA produced the cotton transgenic lines used in the experiments. OAG performed the experiments. KDC supervised the project. OAC wrote the first draft of the manuscript, and KDC edited it. MA provided input to the revised manuscript and advice regarding FAAH isoform analysis and enzyme activity assays. All authors read and approved the final manuscript.
REFERENCES

Amack, S. C., & Antunes, M. S. (2020). CaMV35S promoter—A plant biology and biotechnology workhorse in the era of synthetic biology. Current Plant Biology, 24, 100179.

Aziz, M., & Chapman, K. D. (2020). Fatty acid amide hydrolases: An expanded capacity for chemical communication? Trends in Plant Science, 25(3), 236–249.

Aziz, M., Wang, X., Tripathi, A., Bankaitis, V. A., & Chapman, K. D. (2019). Structural analysis of a plant fatty acid amide hydrolase provides insights into the evolutionary diversity of bioactive acylethanolamides. The Journal of Biological Chemistry, 294(18), 7419–7432.

Bailey, T. L., Williams, N., Misleh, C., & Li, W. W. (2006). MEME: Discovering and analyzing DNA and protein sequence motifs. Nucleic Acids Research, 34(suppl_2), W269–W373.

Blancaflor, E. B., Kilaru, A., Keereetaweep, J., Khan, B. R., Faure, L., & Chapman, K. D. (2014). N-Acylethanolamines: Lipid metabolites with functions in plant growth and development. The Plant Journal, 79(4), 568–583.

Bosier, B., Muccioli, G. G., & Lambert, D. M. (2013). The FAAH inhibitor URB597 efficiently reduces tyrosine hydroxylase expression through CB₁ and FAAH-independent mechanisms. British Journal of Pharmacology, 169(4), 794–807.

Cannon, A. E., & Chapman, K. D. (2021). Lipid signaling through G proteins. Trends in Plant Science, 26(7), 720–728.

Cannon, A. E., Yan, C., Burks, D. J., Rao, X., Azad, R. K., & Chapman, K. D. (2020). Lipophilic signals lead to organ-specific gene expression changes in Arabidopsis seedlings. Plant Direct, 4(7), e002422.

Chakma, S. P., Chileshe, S. M., Thomas, R., & Krishna, P. (2021). Cotton seed priming with brassinosteroid promotes germination and seedling growth. Agronomy, 11(3), 566–578.

Chapman, K. D., Tripathi, A., Venables, B., & Desouza, A. D. (1998). N-acyl ethanolamides: Formation and molecular composition of a new class of plant lipids. Plant Physiology, 116(3), 1163–1168.

Chapman, K. D., Venables, B., Markovic, R., Blair, R. W. Jr., & Bettinger, C. (1999). N-acyl ethanolamides in seeds. Quantification of molecular species and their degradation upon imbibition. The Plant Journal, 120(4), 1157–1164.

Christensen, S. A., Nemchenko, A., Borrego, E., Murray, I., Sobhy, I. S., Bosak, L., DeBlasio, S., Erb, M., Robert, C. A., Vaughn, K. A., Herrfurth, C., Tumlinson, J., Feussner, I., Jackson, D., Turlings, T. C., Engelberth, J., Nansen, C., Meeley, R., & Kolomiets, M. V. (2013). The maize lipoxigenase, ZmLOX10, mediates green leaf volatile, Jasmonate, and herbivore-induced plant volatile production for defense against insect attack. The Plant Journal, 74(1), 59–73.

Couper, M. Q., Teaster, N. D., Blancaflor, E. B., & Chapman, K. D. (2011). N-acyl ethanolamine (NAE) inhibits growth in Arabidopsis thaliana seedlings via ABI3-dependent and -independent pathways. Plant Signaling & Behavior, 6(5), 671–679.

Dereeper, A., Guignon, V., Blanc, G., Audic, S., Buffet, S., Chevenet, F., Dufayard, J. F., Guindon, S., Lefort, V., Lescot, M., Claverie, J. M., & Gascuel, O. (2008). Phylogeny.fr: Robust Phylogenetic Analysis for the Non-specialist. Nucleic Acids Research, 36(Web Server issue), W465–W469.

