INTERACTIONS BETWEEN *N*-ACYLETHANOLAMINE METABOLISM AND ABSCISIC ACID SIGNALING IN *Arabidopsis thaliana* SEEDLINGS

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N-Acylethanolamines (NAEs) are endogenous plant lipids hydrolyzed by fatty acid amide hydrolase (FAAH). When wildtype *Arabidopsis thaliana* seeds were germinated and grown in exogenous NAE 12:0 (35 μM and above), growth was severely reduced in a concentration dependent manner. Wildtype *A. thaliana* seeds sown on exogenous abscisic acid (ABA) exhibited similar growth reduction to that seen with NAE treatment. AtFAAH knockouts grew and developed similarly to WT, but AtFAAH overexpressor lines show markedly enhanced sensitivity to ABA. When low levels of NAE and ABA, which alone have very little effect on growth, were combined, there was a dramatic reduction in seedling growth in all three genotypes, indicating a synergistic interaction between ABA and NAE. Notably, this synergistic arrest of seedling growth was partially reversed in the ABA insensitive mutant *abi3-1*, indicating that a functional ABA signaling pathway is required for the full synergistic effect. This synergistic growth arrest results in an increased accumulation of NAEs, but no concomitant increase in ABA levels. The combined NAE and ABA treatment induced a dose-dependent increase in *ABI3* transcript levels, which was inversely related to growth. The ABA responsive genes *AtHVA22B* and *RD29B* also had increased expression in both NAE and ABA treatment. The *abi3-1* mutant showed no expression of *ABI3* and *AtHVA22B*, but *RD29B* expression remained similar to wildtype seedlings, suggesting an alternate mechanism for NAE and ABA interaction. Taken together, these data suggest that NAE metabolism acts through ABI3-dependent and independent pathways in the negative regulation of seedling development.

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LIST OF ABBREVIATIONS

ABA	Abscisic acid
ABI	ABA insensitve
ABRC	Arabidopsis Biological Resource Center
ATEM1	*Arabidopsis thaliana* late embryogenesis abundant 1
AtFAAH	*Arabidopsis thaliana* fatty acid amide hydrolase
AtHVA22B	*Arabidopsis thaliana* HVA22 homologue B
BSA	Bovine serum albumin
BSTFA	Bis(trimethylsilyl)trifluoro-acetamide
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
faah	FAAH knockout
FFA	Free fatty acid
FW	Fresh weight
GC/MS	Gas chromatography/mass spectrometry
HPLC	High performance liquid chromatography
MAPK	Mitogen-activated protein kinase
MS	Murashige and Skoog
NAE	*N*-acylethanolamine
NAE 12:0	*N*-lauroylethanolamine
NAE 14:0	*N*-myristoylethanolamine
NAE 16:0	*N*-palmitoylethanolamine
NAE 18:0	*N*-stearoylethanolamine
NAE 18:1	*N*-oleoylethanolamine
NAE 18:2	*N*-linoleoylethanolamine
NAE 18:3	*N*-linolenoylethanolamine
OE	*35S::AtFAAH* overexpressor
PNP-A	Plant natriuretic peptide-A
RD29B	Responsive to dessication 29B
RT-PCR	Reverse transcriptase-polymerase chain reaction
SYBR	Synergy Brands, Inc.
TLC	Thin layer chromatography
WT	Wild type
WT (Col)	Wild type, Columbia ecotype
WT (L*er*)	Wild type, Landsberg *erecta* ecotype

CHAPTER 1

INTRODUCTION

N-Acylethanolamines (NAEs) are bioactive lipids thought to play a role in seedling development (Chapman, 2004; Kilaru et al., 2007). The highest NAE levels are found in desiccated seeds and are, in part, hydrolyzed during germination by fatty acid amide hydrolase (FAAH) (Shrestha et al., 2002; Shrestha et al., 2003). This is thought to allow for synchronized cell expansion and growth in germinating seeds and young seedlings (Blancaflor et al., 2003). When exogenous NAE is added to the seedlings, primary root length is significantly reduced and the roots exhibit swollen tips, indicative of a perturbation in cell expansion (Blancaflor et al., 2003; Motes et al., 2005). NAE metabolism mutants have been developed to study the effects of NAE on seedling growth. When *AtFAAH* is overexpressed, seeds are theoretically able to remove NAEs more rapidly and the seedlings grow more rapidly (Wang et al., 2006) and can grow in exogenous NAE (Wang et al., 2006; Teaster et al., 2007). When *AtFAAH* is not expressed (by T-DNA disruption), seeds cannot hydrolyze exogenous NAE as rapidly, and seedling growth is severely inhibited (Wang et al., 2006). It is clear from these studies that *FAAH* expression influences NAE metabolism in Arabidopsis seedlings.

The reduction in early seedling growth mediated by exogenous NAE is similar to that caused by the plant hormone abscisic acid (ABA) (Teaster et al., 2007). ABA functions as a repressor of germination and aids in modulating seedling growth (Finkelstein et al., 2002; Lopez-Molina et al., 2002; Cutler et al., 2009). Activation of the ABA signaling pathway leads to either immediate cellular changes such as the release of intracellular calcium, nitric oxide, sphingolipids or increases in reactive oxygen

species (Coursol et al., 2003; Bright et al., 2006; Chai et al., 2006) or to changes in gene expression (Rock, 2000; Finkelstein et al., 2002; Cutler et al., 2009). Addition of ABA to germinated seeds inhibits seedling growth, leading to reduced root length and smaller overall seedling size (Finkelstein et al., 2002). Under normal seed germination and postgerminative seedling growth, levels of ABA decrease (Finkelstein et al., 2002), in a pattern similar to the reduction in NAE levels (Teaster et al., 2007). The growth responses of seedlings toward ABA are similar to those toward NAEs, and the levels of both of these negative growth regulators are reduced during the course of normal seedling growth. It is therefore possible that NAE metabolism may interact with ABA signaling to produce changes in seedling growth.

Prior work with seedlings grown on solid media has supported the hypothesis that there is an interaction between NAE metabolism and ABA signaling (Motes et al., 2006; Teaster et al., 2007). Low levels of *N*-lauroylethanolamine (NAE 12:0) (10 μM) and low levels of ABA (0.1 μM) alone have little inhibitory effect on wildtype seedling growth (Fig. 1B, 1C). However, the combination of NAE 12:0 and ABA substantially reduces seedling growth (Fig. 1D, 1E), suggesting that the two compounds act together synergistically to reduce seedling growth. It is possible that ABA is operating to modulate NAE metabolism to either produce more NAE or reduce FAAH activity. Alternatively, NAE may interact with the ABA signaling pathway to potentiate its action. Additionally, this synergistic inhibition of growth is enhanced in seedlings overexpressing *AtFAAH* (OE2), and diminished in the *AtFAAH* knockout line (Salk 095108) (Fig. 1B-E). These effects are similar to those seen in seedlings grown in liquid media (Teaster et al., 2007). In liquid media (1/2 strength Murashige and Skoog

(MS)), wild-type seedlings grown separately in 0.1 μM ABA and 10 μM NAE 12:0 have slightly shortened roots and smaller cotyledons. However, when the ABA and NAE are added together, seedling growth is severely reduced, similar to observations on solid media. Also, the *FAAH* overexpressor shows the same heightened sensitivity to ABA in liquid media as it does in solid media. While the use of solid media provides a simple method for quantifying different growth parameters, it is difficult to produce enough tissue for further analysis, so the similarities in growth response under both conditions will support examination of gene expression, enzyme activity and metabolite changes.

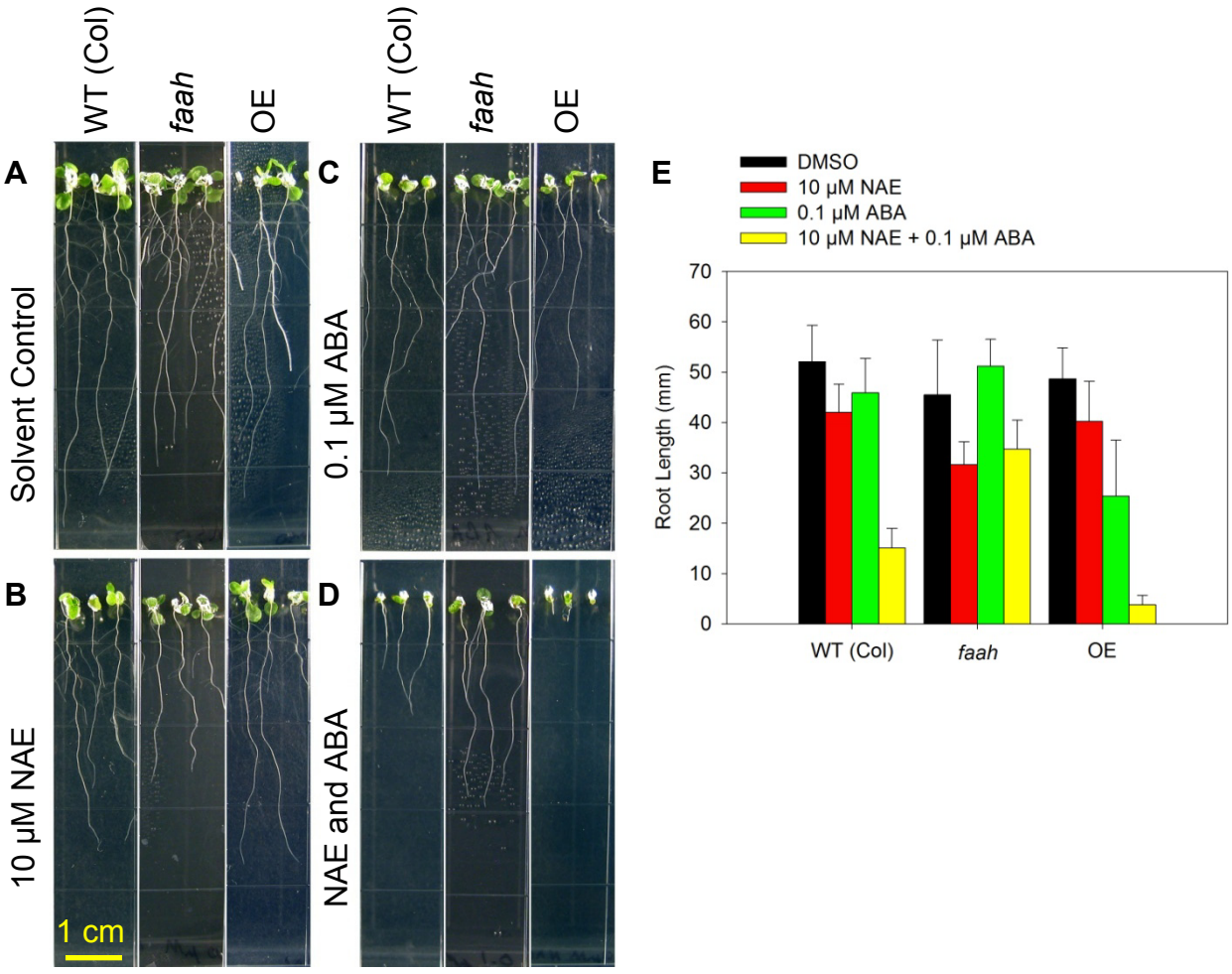

Figure 1: Synergistic effects of ABA and NAE on seedling growth.

(A), (B), (C) and (D) 10 day old wild type, *FAAH* T-DNA insertion mutant (Salk 095108) and *FAAH* overexpressor (OE2) seedlings grown in solidified (0.5X MS) media with (A) solvent only (0.025% DMSO), (B) 10 µM NAE 12:0, (C) 0.1 µM (+)ABA and (D) both 10 µM NAE and 0.1 µM (+)ABA.

(E) Primary root lengths of 10 day old seedlings grown in indicated treatment. Measurements are mean and standard deviation (n>30) from seedlings grown together.

There are several possible mechanisms by which NAEs may interact with ABA. These mechanisms are summarized in Figure 2 (Teaster et al., 2007). The first is that added NAEs may increase endogenous ABA levels, or added ABA may increase endogenous NAE levels. Previous work has shown that exogenous application of ABA has no appreciable effect on overall NAE content in wild type plants, although the composition of the NAE pool is slightly altered by ABA in that unsaturated NAE species are slightly higher (Teaster et al., 2007). In addition, treatment with exogenous NAE 12:0 has no effect on overall ABA levels in wild type plants (Teaster et al., 2007). Because the NAE profile changes when seedlings are grown in ABA, the contributions of the interaction between ABA levels and NAE metabolism to the synergistic effect must be further characterized.

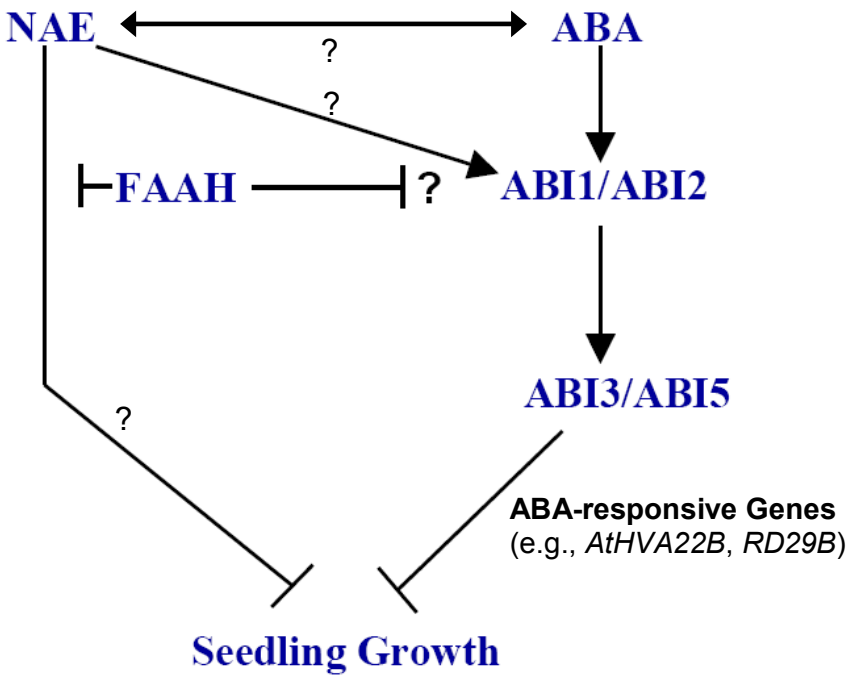

Figure 2: Proposed interactions between ABA signaling and NAE metabolism. NAE metabolism is shown on the left and ABA signaling is shown on the right (modified from Teaster, et.al., 2007).

