Overexpression of fatty acid amide hydrolase induces early flowering in *Arabidopsis thaliana*

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N-acylethanolamines (NAEs) are bioactive lipids derived from the hydrolysis of the membrane phospholipid *N*-acylphosphatidylethanolamine (NAPE). In animal systems this reaction is part of the "endocannabinoid" signaling pathway, which regulates a variety of physiological processes. The signaling function of NAE is terminated by fatty acid amide hydrolase (FAAH), which hydrolyzes NAE to ethanolamine and free fatty acid. Our previous work in *Arabidopsis thaliana* showed that overexpression of *AtFAAH* (*At5g64440*) lowered endogenous levels of NAEs in seeds, consistent with its role in NAE signal termination. Reduced NAE levels were accompanied by an accelerated growth phenotype, increased sensitivity to abscisic acid (ABA), enhanced susceptibility to bacterial pathogens, and early flowering. Here we investigated the nature of the early flowering phenotype of *AtFAAH* overexpression. *AtFAAH* overexpressors flowered several days earlier than wild type and *AtFAAH* knockouts under both non-inductive short day (SD) and inductive long day (LD) conditions. Microarray analysis revealed that the *FLOWERING LOCUS T* (*FT*) gene, which plays a major role in regulating flowering time, and one target MADS box transcription factor, *SEPATALLA3* (*SEP3*), were elevated in *AtFAAH* overexpressors. Furthermore, *AtFAAH* overexpressors, with the early flowering phenotype had lower endogenous NAE levels in leaves compared to wild type prior to flowering. Exogenous application of NAE 12:0, which was reduced by up to 30% in *AtFAAH* overexpressors, delayed the onset of flowering in wild type plants. We conclude that the early flowering phenotype of *AtFAAH* overexpressors is, in part, explained by elevated *FT* gene expression resulting from the enhanced NAE hydrolase activity of AtFAAH, suggesting that NAE metabolism may participate in floral signaling pathways.

Keywords: *Arabidopsis*, fatty acid amide hydrolase, flowering, FLOWERING LOCUS T, lipid signaling, *N*-acylethanalomanine

INTRODUCTION

N-acylethanolamines (NAEs) are bioactive lipids that function in the regulation of various physiological processes in animal systems. This includes the endocannabinoid signaling pathway that regulates several neurobehavioral and neurophysiological activities. Fatty acid derivatives that are amide-linked to an ethanolamine moiety make up the basic molecular structure of NAE, which differ in their acyl chain length and number of double bonds (Kim et al., 2010). NAE bioactivity has been shown to be terminated by the hydrolytic activity of fatty acid amide hydrolase (FAAH) where NAE is hydrolyzed into ethanolamine and its corresponding free fatty acid (McKinney and Cravatt, 2005; Fowler, 2006). In mammals, the importance of FAAH in regulating *in vivo* NAE levels was demonstrated using genetic approaches. For example, it was shown that FAAH knockout mice possess elevated levels of anandamide (NAE 20:4) compared with wild type mice. The higher levels of anandamide in FAAH knockout mice were accompanied by a variety of physiological changes, including hypomotility, increased analgesia, and significant catalepsy

(Cravatt et al., 2001; Clement et al., 2003; Lichtman et al., 2004; Kilaru et al., 2010).

Like animals, recent research in plant systems showed that NAEs impact numerous physiological processes (Kim et al., 2010). In *Arabidopsis thaliana*, a homolog of the mammalian *FAAH* was identified and characterized (*AtFAAH*, locus *At5g64440*), and also was shown to possess NAE hydrolytic activity *in vitro* (Shrestha et al., 2003, 2006). The *Arabidopsis* FAAH encodes a protein of 607 amino acids with 37% identity to rat FAAH within the amidase signature (AS) domain (Shrestha et al., 2003). The AS family, consisting of more than 80 amidases, contain a highly conserved region of approximately 130 amino acids rich in serine, glycine, and alanine (Chebrou et al., 1996; Patricelli et al., 1999; Labahn et al., 2002; Gopalakrishna et al., 2004; Neu et al., 2007). Homologs of FAAH have been identified in multiple plant species and modeling of the AS region revealed a highly conserved active site (Shrestha et al., 2006). These studies suggest that similar to mammalian systems, the molecular machinery for terminating NAE signaling by FAAH also operates in plants (Blancaflor and Chapman, 2006).

Several lines of evidence support a role for FAAH in the catabolism of NAEs in plants. For instance, exogenous NAE at low micromolar concentrations produced a dose-dependent reduction in seedling growth (Blancaflor et al., 2003). Along with the arrested growth, seedlings displayed altered root cell, and cytoskeletal organization when treated with NAE (Blancaflor et al., 2003; Motes et al., 2005). When *AtFAAH* was over expressed in *Arabidopsis* seedlings, the growth inhibitory effects of exogenous NAE treatment were much reduced compared to wild type, whereas SALK T-DNA insertion *AtFAAH* knockout lines displayed an increased sensitivity to NAE-induced growth inhibition (Wang et al., 2006; Teaster et al., 2007; Cotter et al., 2011). The *AtFAAH* overexpressors generally displayed enhanced seedling growth and a tendency to flower earlier than wild type. On the other hand, *AtFAAH* knockouts did not show any other strong phenotype besides their greatly increased sensitivity to exogenous NAE (Wang et al., 2006). In addition to enhanced growth, *AtFAAH* overexpressors showed an increased sensitivity to the plant hormone, abscisic acid (ABA), indicating cross talk between NAE and ABA signaling pathways (Teaster et al., 2007; Cotter et al., 2011). As a trade-off for enhanced growth, *AtFAAH* overexpressors exhibited compromised resistance to a number of plant pathogens, including non-host pathogens that do not normally cause disease in *Arabidopsis* (Kang et al., 2008).

In this paper, we investigated the early flowering phenotype of *AtFAAH* overexpressors. We found that *AtFAAH* overexpressors flowered earlier than wild type and *AtFAAH* knockouts under both inductive long day (LD) and non-inductive short day (SD) conditions. Global gene expression profiling of 14-day-old *Arabidopsis* seedlings and verification by quantitative RT-PCR showed that *FLOWERING LOCUS T* (*FT*), which regulates flowering by activating other floral genes (Kardailsky et al., 1999; Corbesier et al., 2007; Turck et al., 2008; Seo et al., 2011), was elevated in *AtFAAH* overexpressors. We also found that the *AtFAAH* overexpressors grown under SD conditions for 14 days contained on average, about 9% less total NAE than wild type, and the levels of two specific types, NAE 12:0 and NAE 18:2, were reduced by up to 30% in *AtFAAH* overexpressors. Treatment of wild type *Arabidopsis* plants with exogenous NAE 12:0, delayed flowering. Taken together, our data suggest that the early flowering phenotype of *AtFAAH* overexpressors is attributed to altered NAE signaling.