Faure, L., Nagarajan, S., Hwang, H., Montgomery, C. L., Khan, B. R., John, G., Koulen, P., Blancaflor, E. B., & Chapman, K. D. (2014). Synthesis of phenoxyacetyl-ethanolamides and their effects on fatty acid amide hydrolase activity. The Journal of Biological Chemistry, 289(13), 9340–9351.

Fowler, C. J., Börjesson, M., & Tiger, G. (2000). Differences in the pharmacological properties of rat and chicken brain fatty acid amidohydrolase. British Journal of Pharmacology, 131(3), 498–504.

Gachet, M. S., Schubert, A., Calarco, S., Boccard, J., & Gertsch, J. (2017). Targeted metabolomics shows plasticity in the evolution of signaling lipids and uncovers old and new endocannabinoids in the plant kingdom. Scientific Reports, 7, 41177.

Gattiker, A., Gasteiger, E., & Bairoch, A. (2002). ScanProsite: A reference implementation of a PROSITE scanning tool. Applied Bioinformatics, 1(2), 107–108.

Giang, D. K., & Cravatt, B. F. (1997). Molecular characterization of human and mouse fatty acid amide hydrolases. Proceedings of the National Academy of Sciences of the United States of America, 94(6), 2238–2242.

Hamiaux, L., Masquelier, J., Muccioli, G. G., Bouzin, C., Feron, O., Gallez, B., & Lambert, D. M. (2012). The association of N-palmitoylethanolamine with the FAAH inhibitor URB597 impairs melanoma growth through a supra-additive action. BMC Cancer, 12, 92.

Kang, L., Wang, Y. S., Uppalapati, S. R., Wang, K., Tang, Y., Vadapalli, V., Venables, B. J., Chapman, K. D., Blancaflor, E. B., & Mysore, K. S. (2008). Overexpression of a fatty acid amide hydrolase compromises innate immunity in Arabidopsis. The Plant Journal, 56(2), 336–349.

Keereetaweep, J., Blancaflor, E. B., Hornung, E., Feussner, I., & Chapman, K. D. (2013). Ethanolamide oxylipins of linolenic acid can negatively regulate Arabidopsis seedling development. Plant Cell, 25(10), 3824–3840.

Keereetaweep, J., Blancaflor, E. B., Hornung, E., Feussner, I., & Chapman, K. D. (2015). Lipoxigenase-derived 9-hydro (perxo)xies of linoleoylethanolamide interact with ABA signaling to arrest root development during Arabidopsis seedling establishment. The Plant Journal, 82(2), 315–327.

Keereetaweep, J., & Chapman, K. D. (2016). Lipidomic analysis of endocannabinoid signaling: Targeted metabolite identification and quantification. Neural Plasticity, 2016, 2426398.

Kilaru, A., Herrfurth, C., Keereetaweep, J., Hornung, E., Venables, B. J., Feussner, I., & Chapman, K. D. (2011). Lipoxigenase-mediated oxidation of polyunsaturated N-acyl ethanolamines in Arabidopsis. The Journal of Biological Chemistry, 286(17), 15205–15214.

Kilaru, A., Tamura, P., Isaac, G., Welti, R., Venables, B. J., Seier, E., & Chapman, K. D. (2012). Lipidomic analysis of N-acylphosphatidylethanolamine molecular species in Arabidopsis suggests feedback regulation by N-acyl ethanolamides. Planta, 236(3), 809–824.

Kim, S.-C., Faure, L., & Chapman, K. D. (2013). Analysis of fatty acid amide hydrolase activity in plants. In T. Munnik & I. Heilmann (Eds.), Plant lipid signaling protocols (pp. 115–127). Humana Press.