A second potential mechanism for NAE and ABA interaction is that ABA may influence NAE metabolism, changing rates of NAE degradation and/or synthesis. NAEs are formed from *N*-acylphosphatidylethanolamines (NAPEs) by phospholipase D and are removed by both a FAAH pathway to produce free fatty acids and a lipoxygenase pathway, producing oxylipins (Chapman, 2004). It is possible that ABA modulates one of these pathways to change the NAE levels. As indicated in Figure 1, the use of *FAAH* knockouts and overexpressors indicate that adjusting FAAH levels can have a large impact on the synergistic effect, suggesting that this is a possible mechanism for the synergism. ABA may change the activity of FAAH, causing changes in levels of specific NAE species and NAE profiles, without changing overall levels of NAE. Wild type seedlings have been shown to have significantly higher levels of unsaturated NAEs than saturated NAEs (Teaster et al., 2007). There is also some evidence that FAAH interacts with ABA signaling independent of FAAH amidohydrolase activity (Kim et al., 2009). Seedlings with an overexpressed catalytically inactive FAAH still demonstrate hypersensitivity to exogenous ABA. This would suggest that ABA signaling can interact with the FAAH protein itself, without altering NAE levels. The effects of NAE and ABA on the FAAH mediated removal of NAEs can be tested using the available *FAAH* knockouts and overexpressors.

Finally, NAE or FAAH may interact with ABA through modifications of the ABA signaling pathway. NAE may promote the ABA signal cascade that leads to changes in expression of ABA responsive genes. There is evidence that adding NAE promotes the ABA signaling pathway and that mutations in ABA signaling genes are partially tolerant to NAE treatment (Teaster et al., 2007), indicating that NAE can activate the ABA

pathway without directly influencing ABA levels. However, it is not known how this interaction with the ABA signaling pathway occurs. NAEs may be directly interacting with the ABA signaling pathway, through the protein phosphatases ABI1 or 2, or the ABA-regulated transcription factors ABI3 or 5, it may be interacting with the ABA signaling secondary messengers such as Ca^{2+} or inositol triphosphate (Webb et al., 2001; Finkelstein et al., 2002), or it may be interacting with the ABA receptor to activate the signaling pathway (Melcher et al., 2009) or through interaction with PLD alpha and impact on phosphatidic acid (PA) levels, a known modulator of ABI signaling machinery (Mishra et al., 2006).

Research Objectives

The main purpose of this research was to investigate the potential mechanism or mechanisms for the interactions between NAE and ABA in *Arabidopsis* seeds and seedlings. To address this goal, three specific objectives were implemented:

1. Quantify the combined effects of NAE and ABA on seeds and seedling growth
2. Determine the effects of combined NAE and ABA treatments on FAAH activity, NAE levels, and ABA levels
3. Determine the effects of combined NAE and ABA on gene expression.

For the first objective, the effects of NAE and ABA, both separately and together, were examined in seedlings. While these treatments are known to cause decreased primary root elongation in 10 day old seedlings (Fig. 1), the effects had not been well characterized for other growth parameters, such as hypocotyl length and fresh weight. In addition, the effects of NAE and ABA on ABA signaling mutants were also studied. For the second objective, these effects were further characterized by looking at changes

in NAE and ABA levels. This was used to determine whether the growth effects are influenced by ABA through the modulation of NAE profiles by changing FAAH activity. For the third objective, the effects of NAE and ABA on ABA responsive genes, NAE responsive genes and genes that are not responsive to either was studied. This helped to determine if the interaction between NAE and ABA is occurring through a change in gene expression.

CHAPTER 2

MATERIALS AND METHODS

Plant Materials and Growth Assays

The ABI mutant *abi3-1* (CS24), *faah* T-DNA insertion mutant (Salk_095108), and their corresponding parental ecotypes Landsberg *erecta* (L*er*-0) and Columbia (Col) were obtained from the Arabidopsis Biological Resource Center (ABRC). In addition, *35S*::*AtFAAH* overexpressor lines were provided by Elison Blancaflor at the Samuel R. Noble Foundation (Ardmore, OK) (Wang et al., 2006). Plants were propagated in soil for seed production and grown in growth rooms at 22°C with a 16 h light (~50 $\mu mol \cdot m^{-2} \cdot s^{-1}$), 8 h dark cycle. For germination and growth experiments, seeds were surface-sterilized with 95% ethanol followed by 30% bleach with 0.1% Tween-20 for 3 minutes each and rinsed several times with sterile, deionized water. Seeds were stratified for 3 days at 4°C in the dark and grown in either liquid nutrient media (0.5X Murashige and Skoog salts containing 1% sucrose) or solid nutrient media (0.5X MS salts with 1% sucrose and 0.8% phytagel) as previously described (Wang et al., 2006). Germination and growth occurred in a controlled environment room with a 16 h light (~60 $\mu mol \cdot m^{-2} \cdot s^{-1}$), 8 h dark cycle at 20 to 22°C. Seedlings grown on plates were oriented ~30° from vertical to allow roots to grow into media and facilitate reproducible measurements of root elongation. Primary root length was measured at 10 days after imbibition for a minimum of 30 seedlings from images of plants on plates using ImageJ (available at http://rsbweb.nih.gov/ij/; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD). Cotyledon area was measured 10 days after imbibition by removing cotyledons from 30-40 plants, pressing them flat using a microscope slide, and

measuring area with ImageJ. Liquid cultured seedlings were incubated with shaking (~75 rpm) and growth was quantified 10 days after imbibition as fresh weight accumulation after collection with a Buchner funnel and rinsing three times with deionized water to remove exogenous NAE and ABA from the seedlings. ABA and/or NAEs were added from concentrated stocks, dissolved in DMSO, to the appropriate final concentrations after media was allowed to cool to 50°C. Untreated controls contained equivalent final concentrations of DMSO solvent alone, ranging from 0.01 to 0.05%. Concentrations of exogenous ABA (Calbiochem-Novabiochem Corp., catalogue #100111) were calculated based on the active *cis*-isomer, although the ABA used includes an equimolar concentration of the inactive *trans*-isomer. *N*-lauroylethanolamine (NAE 12:0) was synthesized by Dr. Kent Chapman according to (Devane et al., 1992). NAE 12:0 was analyzed by isotope-dilution mass spectrometry for purity and was found to have no contamination from other NAE species. NAE and ABA were both added to media at the beginning of the experiment and were not continuously replaced. To determine if the combined effect of NAE and ABA was additive, synergistic or antagonistic, the expected response from a combination of NAE and ABA was calculated (Colby, 1967). Growth parameters for each treatment were converted to percent of control values. The expected values for an additive response is calculated by the equation Expected % of control = (% of control in treatment A * % of control in treatment B) / 100. If the actual percent of control for the combined treatment is lower than the expected value, the combination is synergistic; if higher, the combination is antagonistic.

NAE Quantification

NAE was quantified by isotope-dilution mass spectrometry (Venables et al., 2005). NAEs were extracted from ~50-100 mg of plant tissue using a BioSpec Mini-Beadbeater (BioSpec, Catalogue #693) into 70°C 2-propanol to inactivate endogenous phospholipases (Chapman et al., 1999). For quantification, 50 µL of 1 ppm deuterated NAE (d4-NAE 16:0 and d4-NAE 20:1) internal standards were added. Total lipids were extracted into chloroform, filtered, and fractionated by normal-phase HPLC. Enriched NAE fractions were collected, dried under N_2 and derivatized in 50 µL of bis(trimethylsilyl)trifluoro-acetamide (BSTFA) at 55°C for 30 minutes. The derivatized samples were dried and taken up in 50 µL hexanes for analysis by GC/MS. Endogenous NAEs were quantified against the internal deuterated standards and calculated in $ng \cdot g^{-1}$ FW.

ABA Quantification

ABA was quantified by isotope-dilution mass spectrometry (Wang et al., 2001). ABA was extracted from ~300 mg of plant tissue using a Biospec Mini-Beadbeater (BioSpec, Catalogue #693) into 30 mM imidazole buffer (pH 7) in 70% 2-propanol. Deuterated D_2-ABA (100 ng) was added as a quantitative standard and the samples were extracted overnight at 4°C and centrifuged. The supernatant was combined with three consecutive extractions (1 ml 100% 2-propanol each) and reduced under N_2 in a dry bath at 70°C to ~1 ml. This aqueous phase was diluted and prepurified on NH_2-columns (Discovery, Catalogue #52637-U) primed with methanol, 5% acetic acid, and water. After sample application, the columns were washed with hexane and methanol and eluted with 3 ml 5% acetic acid in 75% methanol. The eluant was dried and taken

up in 36% methanol and purified by HPLC as described previously (Wang et al., 2001). The ABA fraction was collected, dried, methylated with 0.5 ml of ethereal diazomethane for 5 minutes, dried under N_2, and taken up in 50 µL of ethyl acetate. The methylated samples were analyzed by GC/MS. The relative abundance of representative fragments for ABA (*m/z* 190/194) was used for quantification. The data were calculated in $ng \cdot g^{-1}$ FW for seedlings and seeds.

FAAH Activity Assays

Total proteins were extracted from seeds by grinding ~150-200 mg of seeds with liquid nitrogen, mortar and pestle. Proteins were solubilized on ice in 4 ml of homogenization buffer (10 mM KCl, 1 mM EDTA, 1mM EGTA, 1 mM $MgCl_2$, 400 mM sucrose, and 100 mM potassium phosphate, at pH 7.2) and 0.4 mM DDM. Protein content was estimated using BSA as standard according to Bradford (Bradford, 1976). About 20 µg of protein extract was reacted with 250 µM [^{14}C]NAE 12:0 (20,000 dpm) in bis-tris propane buffer (pH 9.0) for 4 hours at 30°C, in the presence or absence of ABA. Lipids were extracted as previously described (Shrestha et al., 2002). Briefly, the reaction was stopped by incubating the reaction mixture with 70°C 2-propanol for 30 minutes. Lipids were extracted into chloroform overnight at 4°C. Phase separation was induced by adding 1 M KCl, and the aqueous layer was aspirated off. The organic layer was washed twice with 1 M KCl and once with ultrapure water (MilliQ-plus UF). The organic phase was collected and dried under N_2. Lipids were separated by TLC (hexane:ethyl acetate:methanol, 12:8:1; v/v). Identification and quantification of radiolabeled lipids were performed by radiometric scanning (Bioscan AR-2000 Imaging

Scanner). FAAH activities were calculated and reported as µmol substrate consumed per hour.

Quantitative Real Time RT-PCR

Total RNA was isolated from seed and seedling samples using the Qiagen RNeasy Plant Mini Kit (Qiagen, catalogue #74904). RNA was quantified and evaluated for purity by UV spectroscopy and agarose gel electrophoresis (Mehra, 1996). Quantification of mRNA transcripts by quantitative RT-PCR were performed with a Smart Cycler II (Cepheid) instrument using a real-time one-step assay system (Takara Bio) with SYBR Green 1 dye. For the following genes, the gene specific primer pairs given in Table 1 were used: *ABI3* (At3g24650), *AtHVA22B* (At5g62490), *PNP-A* (At2g18660), *AtFAAH* (At5g64440), *RD29B* (At5g62490), and *ATEM1* (At3g51810). All primers were designed to span one intron to distinguish cDNA amplification from genomic DNA contamination. Relative transcript levels in all samples were normalized using 18S rRNA as a constitutively expressed internal control, with intron-spanning primers (F) 5'-TCCTAGTAAGCGCGAGTCATCA-3' and (R) 5'-CGAACACTTCACCGGATCAT-3' (Rider et al., 2003). Quantitative RT-PCR reactions were performed in duplicate with 0.2 µg of total RNA and 0.5 µL of 10 µM gene-specific primers in each 25 µL reaction. The reaction mix was subjected to the following RT-PCR conditions: 42°C for 15 minutes, one cycle; 95°C for 2 minutes, one cycle; 94°C for 10 s, 58°C for 25 s (read cycle), 72°C for 20 s. The number of cycles and annealing temperature were experimentally determined for each set of gene-specific primers. RT-PCR products were examined by gel electrophoresis and by melting curve analysis (60 to 95°C at 0.2°C/s) to rule out anomalous amplification

products. The $2^{-\Delta\Delta CT}$ cycle threshold (C_T) method was used to calculate relative changes in transcript levels determined from quantitative real-time RT-PCR (Livak and Schmittgen, 2001). The data were analyzed using the equation $\Delta\Delta C_T=(C_{T,\ Target}-C_{T,\ 18S})_{Treated}-(C_{T,\ Target}-C_{T,\ 18S})_{Not\ Treated}$, where "Treated" refers to samples treated with ABA and/or NAE and "Not Treated" refers to samples treated with solvent alone. The fold difference between treated and not treated was then calculated using the equation $2^{-\Delta\Delta CT}$. These values were then normalized to the appropriate wild-type solvent control. The normalized values from replicate samples were then averaged together.