MATERIALS AND METHODS

PLANT MATERIAL AND MEASUREMENT OF FLOWERING TIME

Three independent lines overexpressing *AtFAAH* (OE2, OE7, and OE11) and two *AtFAAH* T-DNA knockouts (SALK_118043 and SALK_095108) used in this study were described previously (Wang et al., 2006). For evaluating flowering, seeds were surface-sterilized with 95% ethanol, 30% bleach containing 0.1% Tween 20 and deionized water and planted on agar plates and stratified overnight at 4°C. Seeds were then moved to 22°C growth chambers set to SD (8/16 light/dark) or LD (16/8 light/dark) photoperiod, and grown for an additional 10 days. Individual seedlings were then transplanted to pots containing Metro mix and returned to their respective growth chamber. Plants were monitored daily after transfer to the growth chamber and the number of plants that

flowered was recorded. The number of rosette leaves was recorded on particular days as indicated.

GENE CHIP MICROARRAY EXPERIMENTS AND DATA ANALYSIS

For microarrays, total RNA was extracted from rosette leaves of 14-day-old *AtFAAH* overexpressors and the *AtFAAH* T-DNA insertional mutant SALK_095108, and processed as described previously (Teaster et al., 2007). Seeds of all three genotypes were planted on MS media and seedlings grown for 14 days in 14/10 h light/dark cycle prior to RNA isolation. The microarray experiments were conducted with RNA isolated from three biological replicates using ATH1 Genome Arrays (Affymetrix).

For data analysis, the CEL file for each sample was exported from the Expression Console (Affymetrix) and normalized using robust multi-array average (RMA) as described by Irizarry et al. (2003). The presence/absence call for each probe set was obtained from dCHIP (Li and Wong, 2001). Differentially expressed genes in the AtFAAH overexpressor and SALK_095108 knockout in comparison with wild type control were selected using associative analysis (Dozmorov and Centola, 2003). Type I family wise error rate was reduced using a Bonferroni-corrected *P*-value of 2.19202E-06 representing a threshold of 0.05/N, where N represents number of probe sets present on the chip, which is 22810 for the *Arabidopsis* chip (Abdi, 2007). Microarray data was submitted to the European Molecular Biology Laboratory's European Bioinformatics Institute (EMBL–EBI) ArrayExpress database under accession E-MEXP-3486.

REAL TIME QUANTITATIVE RT-PCR

Total RNA was isolated using an RNeasy Mini Kit (Qiagen) and reverse transcribed to first-strand cDNA with the Qiagen cDNA Synthesis Kit (Qiagen). First-strand cDNA was used as a template for quantitative PCR using gene-specific primers. *Arabidopsis 18s rRNA*, which served as a control for constitutive gene expression in plants, was amplified with primers (F) 5′-TCCTAGTAAGCGCGAGTCATCA-3′ (R) 5′-CGAACACTTCACCGGATCAT-3′ (Dean Rider et al., 2003). Gene-specific primers used were *FT* (At1g65480) (F) 5′-GGAGACGTTCTTGATCCGTTTAATAGATCAAT-3′ (R) 5′-ATAAACACGACACGATGAATTCCTGCAGT-3′; SEP3 (At1g24260) (F) 5′-GTGCCTTCAAGAGAGGCCTTAGCAGTT-3′ (R) 5′-TCTGAAGATCGTTGAGCTGGTCAAGCAT-3′. Relative expression levels using the formula for threshold cycle were calculated by the $(2^{-\Delta\Delta Ct})$ method (Livak and Schmittgen, 2001). Means of four biological replicates with three technical replicates and SE were reported.

NAE QUANTIFICATION

N-acylethanolamines were extracted from 100 to 250 mg of plant tissue and ground by bead beater in warm 2-propanol. The extract was combined with deuterated NAE standards (D4-NAE 16:0, Cayman Chemical Co.; 1 ppm each) and total lipids were extracted into chloroform. The organic phase was collected for further purification by solid phase extraction (SPE). Silica SPE cartridges (100 mg, 1.5 mL; Grace Davison Discovery Sciences) were conditioned with 2 mL methanol followed by 4 mL chloroform. Samples were applied to the column, washed with 2 mL

chloroform, and NAEs were eluted with 2 mL of 1:1 (v/v) ethyl acetate:acetone. The eluate was collected, evaporated under nitrogen, and derivatized with 50 μL BSTFA (Fisher Scientific, Houston, TX, USA) for 30 min at 55°C. After derivatization, the samples were again evaporated under nitrogen and reconstituted in 50 μL hexane. NAEs were identified via selective ion monitoring and quantified against the internal deuterated standards (deuterated NAE 16:0) as TMS-ether derivatives by gas chromatography/mass spectrometry (Agilent model 6890 GC coupled with a 5973 mass selective detector) as described previously (Venables et al., 2005). NAE concentration was calculated based on fresh weight.

EVALUATING EXOGENOUS NAE 12:0 EFFECTS ON FLOWERING

Arabidopsis wild type seeds were germinated in soil and were maintained in a growth chamber at 22°C with 16/8 h light/dark cycle $(60 \mu E\, m^{-2}\, s^{-1})$. Seven day-old seedlings ($n = 15$) were watered with 50 mL of 35 μM NAE 12:0 or 0.05% DMSO solution alone (solvent control) every 3 days until the plants were ready for drying. Time of flowering was recorded and inflorescence height was measured every 4 days. The number of rosette leaves was counted every 4 days from the time of germination until 24 days. The data presented are an average of three experiments with significance tested by Student's *t*-test.

RESULTS

OVEREXPRESSION OF *ATFAAH* INDUCES EARLY FLOWERING UNDER NON-INDUCTIVE SHORT DAYS AND INDUCTIVE LONG DAYS

We evaluated the flowering time of three independent *AtFAAH* overexpressing lines described in Wang et al. (2006). We found that when grown under non-inductive SD conditions, *AtFAAH* overexpressors flowered earlier than wild type (Col-0) and *AtFAAH* knockouts (**Figure 1A**). Quantification of the timing of flowering revealed that more than 50% of all *AtFAAH* overexpressing lines flowered 28 days after planting with one line (*AtFAA-HOE11*) showing more than 70% flowering. In contrast, less

than 20% of wild type and *AtFAAH* knockouts flowered at day 28 (**Figure 1B**). These results were typical of three independent experiments with individual *AtFAAH* overexpressors flowering on average up to 10 days earlier than wild type and *AtFAAH* knockouts. Even under inductive LD conditions *AtFAAH* overexpressor flowered earlier than wild type or *AtFAAH* knockouts. At 16 days, more than 60% of all three *AtFAAH* overexpressors had already flowered whereas wild type and *AtFAAH* knockouts did not. At day 18, only 10% of wild type and *AtFAAH* knockouts flowered while 70–100% of *AtFAAH* overexpressors flowered (**Figure 1B**).