Kim, S. C., Kang, L., Nagaraj, S., Blancaflor, E. B., Mysore, K. S., & Chapman, K. D. (2009). Mutations in Arabidopsis fatty acid amide hydrolase reveal that catalytic activity influences growth but not sensitivity to abscisic acid or pathogens. The Journal of Biological Chemistry, 284(49), 34065–34074.

Ko, J., Park, H., Heo, L., & Seok, C. (2012). GalaxyWEB server for protein structure prediction and refinement. Nucleic Acids Research, 40(W1), W294–W297.

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods, 25(4), 402–408.

Moya-Cuevas, J., Pérez-Alonso, M. M., Ortiz-Garcia, P., & Pollmann, S. (2021). Beyond the usual suspects: Physiological roles of the arabi- dopis amidase signature (AS) superfamily members in plant growth processes and stress responses. Biomolecules, 11(8), 1207–1223.

Muccioli, G. G., Xu, C., Odah, E., Cudaback, E., Cisneros, J. A., Lambert, D. M., López Rodríguez, M. L., Bajaliev, S., & Stella, N. (2007). Identification of a novel endocannabinoid-hydrorylating
enzyme expressed by microglial cells. *The Journal of Neuroscience*, 27(11), 2883–2889.

Palmer, A. G., Senechal, A. C., Mukherjee, A., Ané, J. M., & Blackwell, H. E. (2014). Plant responses to bacterial N-acyl L-homoserine lactones are dependent on enzymatic degradation to L-homoserine. *ACS Chemical Biology*, 9(8), 1834–1845.

Salimath, S. S., Romsdahl, T. B., Konda, A. R., Zhang, W., Cahoon, E. B., Dowd, M. K., Wedegaertner, T. C., Hake, K. D., & Chapman, K. D. (2021). Production of tocotrienols in seeds of cotton (*Gossypium hirsutum* L) enhances oxidative stability and offers nutraceutical potential. *Plant Biotechnology Journal*, 19(6), 1268–1282.

Schneider, C. A., Rasband, W. S., & Eliceiri, K. W. (2012). NIH image to ImageJ: 25 years of image analysis. *Nature Methods*, 9(7), 671–675.

Shaban, M., Ahmed, M. M., Sun, H., Ullah, A., & Zhu, L. (2018). Genome-wide identification of lipoxygenase gene family in cotton and functional characterization in response to abiotic stresses. *BMC Genomics*, 19(1), 599–599.

Shrestha, R., Dixon, R. A., & Chapman, K. D. (2003). Molecular identification of a functional homologue of the mammalian fatty acid amide hydrolase in Arabidopsis thaliana. *The Journal of Biological Chemistry*, 278(37), 34990–34997.

Shrestha, R., Kim, S. C., Dyer, J. M., Dixon, R. A., & Chapman, K. D. (2006). Plant fatty acid (ethanol) amide hydrolases. *Biochimica et Biophysica Acta*, 1761(3), 324–334.

Teaster, N. D., Keereetaweep, J., Kilaru, A., Wang, Y. S., Tran, C. N., Ayre, B. G., Chapman, K. D., & Blancaflor, E. B. (2012). Overexpression of fatty acid amide hydrolase induces early flowering in Arabidopsis thaliana. *Frontiers in Plant Science*, 3, 32.

Teaster, N. D., Motes, C. M., Tang, Y., Wiant, W. C., Cotter, M. Q., Wang, Y. S., Kilaru, A., Venables, B. J., Hasenstein, K. H., Gonzalez, G., Blancaflor, E. B., & Chapman, K. D. (2007). N-acylethanolamine metabolism interacts with abscisic acid signaling in Arabidopsis thaliana seedlings. *Plant Cell*, 19(8), 2454–2469.

Venables, B. J., Waggoner, C. A., & Chapman, K. D. (2005). N-acylethanolamines in seeds of selected legumes. *Phytochemistry*, 66(16), 1913–1918.

Wan, C. Y., & Wilkins, T. A. (1994). A modified hot borate method significantly enhances the yield of high-quality RNA from cotton (*Gossypium hirsutum* L). *Analytical Biochemistry*, 223(1), 7–12.