Table 1: Genes and corresponding primers for quantitative RT-PCR analysis.

Gene	Description	Primers
ABI3	ABA transcription factor	(F)5'-GAGCTGGCTCAGCTTCTGCTATG-3' (R) 5'-AGGCCAAAACCTGTAGCGCATGTTC-3'
AtHVA22B	ABA responsive gene	(F) 5'-CATCGCTGGACCTGCATTA C-3' (R) 5'-GGATATAATGGGATCCATTCGAGG-'3
PNP-A	Expansin-related gene	(F) 5'-CCTACACTAGGTCTGCGTG-3' (R) 5'-GATAACCCGAAAAGCGT-3'
AtFAAH	Fatty acid amide hydrolase	(F) 5'-CCATCTCAAGAACCGGAGCATG-3' (R) 5'-GGTGTTGGAGGCTTGTCATAGC-3'
RD29B	ABA responsive gene	(F) 5'-CATAAAGGTGGAGAAGCTGGAGTA-3' (R) 5'-CCTCCAAATCTTGCCGGAGAATTC-3'
ATEM1	ABA responsive gene	(F) 5'-CTGAAGGAAGAAGCAAGGGAG-3' (R) 5'-TCCATCGTACTGAGTCCTCCTTTAC-3'

CHAPTER 3

RESULTS

Characterization of Combined NAE and ABA Effects on Seedlings

To identify the effects of NAE 12:0 on seedling growth, growth studies of FAAH metabolism mutants and ABA signaling mutants were conducted with exogenously applied NAE 12:0 (Figure 3). In the absence of NAE 12:0, primary root length is similar for all genotypes (Figure 3A). At low levels (10 μM) of applied NAE (Figure 3B), there is no significant difference in primary root length between the FAAH metabolism mutants (*faah* and OE) and wild type (WT (Col)), nor is there a significant difference between an ABA signaling mutant (*abi3-1*) and WT (L*er*). At concentrations of NAE 12:0 above 15 μM, the *FAAH* knockout seedlings have severely reduced primary roots (Figure 3C, 3D, 3E) compared to wild type, while seedlings overexpressing *FAAH* are able to continue growing at least as well as WT (Col). At these higher concentrations, *abi3-1* has similar primary root lengths to WT (L*er*). The dose-dependent reduction in primary root length for all genotypes is shown in Figure 3F.

To identify the effects of ABA on seedling growth, growth studies of FAAH metabolism mutants and ABA signaling mutants were conducted with exogenously applied ABA (Figure 4). In the absence of ABA, primary root length is similar for all genotypes (Figure 4A), with *abi3-1* having slightly reduced primary roots. Upon treatment with ABA, *FAAH* OE demonstrates a hypersensitive reduction in primary root length, while *faah* demonstrates reduced sensitivity to ABA growth inhibition (Figure 4B, 4C). At high levels of ABA (Figure 4D), no genotype shows primary root elongation except *abi3-1*, which is able to produce roots that are ~75% as long as the untreated

roots. The dose-dependent reduction in primary root length for all genotypes is quantified in Figure 4E.

To identify other effects of NAE and ABA on seedlings, cotyledon area was measured in 14-day old seedlings (Figure 5). When treated with NAE 12:0, *faah* again showed a hypersensitive reduction cotyledon area and OE shows tolerance (Figure 5A). At 20 μM NAE, *faah* cotyledon area is significantly less ($p<0.001$) than WT (Col), while *FAAH* OE cotyledon area is significantly more ($p<0.001$) than WT. When treated with ABA, OE showed the hypersensitive reduction seen in primary root length while *faah* showed tolerance (Figure 5B). At 0.25 μM ABA, *faah* had significantly ($p<0.001$) larger cotyledons than wild type, while cotyledon area in OE becomes significantly ($p<0.001$) smaller at 0.05 μM ABA. Cotyledon area was not used for further experiments due to the difficulty in obtaining measurements in seedlings grown in liquid culture.

After quantifying the effects of NAE and ABA separately on seedling growth, the effects of both together were studied (Figure 6). A low level of ABA (0.1 μM), which had little effect on wild type seedling growth (Figure 4B, 5B), was used in combination with different levels of NAE 12:0 (10 μM and 20 μM). These levels were also low enough to not have a severe effect on wild type growth (Figure 3B, 3D, 4A). When added together, NAE and ABA severely reduced primary root length in wild type seedlings (Figure 6E, 6F). Using the primary root length differences in each single treatment and in the combined NAE and ABA treatment, the type of interaction between NAE and ABA was calculated. The primary root length of wild type seedlings in 10 μM NAE was 78% of the length in solvent control and was 58% the length in 0.1 μM ABA. If the combination of NAE and ABA was completely additive, the expected percent of control

in the combined treatment would be ((58% * 78%) / 100) = 45.6%. The measured percent of control in the combined treatment of 10 µM NAE and 0.1 µM ABA is 16.8%. At 20 µM NAE and 0.1 µM ABA, the expected percent of control is calculated to be 15.9%, while the measured value is 3.3%. These lower values are indicative of treatment synergism. The *faah* seedlings were less sensitive (had longer primary roots) to these synergistic inhibitory effects while the *FAAH* OE were more sensitive (had shorter primary roots). In addition, primary root length in *abi3-1* was significantly ($p<0.001$) higher in the combined treatments (Figure 6F, 6G), suggesting that an intact ABA signaling pathway is required for the synergistic inhibition of growth. While the *abi3-1* line did have longer roots, a more qualitative indicator of the *abi3-1* insensitivity to the synergistic effect is the much larger cotyledons of *abi3-1* seedlings as compared to WT (L*er*). The 20 µM NAE + 0.1 µM ABA treatment was used for subsequent experiments because it exhibited a stronger synergistic effect, while also showing clear differences between the FAAH metabolism mutants.

For subsequent experiments seedlings were grown in liquid culture to produce larger quantities of tissues for analyses. Primary root length and cotyledon area are difficult to measure in liquid-grown seedlings. For these liquid cultures, total fresh weight was used to quantify changes in growth due to NAE and ABA synergism. Seedlings were grown in liquid media, harvested and weighed. Synergistic effects can be identified qualitatively by examining the overall morphology of seedlings (Figure 7A). The FAAH metabolism mutants showed the much shorter primary roots and reduced cotyledons in combined treatments when grown in liquid media, while *abi3-1* showed tolerance to the combined treatments. Measurements of fresh weight confirmed the

resistance of OE and the hypersensitivity of *faah* to added NAE (Figure 7B). Conversely, overall growth of *faah* showed the reduced sensitivity to ABA while OE showed only a slight sensitivity to ABA, less than what was seen previously for primary root lengths of seedlings grown in plates (Figure 4).

Figure 3: NAE Effects on Primary Root Length.

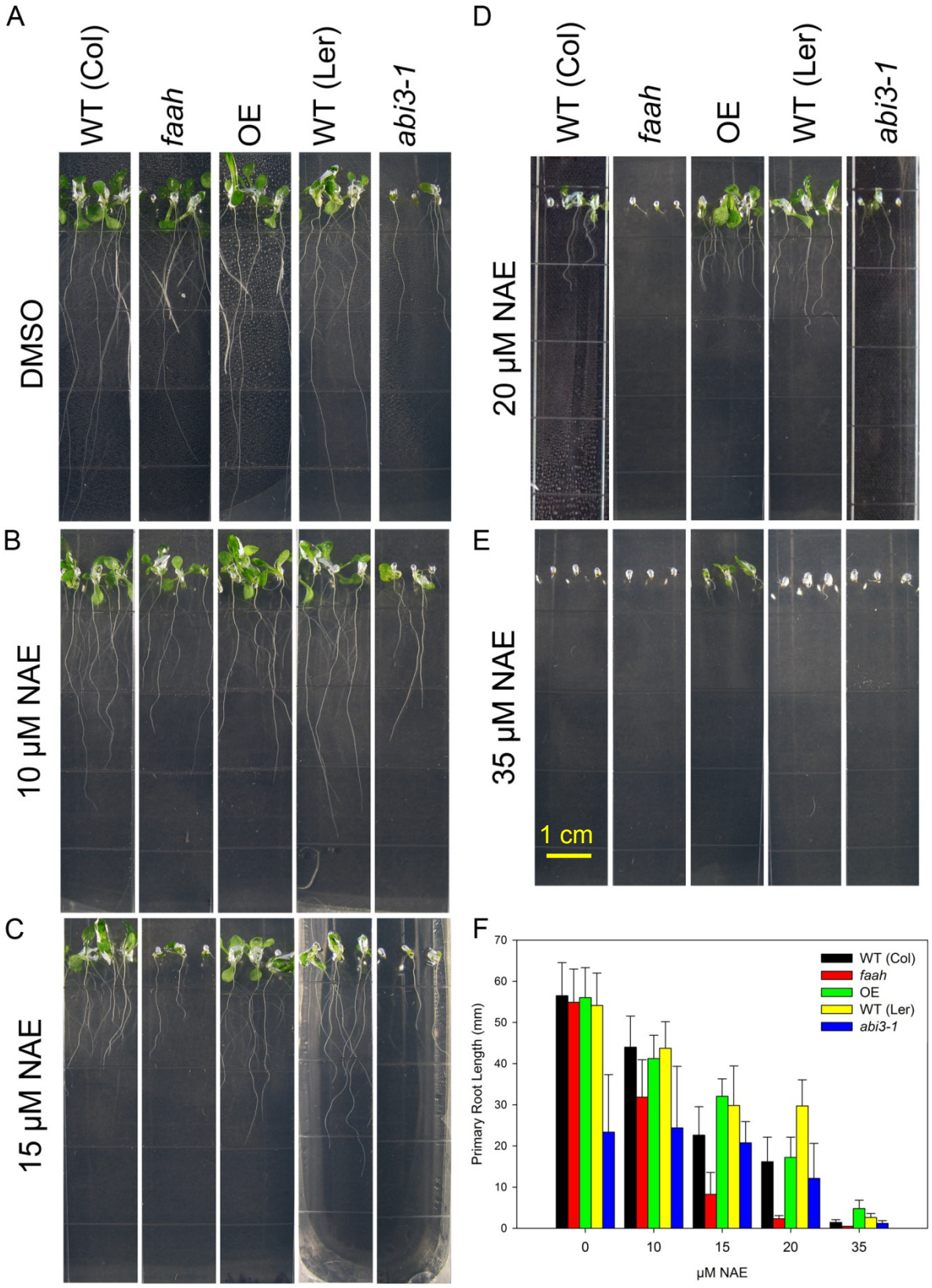

Figure 3: NAE Effects on Primary Root Length.

(A) - (E) Representative images of 10 day old seedlings used for primary root length measurements. Seedlings were grown continuously in 0.02% DMSO or increasing concentrations of NAE 12:0, as indicated.

(F) Quantitative measurements of primary root length of 10 day old seedlings grown in increasing concentrations of NAE 12:0. Measurements are mean and standard deviation (n>30) from one experimental set of plates.

Figure 4: ABA Effects on Primary Root Length

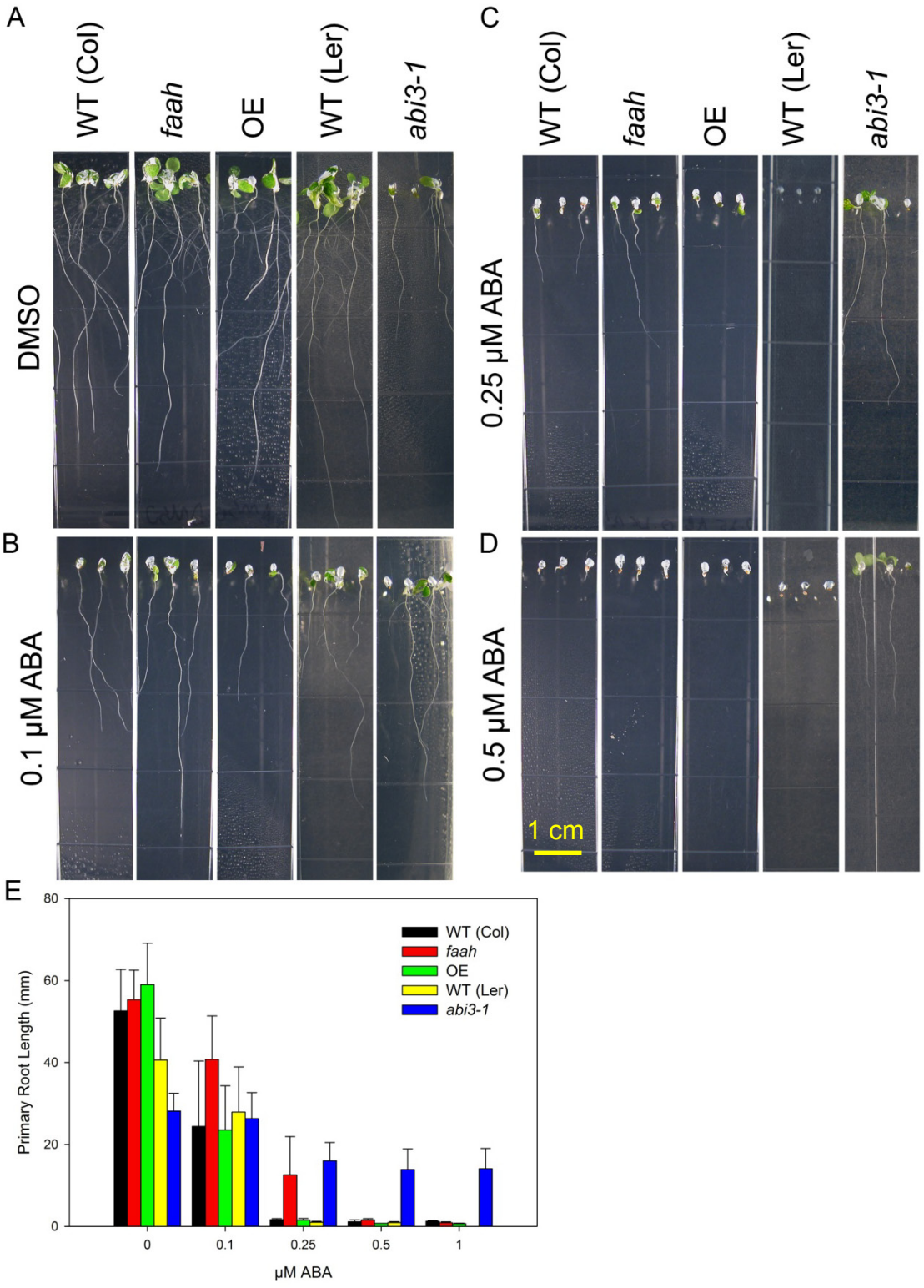

Figure 4: ABA Effects on Primary Root Length

(A) - (E) Representative images of 10 day old seedlings used for primary root length measurements. Seedlings were grown continuously in 0.02% DMSO or increasing concentrations of (+) ABA, as indicated.