We measured the number of rosette leaves and rosette diameter of SD-grown plants, on day 21 and day 28 after planting, to determine if a more rapid growth was responsible for the early flowering phenotype of the *AtFAAH* overexpressors. The total number of rosette leaves and rosette diameter did not differ significantly for wild type and *AtFAAH* overexpressors (**Figures 2A,B**). After 28 days of SD growth, >50% of *AtFAAH* overexpressors had flowered and had 9–10 rosette leaves. Under LD-growth conditions, all plants, regardless of genotype, had flowered, and had seven to nine rosette leaves. Accelerated growth under LDs is expected because of greater photosynthesis, but the similar number of leaves on *AtFAAH* overexpressors and LD-grown plants indicates that the plants flowered at nearly the same plastochron age (i.e., morphological age rather than chronological age) (Erickson and Michelini, 1957).

TRANSCRIPTIONAL PROFILING LINKS ELEVATED *FLOWERING LOCUS (FT)* EXPRESSION TO EARLY FLOWERING OF *ATFAAH* OVEREXPRESSORS

To better understand the molecular basis for the early flowering phenotype of *AtFAAH* overexpressors, microarray analysis was conducted to compare transcript profiles of wild type with that of the *AtFAAH* overexpressors and *AtFAAH* knockouts
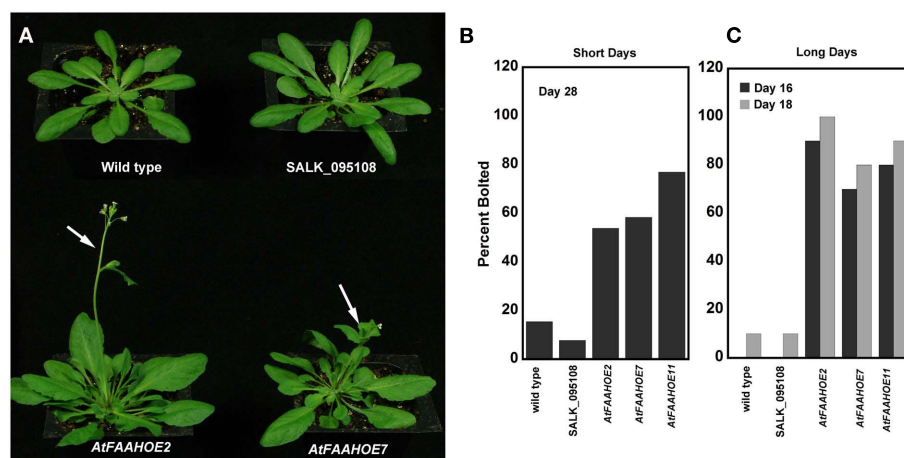
FIGURE 1 | Overexpression of *AtFAAH* induces early flowering of *Arabidopsis* under short and long days. (A) Representative images of 30-day-old wild type, SALK_095108 *AtFAAH* knockout and two *AtFAAH* overexpressors (OE2 and OE7) grown during short day conditions, 8/16 h (light/dark). Note that the two *AtFAAH* overexpressors have already flowered (arrows). The percent of wild type, *AtFAAH* knockout and *AtFAAH* overexpressors that had flowered by day 28 under non-inductive SDs **(B)** and by day 16 and day 18 under inductive LDs **(C)**. The graph is representative of three experiments with *n* > 10.

FIGURE 2 | The number of rosette leaves and rosette diameter is not affected by altered *AtFAAH* expression. Number of rosette leaves **(A)** and rosette diameter **(B)** of wild type and three *AtFAAH* overexpressors under SDs after 21 days, when no plants were flowering, and after 28 days, when some wild type and most *AtFAAH* overexpressors were flowering. **(C)** Number of rosette leaves under longs days 28 days after planting in wild type, two *AtFAAH* knockouts (SALK_118043 and SALK_095108) and three *AtFAAH* overexpressors (OE2, 7, and 11); all plants were flowering.

(SALK_095108). Consistent with the fact that *AtFAAH* knockouts did not have any obvious phenotype (Wang et al., 2006; **Figures 1** and **2**), a less dramatic difference in the overall gene expression profiles were observed between wild type and *AtFAAH* knockouts as indicated by a heat map generated from ratios of transcript levels from *AtFAAH* knockouts (SALK_095108) and wild type (**Figure 3A**). In contrast, there were more distinct differences in the overall transcript profiles when wild type was compared to *AtFAAH* overexpressors (*AtFAAHOE11*; **Figure 3A**). With a Bonferroni-corrected *P*-value threshold of 2.19202E-06, we found 157 genes to be differentially regulated by twofold or more in *AtFAAH* overexpressors compared with wild type. Out of 157 genes, 50 were upregulated and 107 were downregulated in the *AtFAAH* overexpressor line (Table S1 in Supplementary Material). Only 28 genes were differentially expressed by twofold or more between *AtFAAH* knockouts and wild type, of which 7 were upregulated and 21 were downregulated (Table S1 in Supplementary Material). As expected, the *AtFAAH* gene itself was downregulated in *AtFAAH* knockouts and upregulated in *AtFAAH* overexpressors, which helped to validate our microarray results. Seven genes were downregulated and one gene was upregulated by twofold or more in both *AtFAAH* overexpressors and knockouts (**Figure 3B**). The seven genes downregulated by twofold or more in both *AtFAAH* overexpressors and knockouts are presented in Table S2 in Supplementary Material. Among the seven genes downregulated in both *AtFAAH* overexpressors and knockouts, only *circadian clock associated* (*CCA1*) has been clearly implicated in flowering. CCA1 is a transcription factor involved in plant circadian rhythms and overexpressing *CCA1* causes delayed flowering in *Arabidopsis* (Wang and Tobin, 1998). The downregulation of *CCA1* in *AtFAAH* overexpressors could disrupt circadian rhythms important for flowering; however, its downregulation in *AtFAAH*
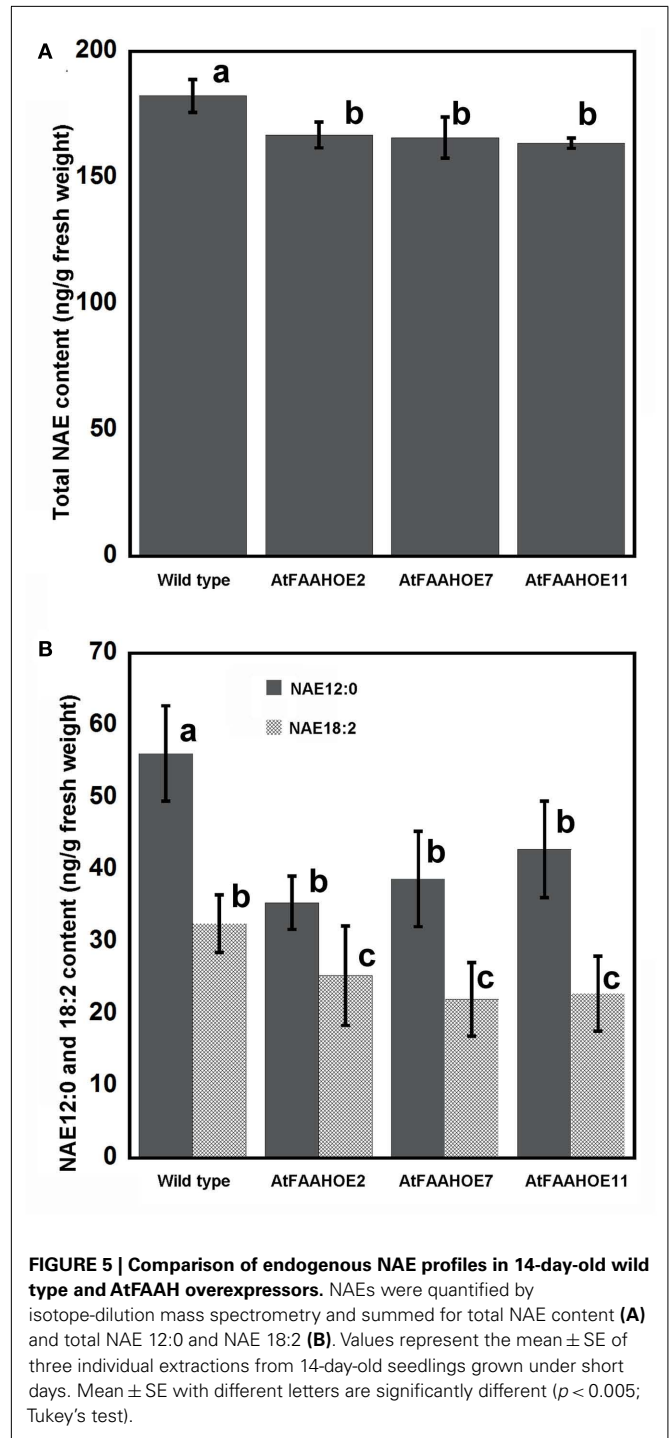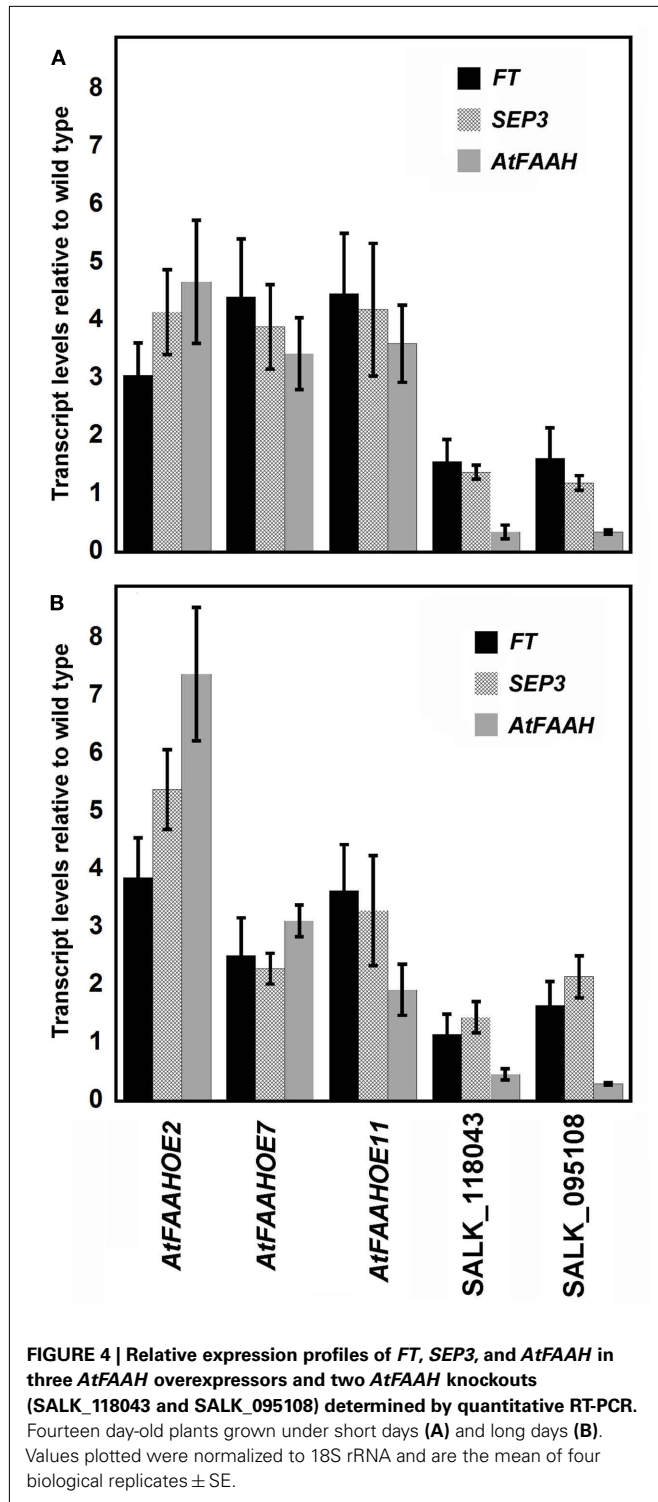
knockouts where no flowering phenotype was apparent is not clear.

Transcriptional profiling did not show an overwhelming representation of flowering-related genes that were different between wild type and *AtFAAH* overexpressors. However, it was noteworthy that *FLOWERING LOCUS T* (*FT*), which is a pivotal gene in the transition from vegetative growth to flowering (Corbesier et al., 2007; Giakountis and Coupland, 2008), was elevated in *AtFAAH* overexpressors. Furthermore, the expression of *SEPTELLA3* (*SEP3*) gene, which encodes a MADs box transcription factor that is regulated by FT in the photoperiodic flowering pathway (Teper-Bamnolker and Samach, 2005), also was elevated in *AtFAAH* overexpressors (**Table 1**; Table S1 in Supplementary Material). To verify results from microarray experiments, we conducted quantitative, real time RT-PCR of *FT* and *SEP3* transcripts using three independent *AtFAAH* overexpressing lines grown for 14 days in LD and SD conditions. Consistent with the microarray results, both LD- and SD-grown *AtFAAH* overexpressing seedlings showed elevated transcript levels of *FT* and *SEP3*. Under SD conditions, *FT* and *SEP3* in three *AtFAAH* overexpressors were threefold higher relative to wild type (**Figure 4A**). A similar trend was observed under LD conditions except for one *AtFAAH* overexpressor (line OE7), which was only twofold elevated relative to wild type (**Figure 4B**).