(F) Quantitative measurements of primary root length of 10 day old seedlings grown in increasing concentrations of (+) ABA. Measurements are mean and standard deviation (n>30) from two different sets of growth experiments: one with WT (Col) background seedlings and one with WT (L*er*) background seedlings.

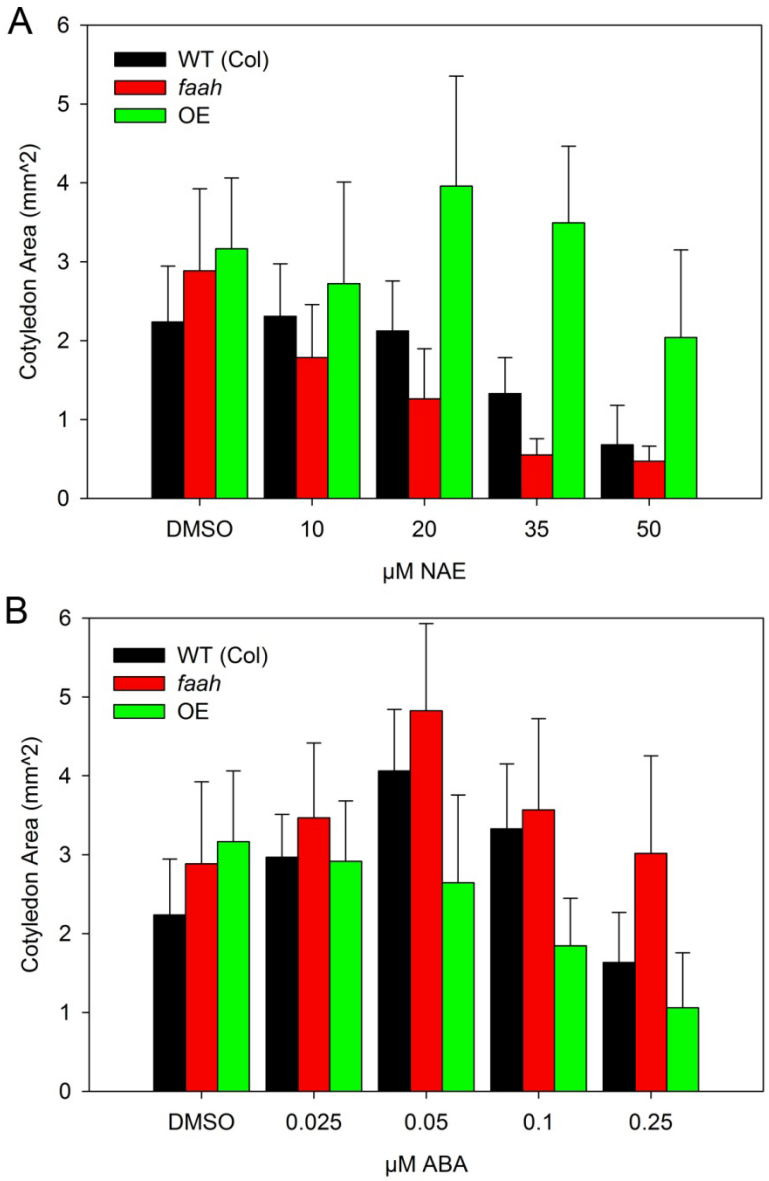

Figure 5: NAE and ABA Effects on Cotyledon Area.

(A) Quantitative measurements of cotyledon area from 14 day old seedlings grown in 0.02% DMSO or increasing concentrations of NAE 12:0, as indicated.

(B) Quantitative measurements of cotyledon area from 14 day old seedlings grown in 0.02% DMSO or increasing concentration of (+) ABA, as indicated. Measurements are mean and standard deviation (n>50) from seedlings grown together in one experiment.

Figure 6: Synergistic Effects of NAE and ABA on Primary Root Length

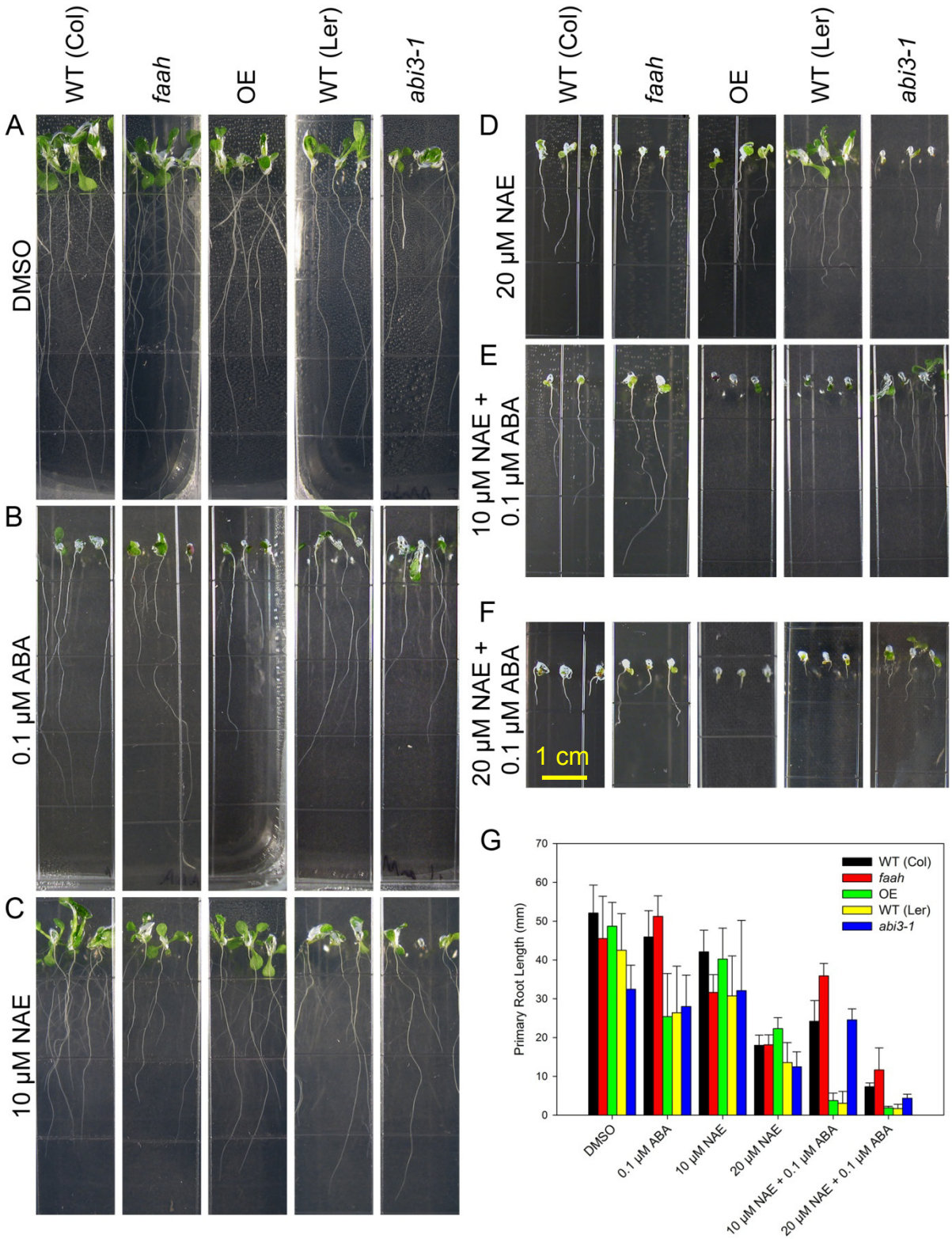

Figure 6: Synergistic Effects of NAE and ABA on Primary Root Length

(A) - (F) Representative images of 10 day old seedlings used for primary root length measurements. Seedlings were grown in NAE 12:0, (+) ABA or both, as indicated. (G) Quantitative measurements of primary root length of 10 day old seedlings grown in NAE 12:0, (+) ABA or both. Measurements are mean and standard deviation (n>30) from one complete experiment.

Figure 7: Synergistic Effects of NAE and ABA on Fresh Weight.

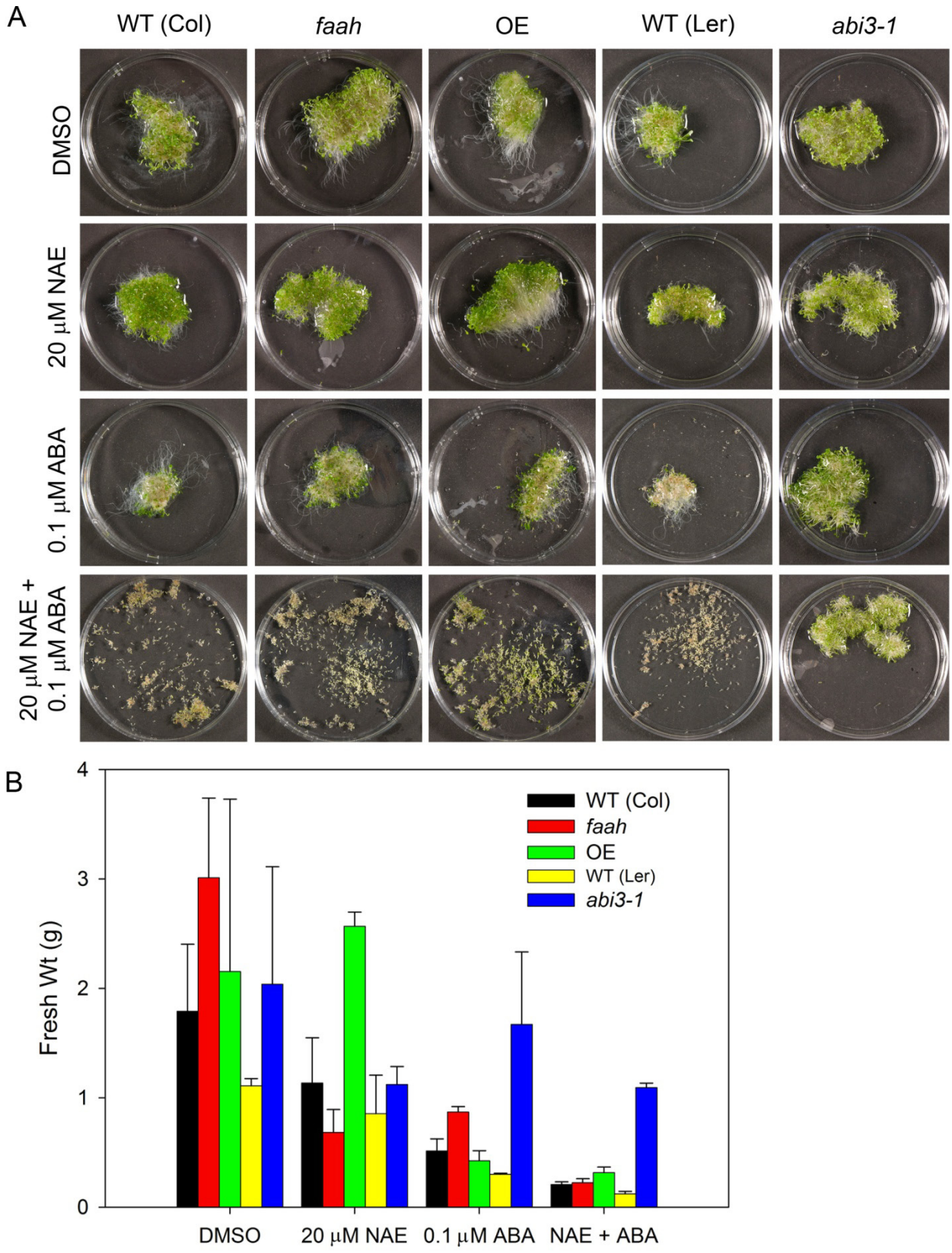

Figure 7: Synergistic Effects of NAE and ABA on Fresh Weight.

(A) Representative images of total fresh weight of 10 day old seedlings grown in NAE 12:0, (+) ABA or both, as indicated.

(B) Quantitative measurements of fresh weight of 10 day old seedlings grown in NAE 12:0, (+) ABA or both. Measurements are mean and standard deviation (n>30) from one complete experiment.