FT and *SEP3* were the only upregulated genes in *AtFAAH* overexpressors that had a known association with flowering (**Table 1**). To determine whether other genes differentially regulated by *AtFAAH* overexpression are related to flowering, we compared *AtFAAH* overexpression transcript data (Table S1 in Supplementary Material) with data from global expression analysis of the shoot apical meristem upon photoperiodic induction (i.e., transfer to LDs; Schmid et al., 2003). This study uncovered a large

FIGURE 3 | *AtFAAH* overexpression triggers a more dramatic change in gene expression than *AtFAAH* knockouts. (A) Heat map of gene expression in wild type, *AtFAAH* knockout (SALK_095108) and *AtFAAH* overexpressor (OE11). **(B)** Venn diagrams showing the number of genes differentially regulated in *AtFAAH* knockouts and *AtFAAH* overexpressors relative to wild type.

number of floral repressors that were downregulated upon floral photoperiodic induction. Interestingly, four genes namely *At3g16460*, *At3g27200*, *At5g43270*, and *At3g20370*, which were downregulated in *AtFAAH* overexpressors (Table S1 in Supplementary Material), were downregulated upon floral induction by photoperiod and therefore suggested to be potential flowering repressors (Schmid et al., 2003). One gene, namely *At3g28500*, was upregulated in *AtFAAH* overexpressors (**Table 1**) and by

photoperiodic flower induction (Schmid et al., 2003). Interestingly *in silico* analysis using the Genevestigator database[1,2] (Zimmermann et al., 2004) and the *Arabidopsis* eFP Browser[3] (Winter et al., 2007), indicated that *At3g28500*, which encodes

[1]http://www.plantphysiol.org/content/155/3/1237.full – ref-44
[2]https://www.genevestigator.ethz.ch/
[3]http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi

Table 1 | Genes induced twofold or more in *AtFAAH* overexpressors compared to wild type.

Locus ID	Predicted function	Ratio *AtFAAH* OE/WT
At3g28320	Protein of unknown function	17.18
At3g28310	Protein of unknown function	7.46
At1g20490	AMP-dependent synthetase and ligase family protein	6.12
At5g44420	Encodes an ethylene- and jasmonate-responsive plant defensin	4.50
At5g66970	P-loop containing nucleoside triphosphate hydrolases superfamily protein	4.14
At1g02300	Cysteine proteinases superfamily protein	4.03
At1g26380	FAD-binding Berberine family protein	3.74
At2g45220	Plant invertase/pectin methylesterase inhibitor superfamily	3.58
At1g13470	Protein of unknown function	3.53
At4g37990	Encodes an aromatic alcohol: NADP+ oxidoreductase	3.51
At5g64510	Unknown protein	3.49
At1g61800	Glucose-6-phosphate/phosphate transporter 2	3.49
At3g47090	Leucine-rich repeat protein kinase family protein	3.47
At1g15520	ABC transporter family involved in ABA transport and resistance to lead	3.30
At1g73260	Encodes a trypsin inhibitor involved in modulating programmed cell death in plant–pathogen interactions	3.20
At3g22600	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein	3.17
At1g26530	PIN domain-like family protein	3.11
At1g23850	Unknown protein	3.03
At1g09420	Encodes a protein similar to glucose-6-phosphate dehydrogenase	3.03
At5g64050	Glutamate-tRNA ligase. Targeted to mitochondria and chloroplast	3.02
At5g55570	Unknown protein	2.82
At5g45840	Leucine-rich repeat protein kinase family protein	2.82
At1g12080	Vacuolar calcium-binding protein-related	2.79
At1g23120	Polyketide cyclase/dehydrase and lipid transport superfamily protein	2.77
At1g08430	Encodes a Al-activated malate efflux transporter	2.76
At5g45040	Cytochrome *c*; functions in: electron carrier activity, iron ion binding, heme binding	2.75
At5g58575	Sgf11, transcriptional regulation	2.71
At5g45280	Pectin acetylesterase family protein; functions in: carboxylesterase activity	2.65
At4g11650	Osmotin-like protein	2.48
At1g10920	Encodes LOV1, a disease susceptibility gene	2.44
At1g71990	Lewis-type alpha 1,4-fucosyltransferase	2.42
At1g22550	Major facilitator superfamily protein	2.41
At1g17860	Kunitz family trypsin and protease inhibitor protein	2.40
At5g64440	AtFAAH (fatty acid amide hydrolase) modulates endogenous NAEs (*N*-acylethanolamines) levels in plants	2.40
At1g22440	Zinc-binding alcohol dehydrogenase family protein	2.38
At1g24260	Member of the MADs box transcription factor family. SEP3 is redundant with SEP1 and 2	2.33
At1g11080	Serine carboxypeptidase-like 31 (scpl31)	2.31
At3g60980	Tetratricopeptide repeat (TPR)-like superfamily protein	2.30
At3g46900	encodes a member of copper transporter family	2.29
At5g58120	Disease resistance protein (TIR–NBS–LRR class) family	2.28
At4g15210	cytosolic beta-amylase expressed in rosette leaves and inducible by sugar	2.26
At5g43580	Predicted to encode a PR (pathogenesis-related) peptide that belongs to the PR-6 proteinase inhibitor family	2.25
At1g01670	RING/U-box superfamily protein; functions in: ubiquitin-protein ligase activity	2.25
At5g01180	Encodes a dipeptide transporter expressed in pollen and ovules during early seed development	2.22
At2g24960	Unknown protein	2.19
At5g58310	Encodes a protein shown to have methyl IAA esterase activity *in vitro*	2.18
At5g45310	Unknown protein	2.18
At3g48920	Member of the R2R3 factor gene family	2.12
At3g28500	60S acidic ribosomal protein family	2.11
At3g43430	RING/U-box superfamily protein	2.09
At1g72030	Acyl-CoA *N*-acyltransferases (NAT) superfamily protein	2.08
At5g64640	Plant invertase/pectin methylesterase inhibitor superfamily	2.06
At1g65480	FT, together with LFY, promotes flowering	2.02

FIGURE 4 | Relative expression profiles of *FT*, *SEP3*, and *AtFAAH* in three *AtFAAH* overexpressors and two *AtFAAH* knockouts (SALK_118043 and SALK_095108) determined by quantitative RT-PCR. Fourteen day-old plants grown under short days **(A)** and long days **(B)**. Values plotted were normalized to 18S rRNA and are the mean of four biological replicates ± SE.

FIGURE 5 | Comparison of endogenous NAE profiles in 14-day-old wild type and AtFAAH overexpressors. NAEs were quantified by isotope-dilution mass spectrometry and summed for total NAE content **(A)** and total NAE 12:0 and NAE 18:2 **(B)**. Values represent the mean ± SE of three individual extractions from 14-day-old seedlings grown under short days. Mean ± SE with different letters are significantly different ($p < 0.005$; Tukey's test).

a 60S acidic ribosomal protein, was preferentially expressed in all stages of flower development as well as the inflorescence shoot apex (**Figure A1** in Appendix). Hence there may be additional clues yet to mine related to the molecular regulation of floral transition in the microarray data from *AtFAAH* overexpressors.

ENDOGENOUS NAE LEVELS ARE LOWER IN *AtFAAH* OVEREXPRESSORS

To determine if increased NAE hydrolytic activity could explain the early flowering phenotype in *AtFAAH* overexpressors, we quantified endogenous NAE levels of 14-day-old wild type and three *AtFAAH* overexpressor lines grown under SDs. Consistent with the overexpression of *FAAH*, total endogenous NAE content in the three *AtFAAH* overexpressors was less than that of wild type (**Figure 5A**). We then examined the levels of the different NAE

Table 2 | *N*-acylethanolamines profiles of 14-day-old *Arabidopsis* seedlings grown under short days.

Background (ng/g fr. weight)	Wild type	*AtFAAHOE2*	*AtFAAHOE7*	*AtFAAHOE11*
NAE 12:0	56.1 ± 6.6	35.4 ± 3.7	38.7 ± 6.6	42.8 ± 6.7
NAE 14:0	18.6 ± 4.6	22.5 ± 3.9	22.8 ± 4.0	18.3 ± 3.8
NAE 16:0	20.3 ± 6.7	18.6 ± 4.0	21.6 ± 4.8	19.1 ± 4.0
NAE 18:0	20.2 ± 3.7	25.0 ± 5.5	23.1 ± 4.9	24.1 ± 5.5
NAE 18:1	19.0 ± 1.8	26.3 ± 3.6	24.2 ± 3.7	23.8 ± 5.8
NAE 18:2	32.5 ± 4.0	25.3 ± 6.9	22.0 ± 5.1	22.8 ± 5.2
NAE 18:3	15.8 ± 2.3	13.8 ± 2.3	13.3 ± 2.6	12.6 ± 1.8

Values are means ± SE of three biological replicates.

species to determine which NAE type contributed most to the decline in total NAE levels in *AtFAAH* overexpressors. We found that the levels of NAE 14:0, NAE 16:0, NAE 18:0, NAE 18:1, and NAE 18:3 were similar between wild type and the three *AtFAAH* overexpressors (**Table 2**). On the other hand, endogenous NAE 12:0 and NAE 18:2 were significantly less (up to a 30% reduction) in *AtFAAH* overexpressors compared to wild type (**Table 2; Figure 5B**), suggesting that these two NAE types may play a role as lipid mediators in the regulation of flowering time.

EXOGENOUS NAE 12:0 DELAYS THE ONSET OF FLOWERING IN WILD TYPE *ARABIDOPSIS* PLANTS

Given that NAE 12:0 is about 30% reduced in *AtFAAH* overexpressors, we asked if exogenous application of NAE 12:0 delays flowering time in wild type *Arabidopsis* plants. Seven days after planting directly onto soil, 50 mL of 35 μM NAE 12:0 was applied directly to the base of the plants every 3 days until senescence. We found that at 24 days, rosette leaf number in NAE 12:0-treated wild type plants were slightly less than solvent controls (**Figure 6A**). NAE-treated plants showed a delay in flowering by at least 6 days and this occurred whether the treatment began at day 7 or on day 20 (few days prior to flowering under these conditions). We also quantified inflorescence stem height when NAE 12:0 was applied beginning at 7 days or beginning at 20 days. We found that plants treated with exogenous NAE 12:0 regardless of the age of the plant, had shorter inflorescence stems compared to plants treated with the solvent control solution (**Figures 6B,C**). Hence, it appeared that application of NAE to plants, either early or late, had two general effects – one, a reduction in growth and two, a delay in flowering. The similar impact on both flowering and inflorescence elongation between early (7 day) and late (20 day) treatments suggests that these impacts perhaps result from a signal transduction-mediated transition event and not simply a delay in development.

We also treated 7-day-old seedlings with 50 μM NAE 12:0 and examined the expression of *FT* and *SEP3* by quantitative, real time RT-PCR. Consistent with the delayed flowering time of NAE 12:0-treated plants and elevated expression of *FT* in *AtFAAH* overexpressors, we found that exogenous NAE 12:0 reduced *FT* expression by about threefold. *SEP3* expression on the other hand was reduced by only 1.3-fold (**Figure A2** in Appendix).

DISCUSSION

Although early flowering in *AtFAAH* overexpressors was noted previously (Wang et al., 2006), it was unclear how the development

of this phenotype is triggered. In this paper, we showed that early flowering of *AtFAAH* overexpressors was associated with increased expression of the key flowering integrator gene *FT*, under both inductive LD and non-inductive SDs. These results are not entirely surprising, given that FT is well established to be a potent promoter of the transition to flowering by coordinating the expression of a complex network of genes within the shoot apex (for review see Giakountis and Coupland, 2008). Some of the genes in the *FT* pathway act as repressors of *FT* transcription during non-inductive SDs (Gómez-Mena et al., 2001; Piñeiro et al., 2003; Takada and Goto, 2003) or are transcriptionally activated by FT during inductive LDs (Ruiz-Garcia et al., 1997; Teper-Bamnolker and Samach, 2005). However, despite the elevated expression of *FT*, many of the genes that have been associated with FT function did not change dramatically in *AtFAAH* overexpressors (Table S1 in Supplementary Material). Only the floral organ identity gene, *SEP3*, was elevated in *AtFAAH* overexpressors by twofold or more, which is consistent with a previous report showing that *FT* regulates *SEP3* accumulation (Teper-Bamnolker and Samach, 2005). These observations suggest that FT might have other downstream target genes that promote flowering or unknown repressors that regulate *FT* transcription in the *AtFAAH* overexpression background. One possible target of FT is the 60S acidic ribosomal protein encoded by the *At3g28500* gene, which was upregulated twofold in our microarrays (**Table 1**) and also reported to be induced in LDs in an FT-dependent manner (Schmid et al., 2003). Moreover, the *At3g28500* gene was upregulated fourfold when *HEME ACTIVATOR PROTEIN 3b* (*HAP3b*), which encodes a CCAAT-binding transcription factor, is overexpressed and down-regulated more than twofold in *hap3b* mutants. *HAP3b* overexpressors display early flowering whereas *hap3b* mutants are late flowering under LDs (Cai et al., 2007) suggesting that *At3g28500* is an important component of flowering under LDs. Although the significance of overexpressing a *60S acidic ribosomal protein* gene for promoting flowering is unknown, it is tempting to speculate that the translation of select mRNA in the cell particularly those involved in flowering might be affected, and thus partly explain early flowering in *AtFAAH* overexpressors.