Endogenous NAE and ABA Content in Seedlings Treated with NAE and ABA

The effects of NAE and ABA exposure, individually and combined, on endogenous NAE levels were quantified using isotope-dilution mass spectrometry (Figure 8). FAAH metabolism mutants showed different NAE profiles in the various treatments. The *faah* seedlings had very high levels of NAE 14:0 (up to ~4000 ng/g FW) in NAE 12:0 treatment compared to wild type seedlings (Figure 8B). To determine whether these high levels of NAE 14:0 were due to contamination of the NAE 12:0 solution, samples of NAE 12:0 were analyzed by GC/MS. The NAE 12:0 solution had no other NAE species, and specifically had no NAE 14:0. In addition, NAE 18:2 was much higher in NAE 12:0 treated *faah* than in WT. The amounts of other NAE species were similar to WT (Figure 8A). The *FAAH* OE seedlings had much lower levels of all NAE species when treated with NAE or ABA (Figure 8C). In the combined treatment, however, OE had an NAE profile similar to WT. The ABA signaling mutant *abi3-1* showed higher levels of all NAE species compared to WT (L*er*) when untreated (Figure 8D, 8E). When treated with NAE 12:0 or ABA, levels of all NAE species were lower in the *abi3-1* seedlings. However, the NAE levels were only slightly increased in the combined treatment, similar to the FAAH metabolism mutants. Levels of all NAE species were much higher in WT (L*er*) seedlings in the combined treatment. These seedlings also had the lowest growth in the combined treatment (Figure 6). All NAE species were lower in seedlings than in desiccated seeds (Figure 8F). However, with the exception of high levels of NAE 14:0 in *faah* and higher levels of NAE 18:2 in *abi3-1*, the NAE profiles, or the relative amounts of each NAE species, were similar between seeds and seedlings, especially those grown in the combined NAE and ABA treatment.

NAE 12:0 amounts were excluded from the above NAE profiles due to extremely high levels in NAE 12:0 treated seedlings (Figure 9), as might be expected. These levels were 50-100 times higher than any other NAE species. The *faah* seedlings had the highest levels of NAE 12:0 when treated with NAE 12:0, while *FAAH* OE had the lowest levels, confirming that FAAH can metabolize exogenously applied NAE. In all genotypes except the *FAAH* overexpressors, NAE 12:0 levels were much higher in the combined treatment, suggesting that perhaps the absolute endogenous levels of NAE are not solely responsible for the differences in growth in seedlings treated with ABA or combinations of NAE and ABA.

The overall NAE levels (not including NAE 12:0) in seedlings also differed by genotype. In untreated seedlings, *abi3-1* had the highest levels of NAE while *FAAH* OE had the lowest (Figure 10A). Upon treatment with NAE 12:0, total NAE levels increased in *faah* and WT (L*er*), remained the same in WT (Col) and were slightly reduced in *abi3-1*. *FAAH* OE seedlings were able to completely metabolize NAEs in the NAE 12:0 treatment. A similar pattern was also seen in ABA treated seedlings. In the combined treatment, NAE levels were greatly increased in all genotypes. The *faah* and WT (L*er*) seedlings had the highest levels, while WT (Col), OE and *abi3-1* all had similar levels. When NAE levels in seedlings were compared to total NAE levels in seeds a significant change in levels was seen (Figure 10B). Of the FAAH metabolism mutants, *faah* had the highest level of NAE in seeds and WT and OE had similar levels. In the ABA signaling mutant, seed levels of NAE were slightly higher than WT (L*er*).

ABA levels also were measured using isotope-dilution mass spectrometry. Total ABA levels in seedlings differed greatly between the different genotypes (Figure 10C).

The FAAH metabolism mutants had similar levels of ABA in untreated and NAE 12:0 treated seedlings. In ABA treated seedlings, levels of endogenous ABA increased for the three genotypes; however, the levels of ABA in *faah* were significantly ($p<0.001$) higher than in WT or OE. This was also true of the combined treatment. The ABA signaling mutant *abi3-1* had lower levels of ABA in NAE 12:0 treated seedlings, but the levels increased greatly in ABA and combined treatment seedlings. ABA levels in the *abi3-1* seedlings remained significantly lower ($p>0.01$) than WT (L*er*) in all treatments.

To determine if the change in NAE profiles in ABA treated seedlings was due to interactions between ABA and FAAH, FAAH amidohydrolase activity was measured in the presence of ABA. Treatment with high levels (5 μM ABA) had no effect on the endogenous amidohydrolase activity in total protein extracts from WT seeds (Figure 11A, 11B). The reaction rates of the FAAH metabolism mutant protein extracts showed the expected profile, with no FAAH activity in the *FAAH* knockouts (*faah*) and very high activity in the *FAAH* overexpressor (OE). These rates were not affected by either low levels or high levels of ABA (Figure 11C). The ABA signaling mutant had a slightly higher FAAH activity than WT (L*er*). This rate was also not affected by the addition of ABA.

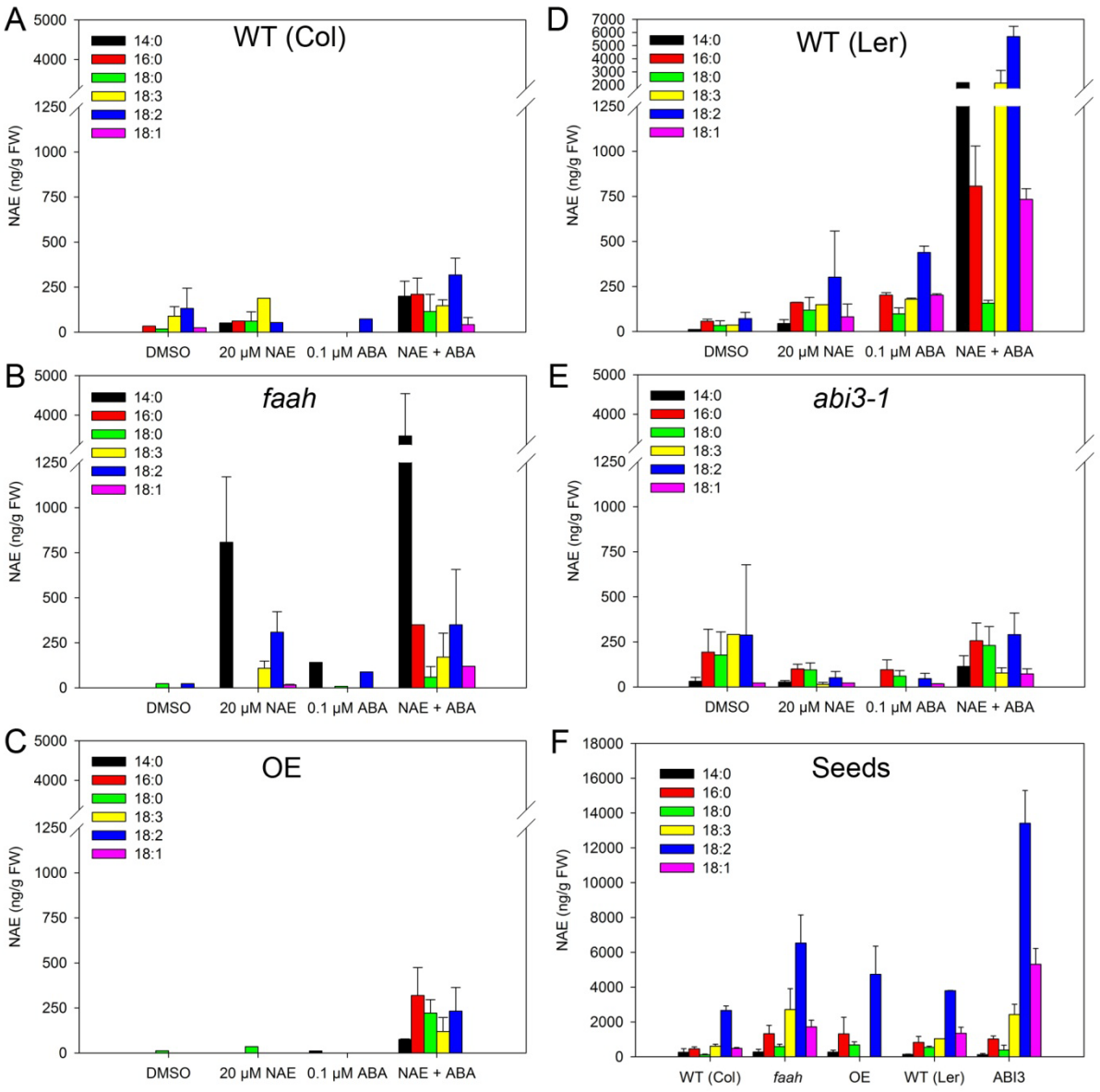

Figure 8: NAE profiles in seedlings grown in NAE and ABA.

(A), (B), (C), (D), (E) and (F) Quantification of NAE 14:0, 16:0, 18:0, 18:3, 18:2 and 18:1 levels by isotope-dilution mass spectrometry in 10 day old (A) WT (Col), (B) *faah*, (C) OE, (D) WT (L*er*), and (E) *abi3-1* seedlings and (F) desiccated seeds.

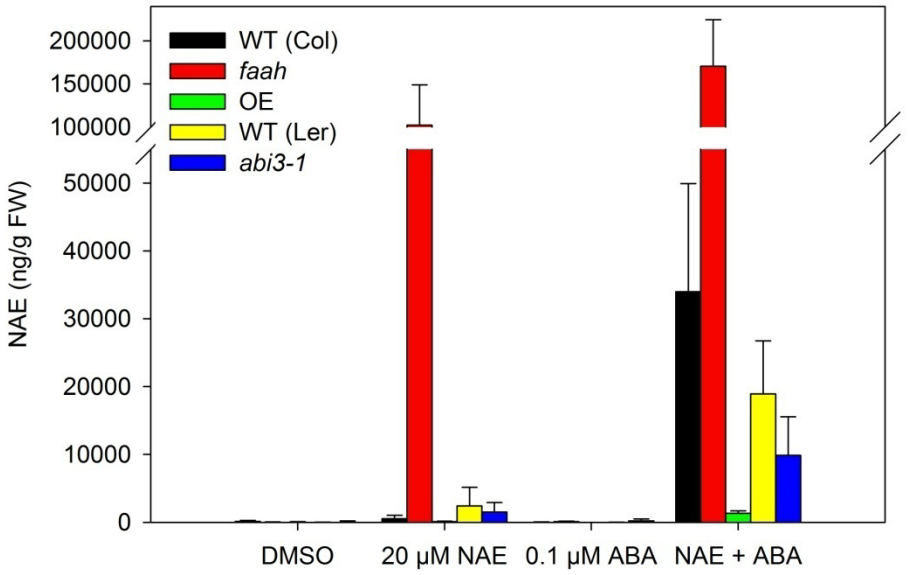

Figure 9: NAE 12:0 levels in seedlings grown in NAE and ABA.

Quantification of NAE 12:0 levels by isotope-dilution mass spectrometry in 10 day old seedlings treated with 0.05% DMSO, 20 µM NAE 12:0, 0.1 µM ABA or both NAE and ABA.

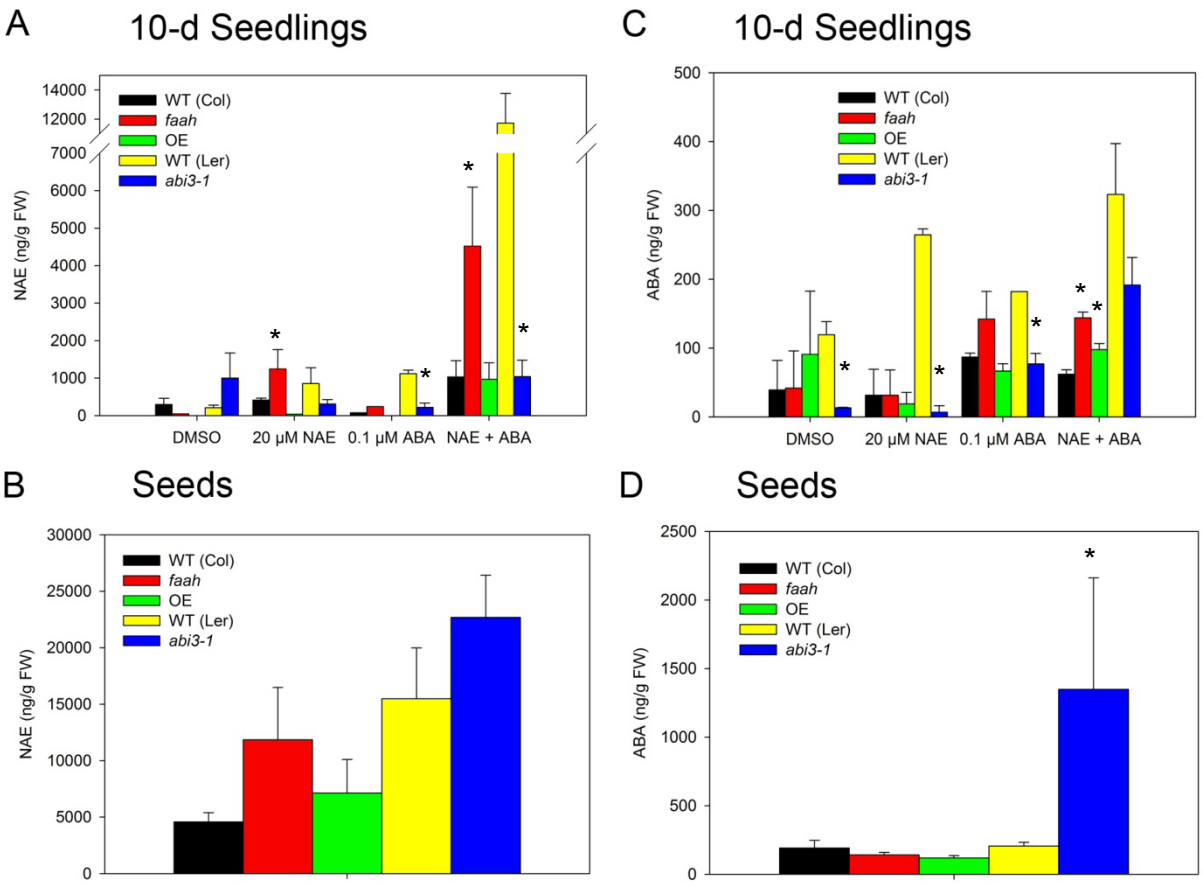

Figure 10: Total NAE and ABA levels in seedlings grown in NAE and ABA.