It should be noted that the early flowering phenotype exhibited by *AtFAAH* overexpressors is not typical of other early flowering plants in *Arabidopsis*. For example, early flowering in *FT* over-expressors under SDs is typically accompanied by smaller rosette leaves and a reduced number of rosette leaves (Kardailsky et al., 1999; Kobayashi et al., 1999; Abe et al., 2005). A reduced number
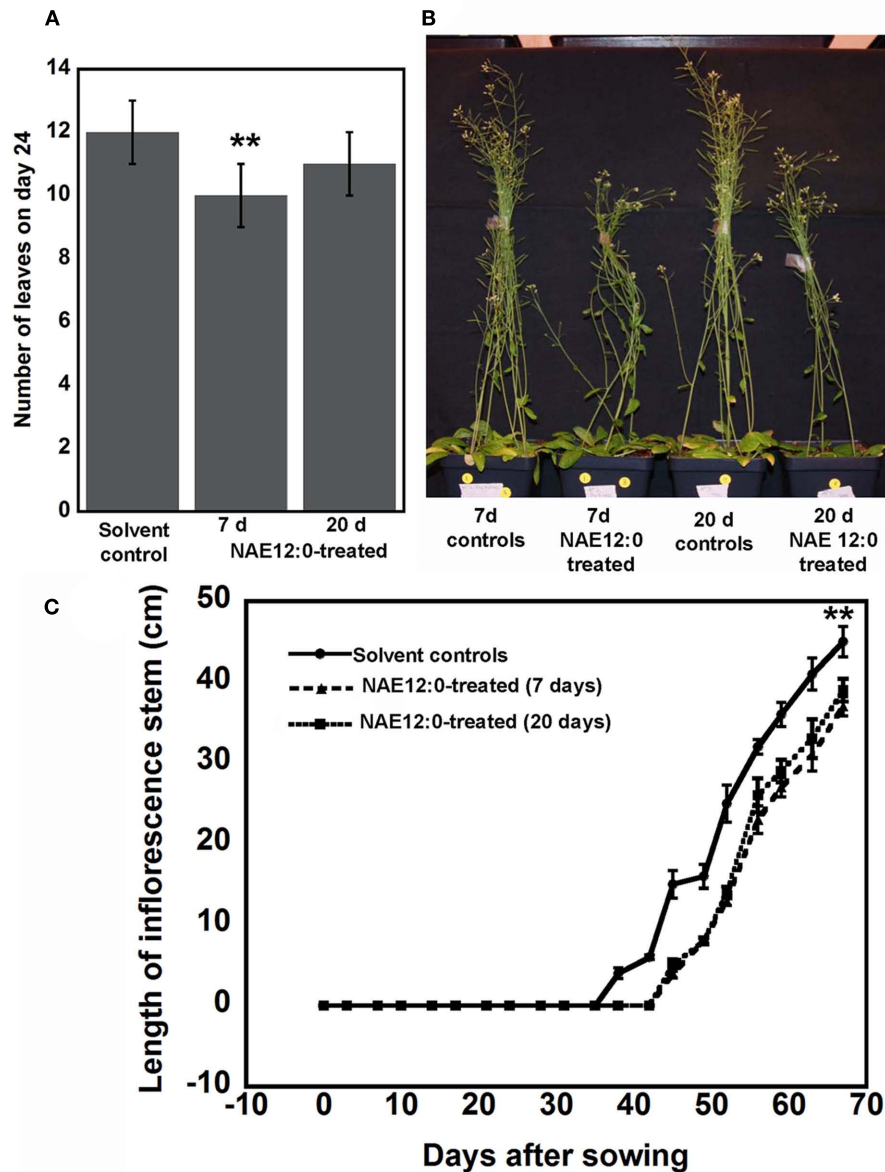
FIGURE 6 | Exogenous NAE 12:0 application delays flowering time in wild type plants. (A) Number of leaves of in 24-day-old plants treated with solvent control solution of 35 μM NAE 12:0 at 7 or 20 days after planting. **(B)** Representative images of wild type *Arabidopsis* plants exposed continuously to NAE 12:0 beginning at 7 or 20 days after planting. Note the shorter inflorescence stems of NAE 12:0-treated plants. **(C)** Quantification of the length of the inflorescence stem after the initiation of flowering. Note that flowering in NAE 12:0-treated plants was delayed by about 6 days regardless of whether the plant was treated at 7 or 20 days after planting. Asterisks indicate statistically significant difference according to Student's *t*-test ($p < 0.01$).

of rosette leaves is also a characteristic found in several flowering mutants that enhance the expression of *FT* (Piñeiro et al., 2003; Yoo et al., 2005; Cai et al., 2007; Seo et al., 2011), and reflects the shorter period of vegetative growth. The number of leaves of *AtFAAH* overexpressors and wild type at 21 and 28 days, under SD conditions, were the same, indicating that vegetative development was not accelerated. However, by 28 days most *AtFAAH* overexpressors had transitioned to flowering with 9–10 rosette leaves, while most wild type plants were vegetative and still producing leaves. *AtFAAH* overexpressors therefore behaved more like

plants grown under LD conditions, which, irrespective of genotype, produced seven to nine leaves before flowering (**Figure 2**). These data indicate that the early flowering in *AtFAAH* overexpressors is independent of the rate of rosette leaf production and is not a product of enhanced growth, but results from uncoupling flowering from photoperiod, presumably via FT activation. In addition to *FT*, overexpression of other genes in the flowering pathway uncouple flowering from photoperiod including CONSTANS (CO), a transcription factor that activates FT (Takada and Goto, 2003), and the downstream meristem identity genes *LEAFY*

(*LFY*; Schultz and Haughn, 1991) and *APETALA1* (*AP1*; Mandel et al., 1992). If *AtFAAH* overexpression bypasses photoperiodic induction by elevating *FT* directly, elevated *LFY*, and *AP1* would be expected. However, these genes did not change significantly in microarray studies of *AtFAAH* overexpressors.

Recent work in tomato (Shalit et al., 2009) and maize (Danilevskaya et al., 2010), define *FT* and its orthologs as general determinacy factors that function to limit vegetative growth – with smaller leaves and the transition to reproductive growth being only two manifestations of the broader impacts. However, the reduced leaf size that commonly accompanies *FT* overexpression was not observed in *AtFAAH* overexpressors. It is possible that elevated *AtFAAH*, which previously was shown to enhanced vegetative growth in *Arabidopsis* (Wang et al., 2006), can overcome the negative effects of *FT* overexpression on leaf development by reducing endogenous levels of NAE (**Figure 5**). Consistent with this notion is the finding that exogenous application of NAE 12:0 to wild type plants, which was more than 30% less in *AtFAAH* overexpressors than wild type, delayed flowering in wild type plants (**Figure 6**).

A global expression study of *Arabidopsis* shoot apices uncovered a large number of potential floral repressors that were downregulated upon photoperiodic induction compared to upregulated genes (Schmid et al., 2003). A similar trend was observed in *AtFAAH* overexpressors in that there were more genes downregulated than upregulated (**Figure 3**; **Table 1**). Interestingly, four of the potential floral repressors identified by Schmid et al. (2003) were also downregulated in *AtFAAH* overexpressors including genes encoding *mannose-binding lectin superfamily protein* (*At3g16460*), *cupredoxin superfamily protein* (*At3g27200*), *squamosa-promoter binding protein-like* (*SPL*; *At5g43270*), and *TRAF-like family protein* (*At3g20370*). Although members of the *SPL* gene family are known to regulate floral transition through microRNAs (miRNAs) and FT activation (Wang et al., 2009), the significance of the downregulation of the aforementioned four genes for the early flowering phenotype of *AtFAAH* overexpressors is not clear. Activation of *FT* expression also is well known to be regulated by the B-box zinc finger transcription factor CO, particularly under LD conditions (An et al., 2004; Ayre and Turgeon, 2004). Expression of *CO* was not significantly changed in *AtFAAH* overexpressors but a gene homologous to *CO* (At3g02380) was twofold downregulated (Table S1 in Supplementary Material). Because it is the timing of *CO* expression and CO protein stability that is crucial for photoperiodic flower induction (Imaizumi, 2010), we cannot rule out the possibility that microarray analysis of *AtFAAH* overexpressors was not able to detect significant changes in *CO* expression given that RNA from only one time point was collected. Alternatively, there are reports that induction of *FT* expression and flowering could be facilitated by CO-independent pathways. For example, the miRNA172 pathway was shown to induce *FT* expression and flowering in *Arabidopsis* despite the absence of functional CO (Jung et al., 2007). The depletion of endogenous NAEs by *AtFAAH* overexpression could bypass the requirement for CO to trigger *FT* expression through yet to be characterized CO-independent pathways.

N-lauroylethanolamine (NAE 12:0) elicits a number of growth inhibitory effects when applied to *Arabidopsis* seedlings (Blancaflor et al., 2003; Motes et al., 2005). This NAE type is comparatively low in concentration in desiccated seeds, but is among the most prevalent NAE type in seedling and leaf tissues (Wang et al., 2006; **Figure 5**). Seed germination and post-germinative growth is characterized by the targeted depletion of the predominant polyunsaturated NAEs such that the NAE composition and content of vegetative tissues differs substantially from that of seeds (Kim et al., 2010). While there is much to be learned about the relationship of individual NAE species with physiological function in plants, this manuscript provides new information about NAE 12:0, namely the delay in flowering time in *Arabidopsis*. Plants watered with NAE 12:0-containing solutions ($35 \mu M$) were delayed in flowering (**Figure 6**). This delay was observed whether the exogenous application of NAE 12:0 began at 7 or 20 days after sowing indicating that the timing (prior to flowering) but not the length of exposure to NAE 12:0 was required to delay their transition to flowering. Perhaps more significantly, the endogenous level of NAE 12:0 was the NAE type that was most altered in the *AtFAAH* overexpressing lines, suggesting that the specific metabolism of NAE 12:0 by *AtFAAH* is related to flowering transition time. Response to application of NAE 12:0 solutions to the soil implies that NAE 12:0 is likely taken up through the roots and translocated to the shoots to where it exerts its effect. It is tempting to speculate that FT may be a direct target of NAE 12:0, since FT has homology to known phosphatidylethanolamine binding proteins (PEBP). To our knowledge there have been no studies to examine the interaction of acylethanolamines with FT or FT-related proteins. In fact the annotation of FT as a member of the PEBP family comes from sequence homology rather than from functional binding data. Certainly future experiments to probe the role of NAE signaling in flowering time via FT should include assays of direct interaction between FT and NAE 12:0 and other ethanolamine-containing lipids. Whether the action of NAE 12:0 on FT is direct or indirect, the data presented here provide the first indication that NAE metabolism may play a role in the signal transduction events that lead to FT-mediated transition from vegetative to reproductive growth in *Arabidopsis*. It remains to be tested whether or not the NAE pathway represents a bypass of other floral transition mechanisms such as CO.

AUTHOR CONTRIBUTIONS

N. D. Teaster performed flowering assays, quantitative RT-PCR and assisted in gathering plant material for NAE quantification. J. Keereetaweep conducted the NAE quantification assays. Y.-S. Wang and Y. Tang conducted the microarray studies and A. Kilaru and C. N.-Q. Tran performed the external NAE experiments on flowering time. N. D. Teaster wrote the paper and analyzed data with the assistance of B. G. Ayre, K. D. Chapman, and E. B. Blancaflor.

fellowship from the Texas Academy of Math and Sciences, University of North Texas.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Plant_Physiology/10.3389/fpls.2012.00032/abstract

Table S1 | Differentially expressed genes in *AtFAAH* overexpressor and *AtFAAH* knockouts compared to wild type. These data were deposited with the EMBL–EBI ArrayExpress database under accession E-MEXP-3486.

Table S2 | Genes downregulated twofold or more in both *AtFAAH* overexpressors and *AtFAAH* knockouts.

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APPENDIX

FIGURE A1 | *At3g28500* **is strongly expressed in flowers and inflorescence meristem (arrows).** *In silico* expression profiling of *At3g28500* based on publicly available microarray data sets from Genevestigator [**(A)**, Zimmermann et al., 2004] and *Arabidopsis* eFP browser [**(B)**, Winter et al., 2007].

FIGURE A2 | **Relative expression of** *FT* **and** *SEP3* **of 7-day-old** *Arabidopsis* **seedlings treated with 50 μM NAE 12:0 for 24 h as determined by quantitative RT-PCR.**