(A) Quantification of NAE from 10 day old seedlings grown in 0.05% solvent control, 10 µM NAE, 0.1 µM ABA or a combination of NAE and ABA. Sum of NAE 14:0, 16:0, 18:0, 18:3, 18:2, and 18:1 species. * $p<0.05$ compared to wild-type.

(B) Quantification of NAE from desiccated seeds by isotope-dilution mass spectrometry. Sum of NAE 12:0, 14:0, 16:0, 18:0, 18:3, 18:2, and 18:1 species.

(C) Quantification of ABA from 10 day old seedlings grown in 0.05% solvent control, 10 µM NAE, 0.1 µM ABA or a combination of NAE and ABA. * $p<0.05$ compared to wild-type.

(D) Quantification of ABA from desiccated seeds by isotope-dilution mass spectrometry. * $p<0.05$ compared to wild-type.

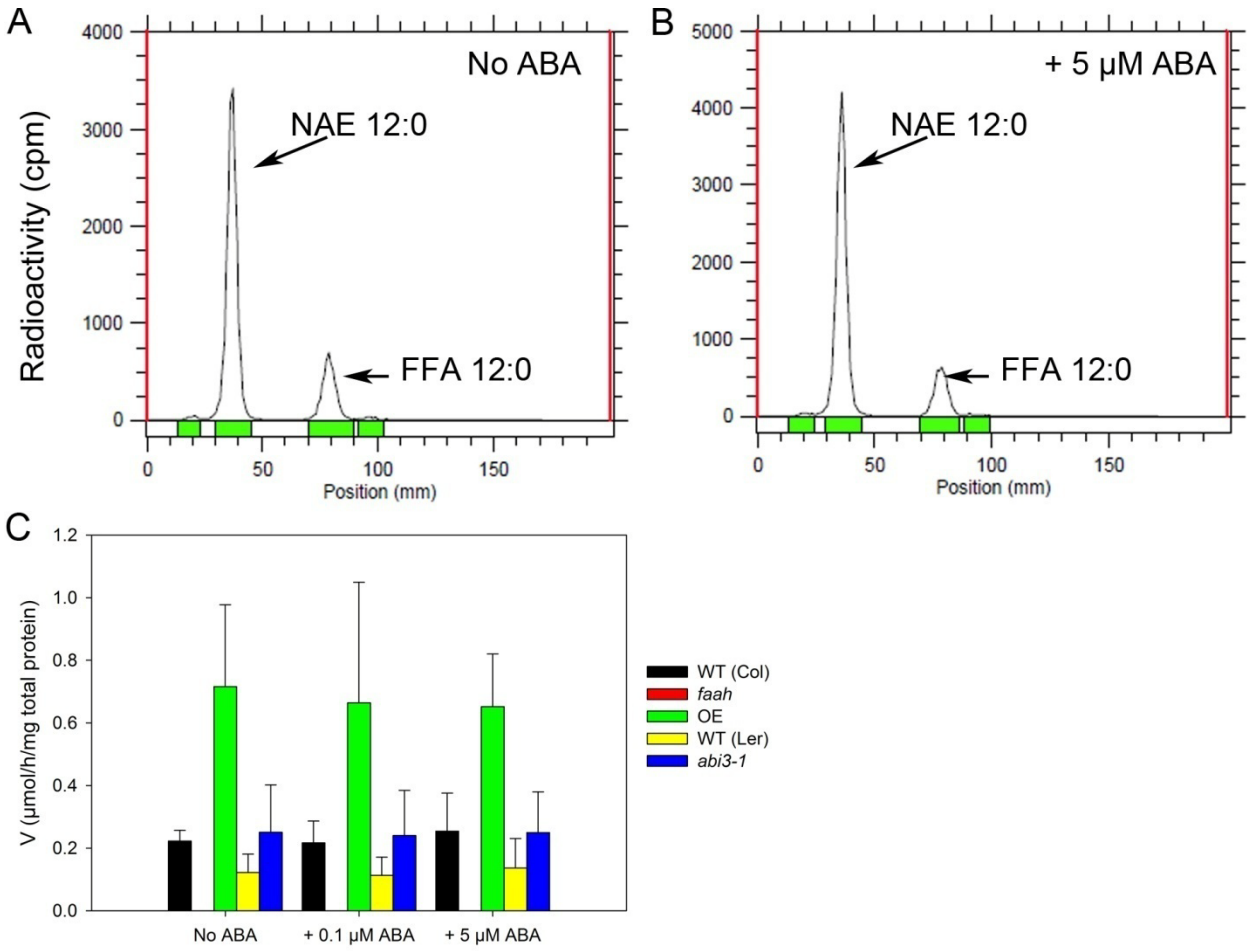

Figure 11: FAAH activity in the presence of ABA.

(A) and (B) Characterization of FAAH amidohydrolase activity in WT seeds (A) without ABA and (B) with 5 µM ABA.

(C) Quantification of FAAH amidohydrolase activity in seed protein extracts in the absence and presence of ABA.

Effects of Combined NAE and ABA Treatment on ABA-Regulated Gene Expression

The effects of NAE treatment on ABA regulated gene expression were characterized by semi-quantitative real time RT-PCR on FAAH metabolism mutant seedlings grown in various concentrations of NAE 12:0 (Figure 12). Seedlings grown for analysis showed the appropriate NAE dose dependent reduction of growth, including the hypersensitivity of *faah* and the insensitivity of *FAAH* OE (Figure 12A). Three ABA-regulated genes were previously identified: *ABI3*, *AtHVA22B* and *RD29B* (Teaster et al., 2007). *ABI3* transcript was present in all three genotypes at all times, but levels increased with increasing levels of NAE 12:0. At 35 and 50 μM NAE, levels were much higher (Figure 12B). *AtHVA22B* and *RD29B* transcript levels were very low in low levels of NAE (10 and 20 μM) but were greatly increased in higher levels (35 and 50 μM NAE). The *faah* seedlings had higher levels of both *AtHVA22B* and *RD29B* at all levels of NAE while the *FAAH* OE had much lower levels of both genes even at 35 and 50 μM NAE.

The effects of ABA treatment on transcript levels of the same three genes was also characterized (Figure 13). Again, seedlings grown for analysis showed the appropriate dose ABA dose dependent reduction in growth, including hypersensitivity of *FAAH* OE (Figure 13A). Levels of *ABI3* transcript were lower in *faah* seedlings and higher in OE seedlings compared to WT at all levels of ABA treatment (Figure 13B). Levels of *AtHVA22B* were slightly higher in *faah* seedlings (appearing at 0.1 μM ABA) and slightly lower in OE seedlings. Levels of *RD29B* in *faah* were similar to WT (seen at all levels of ABA) but were much lower in OE seedlings (not apparent until treated with 0.1 μM or more ABA).

The effects of the combined treatments on these three gene transcript levels were also characterized (Figure 14). The growth of seedlings used for analysis was similar to previous seedlings grown in NAE and ABA, including hypersensitive *faah* seedlings and slightly resistant OE seedlings (Figure 14A). Transcript levels of all three genes were increased in the combined treatment in the three genotypes (Figure 14B). *ABI3* levels in all three genotypes were similar in both ABA treatment and in the combined treatment. In wild type seedlings, the levels of *RD29B* in the combined treatment was also similar to levels of ABA treatment alone (Table 2). The levels of *AtHVA22B* in the combined treatment was slightly higher than in the ABA treatment alone in WT seedlings (Table 2). In *faah* seedlings, *AtHVA22B* and *RD29B* levels were much higher in the combined treatment than in ABA alone (Table 2). In the OE seedlings, levels of *AtHVA22B* were much higher in the combined treatment but the levels of *RD29B* were only slightly higher than in the ABA treatment (Table 2).

The combined NAE and ABA reduction in seedling growth could be occurring through the ABA signaling pathway, so the effects of synergism on ABA-dependent gene transcripts in ABA-signaling mutants were characterized (Figure 15). The synergistic effect was not present in the seedlings used for this study, as expected (Figure 15A). The *abi3-1* mutant had no *ABI3* transcript, as expected (Figure 15B). It also showed no induction of the ABA-regulated gene *AtHVA22B*. However, another ABA-regulated gene, *RD29B*, did show an increase in transcript when treated with ABA and in the combined treatment. The levels of transcript were slightly lower in the combined treatment than in the ABA treatment.

Relative gene transcript levels were measured by quantitative real time RT-PCR for the combined treatment (Table 2). *FAAH* showed no substantial change in transcript levels in NAE, ABA or combined treatment. The ABA responsive genes *AtHVA22B*, *RD29B* and *ATEM1* all showed higher transcript levels in ABA and combined treatments, except *AtHVA22B* in the *abi3-1* mutants, which exhibited very little increase in transcript levels. An NAE responsive gene *PNP-A* showed slight increases in transcript levels in WT (Col) and *FAAH* OE seedlings when treated with NAE, but levels were much higher in *faah* seedlings. In addition, ABA treatment slightly lowers levels of *PNP-A* in all three genotypes. In the ABA signaling mutant *abi3-1*, levels are not responsive to NAE treatment, but drop severely in ABA treament.

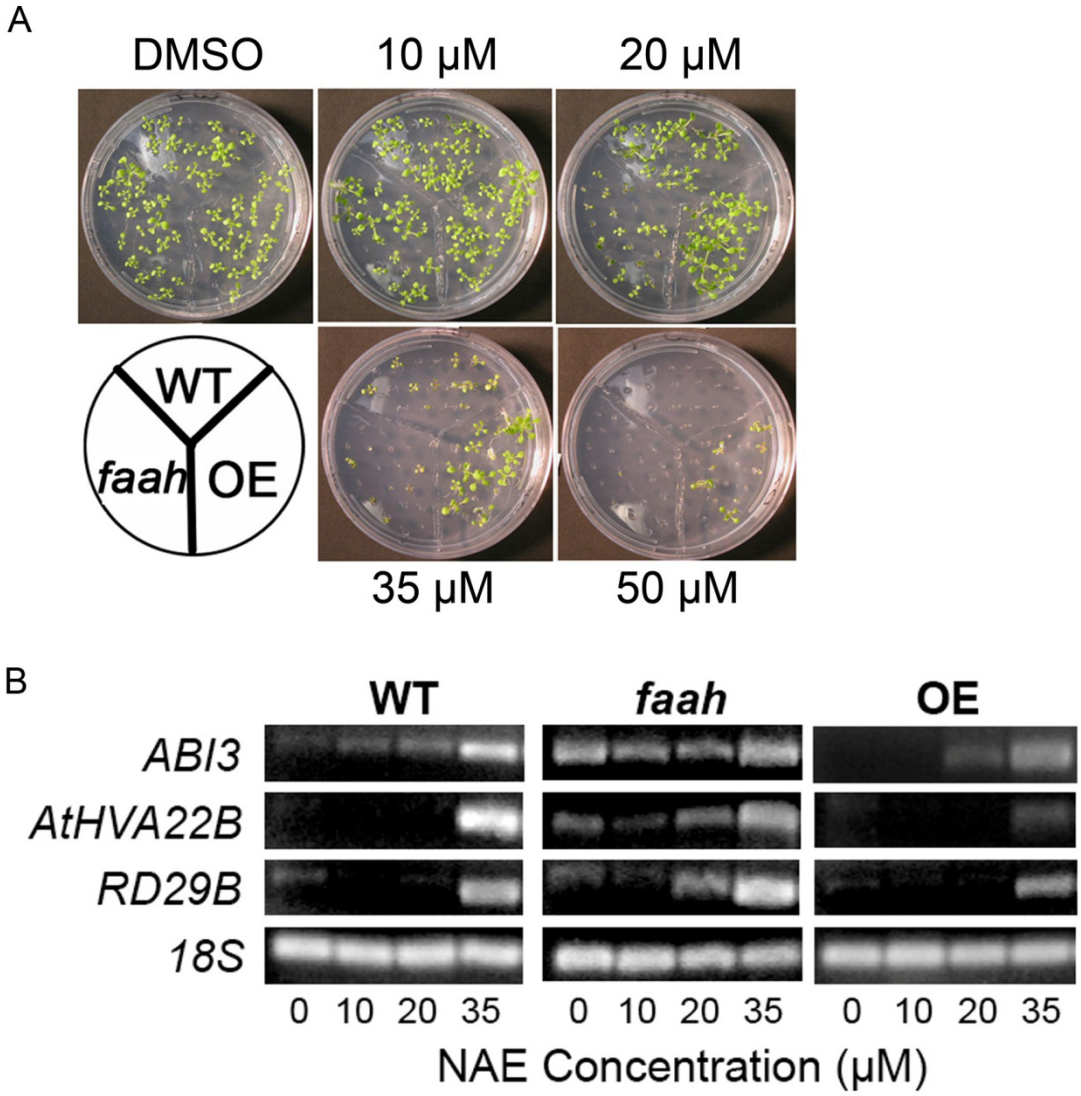

Figure 12: Impact of NAE Dose Dependent Inhibition of Seedling Growth on ABI Signaling-related Transcript Levels.

(A) Representative images of 10-d-old seedlings at increasing concentrations of NAE 12:0 exhibit the tolerance of the *FAAH* overexpressor and the sensitivity of the *FAAH* knockout. (Contributed by Dr. Neal Teaster)

(B) Agarose gel analysis of RT-PCR using gene specific primers to analyze gene expression in 10-d-old seedlings (treated with increasing concentrations of NAE 12:0). *ABI3* was run for 40 cycles, *AtHVA22B* and *RD29B* for 22 cycles and *18S* for 25 cycles.

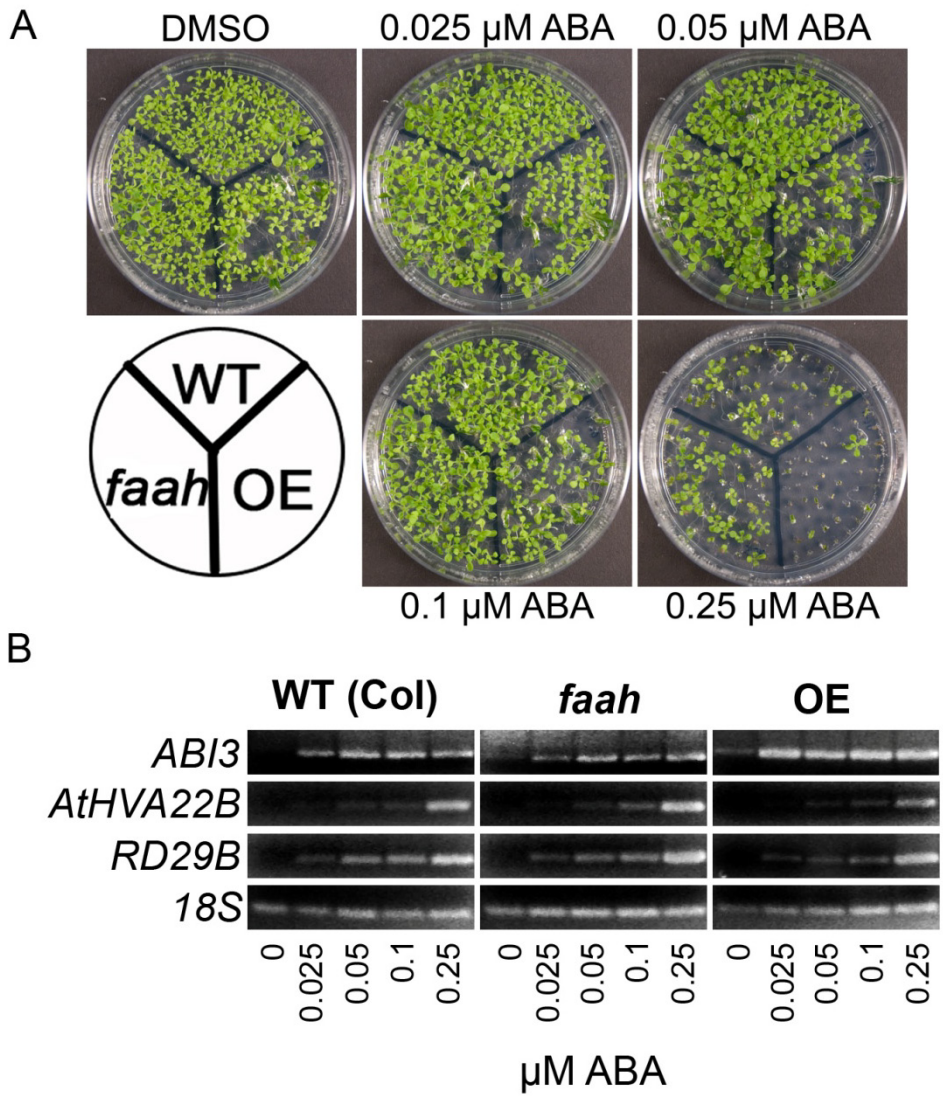

Figure 13: Impact of ABA Dose Dependent Inhibition of Seedling Growth on ABI Signaling-related Transcript Levels.

(A) Representative images of 10-d-old seedlings at increasing concentrations of ABA exhibit the tolerance of the *FAAH* knockout and the sensitivity of the *FAAH* overexpressor.

(B) Agarose gel analysis of RT-PCR using gene specific primers to analyze gene expression in 10-d-old seedlings (treated with increasing concentrations of (+) ABA). *ABI3* was run for 40 cycles, *AtHVA22B* and *RD29B* for 22 cycles and *18S* for 25 cycles.

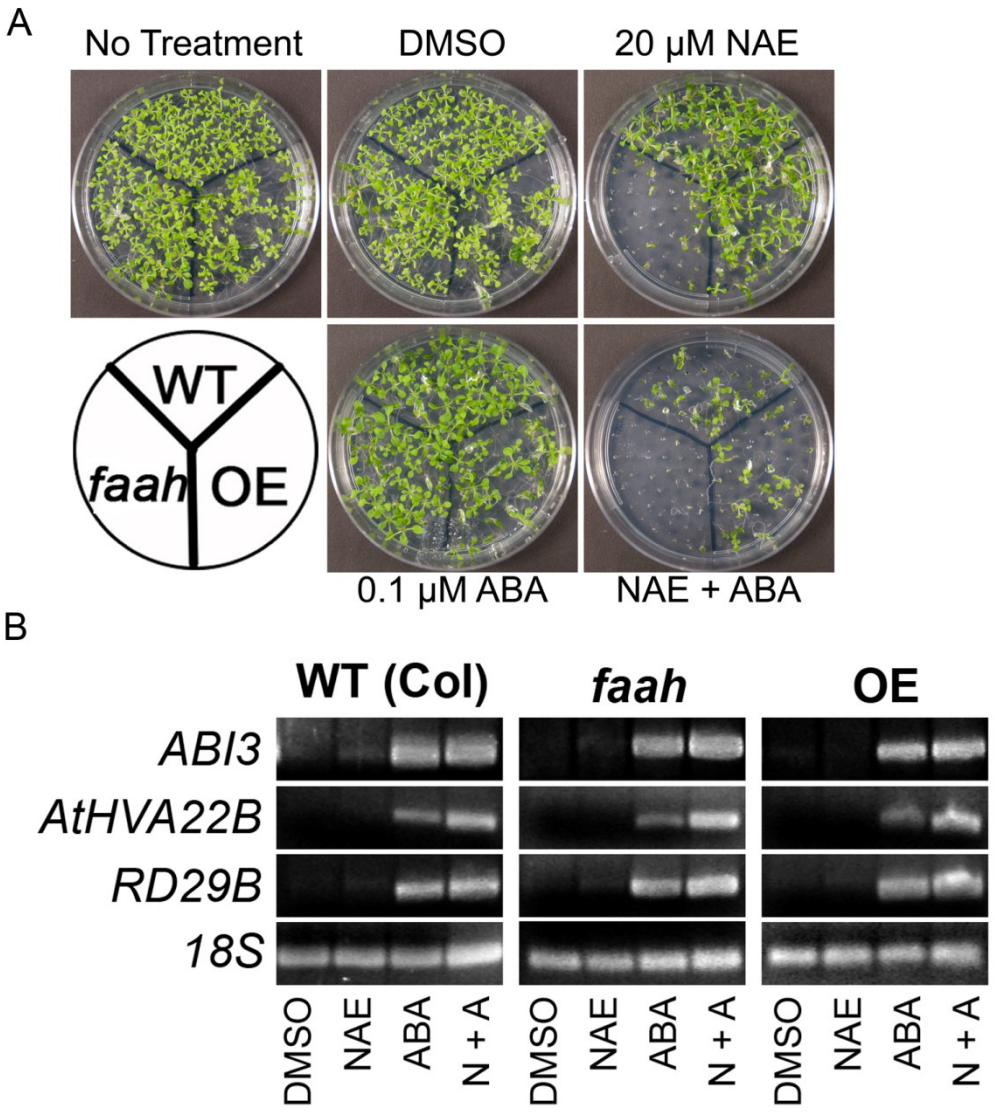

Figure 14: Impact of NAE and ABA Dose Dependent Inhibition of Seedling Growth on ABI Signaling-related Transcript Levels.

(A) Representative images of 10-d-old seedlings grown in NAE 12:0 and ABA exhibit the tolerance of the *FAAH* overexpressor and the sensitivity of the *FAAH* knockout. (B) Agarose gel analysis of RT-PCR using gene specific primers to analyze gene expression in 10-d-old seedlings (treated with 20 µM NAE, 0.1 µM ABA or NAE + ABA). *ABI3* was run for 40 cycles, *AtHVA22B* and *RD29B* for 22 cycles and *18S* for 25 cycles.

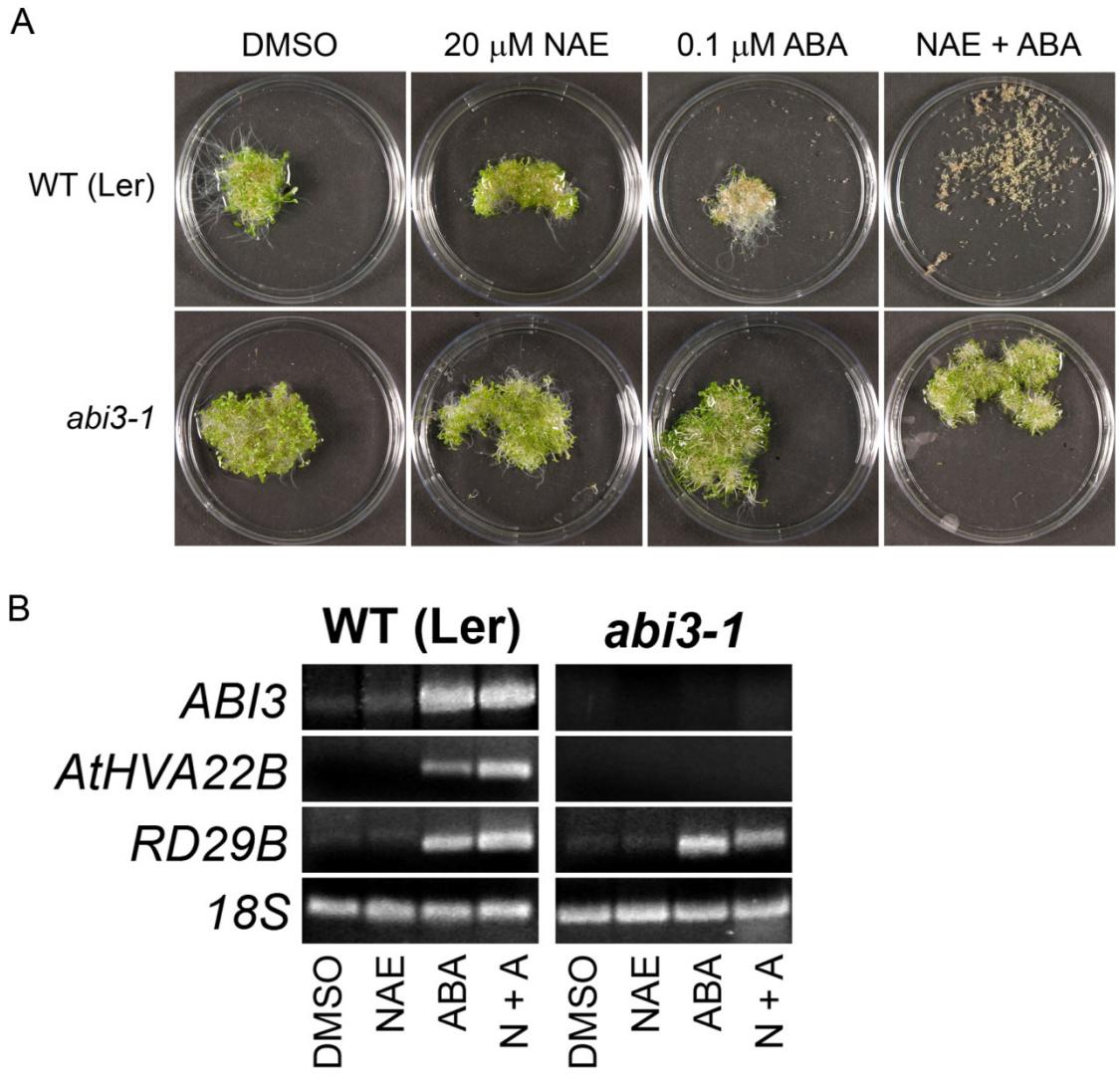

Figure 15: An intact ABA signaling pathway is required for synergistic growth arrest.

(A) Representative images of 10d-old seedlings grown in NAE 12:0, ABA or NAE + ABA.

(B) Agarose gel analysis of RT-PCR using gene specific primers to analyze gene expression in 10-d-old seedlings (treated with 20 μM NAE, 0.1 μM ABA or a combination of both NAE + ABA). *ABI3* was run for 40 cycles, *AtHVA22B* and *RD29B* for 22 cycles and *18S* for 25 cycles.

A	*FAAH*			*AtHVA22B*			*RD29B*			*ATEM1*			*PNP-A*		
	WT	***faah***	**OE**	**WT**	***faah***	**OE**	**WT**	***faah***	**OE**	**WT**	***faah***	**OE**	**WT**	***faah***	**OE**
DMSO	1.0	-	8.6	1.0	0.5	1.8	1.0	0.3	2.6	1.0	0.5	0.9	1.0	1.3	9.2
20 µM NAE	0.7	-	7.0	0.8	1.6	0.7	1.4	2.5	0.5	0.9	1.2	1.9	7.4	21.5	7.0
0.1 µM ABA	0.4	-	3.8	48.8	24.5	82.2	91.0	63.3	358	7.5	4.3	23.9	0.2	0.3	0.5
20 µM NAE + 0.1 µM ABA	0.3	-	6.0	109	134	167	140	188	474	14.1	22.9	25.5	2.3	1.4	2.5

B	*FAAH*		*AtHVA22B*		*RD29B*		*ATEM1*		*PNP-A*	
	L*er*	***abi3-1***	**L*er***	***abi3-1***	**L*er***	***abi3-1***	**L*er***	***abi3-1***	**L*er***	***abi3-1***
DMSO	1.0	0.7	1.0	0.8	1.0	2.3	1.0	0.5	1.0	11.7
20 uM NAE	2.7	1.2	0.6	1.0	3.4	2.2	0.9	1.5	0.4	2.0
0.1 uM ABA	1.8	1.4	34.4	5.1	114.3	92.9	12.9	6.9	0.0	1.2
20 uM NAE + 0.1 uM ABA	1.5	1.4	135.9	4.6	261.9	123.4	18.8	1.3	0.1	0.6

Table 2: Effects of NAE 12:0 and/or ABA on Relative Transcript Levels of Selected Genes in 10 Day Old Seedlings.

(A) Changes in gene transcript levels in 10 day old WT (Col) (WT), *FAAH* knockout (*faah*) and *FAAH* overexpressor (OE) seedlings shown as average fold change relative to WT DMSO for each gene (n=3).

(B) Changes in gene transcript levels in 10 day old WT (L*er*) and ABI3 knockout (*abi3-1*) seedlings shown as average fold change relative to WT (L*er*) DMSO for each gene (n=3).

CHAPTER 4

DISCUSSION

Previous studies have shown that *Arabidopsis thaliana* seeds germinated and grown in *N*-lauroylethanolamine (NAE 12:0) display a severe reduction in growth (Blancaflor et al., 2003; Motes et al., 2005). However, the mechanism of this NAE-induced growth reduction is unknown. The similarities in growth defects between NAE and ABA treated seedlings suggested a similar mechanism between the two (Teaster et al., 2007). The evidence presented here suggests NAE-induced growth arrest occurs primarily through an ABA-regulated pathway, although it can also occur through an ABA-independent mechanism that it currently unknown. In addition, my thesis work provides clues for the further elucidation of the mechanism of NAE-induced growth arrest.

Evidence presented here demonstrates that the interaction between NAE and ABA does not occur through modulating levels of either NAE or ABA. ABA levels did not significantly increase in the combined NAE and ABA treatment, even when growth was severely reduced, as in the *FAAH* overexpressor line (Figure 10C). The levels of ABA in the *abi3-1* seedlings were higher in the combined treatment as compared to the control, but this is likely due to a combination of higher levels in seeds and the ABA treatment added to the seedlings. Total NAE levels were higher in the combined treatment for the *faah* and WT (L*er*) seedlings (Figure 10A). In order for seedlings to grow normally, it is postulated that the high levels of NAE found in seeds must be depleted (Blancaflor et al., 2003; Teaster et al., 2007). The *faah* seedlings are less capable of removing the NAEs, so they exhibit slower growth. In addition, the levels of

NAE 14:0 were much higher than in wild-type seedlings. The significance of this elevated NAE 14:0 is unknown, but this remains an interesting alteration in NAE profiles that should be followed up in future studies. This extra NAE 14:0 is not due to contamination of the NAE 12:0 used to treat seedlings, so it must be produced endogenously. One possible reason for this accumulation is that the seedlings are attempting to remove the extra NAE 12:0 by elongating the NAE 12:0 through an unknown mechanism. It is also possible that NAE 14:0 may play a protective role in seedling development that is currently unknown. Seedlings could be increasing the amount of NAE 14:0 from NAE14:0-PE in an attempt to alleviate the NAE 12:0 induced stress. The WT (L*er*) seedlings also grew poorly in the combined treatment and the NAE levels remained high in those seedlings, suggesting some possible ecotype differences in NAE-induced inhibition of seedling growth. The pattern of the different NAE levels in seedlings did not differ significantly from the pattern in seeds (Figure 8F). The levels are much lower in seedlings than in seeds, but the overall pattern remained similar, except for the much higher levels of NAE 14:0 in the *FAAH* knockout seedlings. Because total NAE levels and NAE profiles do not change significantly in the combined NAE and ABA treatment, it is unlikely that the mechanism of NAE-induced growth arrest is through modulation of NAE levels.

There are several limitations in using NAE 12:0 in these experiments. The NAE 12:0 levels typically found in *Arabidopsis* is 40-80 ng/g fresh weight in seeds and 60-100 ng/g fresh weight in seedlings (Wang et al., 2006). The amount of NAE 12:0 added in the combined NAE and ABA treatment (20 μM NAE and 0.1 μM ABA) is 4.8 μg/g of media, 100 times more than typically found in plants. These high levels of NAE 12:0

are likely capable of causing other pleiotropic effects beyond any effects on NAE and ABA signaling. NAE 12:0 has been shown to be a potent inhibitor of lipoxygenase (LOX), which is normally able to remove high levels of unsaturated NAEs (Keereetaweep et al., 2010). When LOX activity is inhibited, plants have higher levels of unsaturated NAEs (especially NAE 18:2 and NAE 18:3). In addition, NAE 12:0 was able to block wound-induced formation of jasmonic acid. This would suggest that NAE 12:0 is able to interact with other stress response pathways to inhibit growth. Another limitation of using NAE 12:0 is that it can be removed from the media by the seedlings. NAE 12:0 levels in media with WT seedlings grown in a starting concentration of 50 μM NAE 12:0 is reduced to 45 μM by 6 days after sowing seeds in the media and 0.5 μM by 12 days (Teaster, 2009). In the experiments presented here, NAE 12:0 is not replenished in the media. However, NAE 12:0 has less of an effect on seedling growth after 5 days (Teaster et al., 2007). Because levels remain high in media up to that time, it is unlikely that the lower levels at 10 days would have much of an effect on growth. To eliminate the possibility of the effects of NAE 12:0 removal from the media, it may be necessary to repeat these experiments in media that is refreshed every 3-5 days with new media and NAE.

Exogenous application of NAE and ABA apparently inhibit seedling growth in a similar overall manner. Wild-type seedlings grown in NAE 12:0 showed a dose-dependent reduction in primary root length, similar to the reduction in seedlings grown in ABA (Figure 3). At low levels of NAE and ABA, there was little reduction in growth, but at higher levels, root length was greatly reduced (Figures 3 and 4). The *FAAH* overexpressor showed a hypersensitive response to ABA and the *FAAH* knockout had a

slight tolerance toward exogenous ABA. When low levels of both NAE and ABA are added together, seedlings demonstrate a synergistic response that is greater than the sum of the reduction in growth in either compound separately (Figure 6). The pattern of growth reduction in seedlings treated with low levels of both NAE and ABA was similar to the reduction in growth seen in ABA-treated seedlings (Figure 4), suggesting that the effect of ABA on growth arrest is dominant over any NAE effects on growth arrest. Perhaps NAE helps to modulate ABA responses in seedling growth arrest, but this interaction requires further investigation.

The reduction of seedling growth can be associated with changes in gene expression at the molecular level by observing the levels of *ABI3* transcripts in the seedlings. When seedlings developed normally, the level of *ABI3* transcripts in tissues was low. When seedling growth was reduced, the *ABI3* transcript levels were high (Figures 12-14). While this inverse association between *ABI3* transcript levels and growth had been suggested in previous work (Teaster et al., 2007), the evidence presented here demonstrates this effect more clearly. In the combined NAE and ABA treatment, where seedlings were unable to grow, the levels of *ABI3* transcript were much higher (Figure 14). In addition, ABI3 regulated genes (*AtHVA22B* and *RD29B*) showed a similar pattern of increased levels at lower growth. The changes in expression of these ABA-regulated genes were much higher than changes in expression of an NAE-regulated gene *PNP-A* (Table 2). It is unknown whether high levels of *ABI3* inhibited seedling growth or whether inhibiting seedling growth resulted in higher *ABI3* levels. Previous work indicates that *ABI3* levels increase over time in NAE treated seedlings (Teaster et al., 2007). This increase begins at 5 days after sowing in

liquid media, which is before differences in growth can be measured. This would suggest that *ABI3* transcript levels increase first, which in turn leads to inhibition of seedling growth, but this needs to be further examined. Regardless of which occurs first, it is evident that ABI3 plays an important role in seedling growth arrest and that both NAE metabolism and ABA application can influence the overall transcript levels of this important transcriptional regulator. This is further evidence of the importance of the ABA-induced growth arrest mechanism in the combined NAE and ABA treatments.

The ABA signaling pathway plays an important role in NAE-induced growth arrest, as demonstrated by the requirement for an intact ABA signaling pathway for the synergistic reduction in growth from combined low levels of NAE and ABA (Figures 6 and 7). When the ABA signaling pathway was disrupted, as in the *ABI3* knockout seedlings, there was no synergistic reduction in growth by NAE and ABA (Figure 6). This was also previously observed for other ABA signaling mutants *abi1, abi2, abi4* and *abi5* (Teaster et al., 2007). There was also no increase in the ABA-regulated *AtHVA22B* gene transcripts in the combined treatment (Figure 15). All of these proteins have been previously identified as part of the secondary dormancy pathway that was discovered by Chua and coworkers (Lopez-Molina et al., 2002; Lois et al., 2003). Secondary dormancy refers to the ability of *Arabidopsis* seedlings that have already passed the primary dormancy stage, and have germinated, to halt growth in unfavorable conditions. This secondary dormancy pathway is especially important in early seedling development, when abiotic stress perception is key to survival. In addition, NAE 12:0 has been found to only induce growth arrest within a similar narrow window during early seedling development (Teaster et al., 2007). When low levels of ABA and NAE are

combined in this early developmental stage, it is possible these two compounds both activate the secondary dormancy mechanism, operating through ABI3, inhibiting seedling growth. Altogether, these data provide strong evidence that an intact ABA signaling pathway is required for NAE-induced growth arrest.

Although the ABA signaling pathway appears to play a major role in NAE-induced growth arrest, NAE 12:0 also appeared to induce seedling growth arrest through an ABA-independent mechanism since growth of *abi3-1* seedlings was inhibited at higher levels of NAE (Figure 3). If NAE 12:0 was capable of inducing growth arrest only through the ABA signaling pathway, the *abi3-1* seedlings would be expected to grow normally in exogenous NAE. Instead, *abi3-1* seedlings have a 50% reduction in primary root length compared to WT (L*er*) (Figure 3F). The observed growth reduction in *abi3-1* seedlings treated with NAE was also associated with lower levels of the ABA-regulated gene *AtHVA22B* compared to untreated seedlings (Figure 15). This demonstrates that NAE can inhibit growth somewhat independent of the normal ABA response pathway. However, this inhibition is not as strong as that seen when the ABA-signaling pathway is intact. While *AtHVA22B* showed very low levels of transcipt in *abi3-1* seedlings, *RD29B* had much higher expression in ABA and NAE treated seedlings as compared to untreated seedlings (Table 2). *RD29B* is a general drought response gene that is strongly regulated by the ABI3 transcription factor (Nakashima et al., 2006), but can be activated independently of ABA through MAPK mechanisms (Hua et al., 2006). This suggests that NAE partially functions through a different pathway that is partly, but not entirely, dependent on ABA.

In the combined NAE and ABA treatment, the hypersensitive reduction in growth seen in the *FAAH* OE seedlings is counter-intuitive. If NAE induced growth arrest through both the ABI3-dependent and -independent mechanisms equally, it would be expected that because *FAAH* OE seedlings can remove NAE, they should have similar growth in the combined treatment as they do when grown in ABA alone. The opposite would be expected of the *faah* KO seedlings - they have higher levels of NAE, so they would be expected to be more sensitive to the combined treatment. However, this is not what was observed; instead, the *FAAH* OE seedlings are more sensitive and the *faah* knockout seedlings are less sensitive. There are several possible explanations for these results. The first is that the NAE levels are not as important in the NAE-induced growth arrest as the levels of FAAH protein itself. There is some evidence that FAAH itself can directly interact with ABA signaling, independent of its catalytic activity (Kim et al., 2009). When seedlings with overexpressed catalytically inactive *FAAH* are grown in the presence of ABA, they are unable to grow. This suggests that FAAH is able to interact with ABA signaling independent of its amidohydrolase activity. The mechanism of this interaction between ABA and FAAH is not yet known. It is also possible that the NAE-induced growth arrest occurs early in seedling development, when NAE levels are still high in the media and have not yet been reduced by FAAH (Teaster, 2009). In this case, it is the initial level of NAE 12:0 that is responsible for the reduction in growth, and the removal of NAE is not as important for allowing seedling growth. Because the *FAAH* overexpressor seedlings are hypersensitive to ABA, it is possible that the high levels of NAE 12:0 present during germination and early seedling development interact

with the ABA-signaling pathway to increase this hypersensitive reduction in growth in the combined NAE and ABA treatment.

A complicating factor in the comparison between seedlings in the WT (Col) and WT (L*er*) background is the different growth rates of the various *Arabidopsis* ecotypes (Beemster et al., 2002). Landsberg *erecta* seedlings, in general, grow slower than the Columbia ecotype, but eventually plants grow larger. Seedlings in the L*er* background, including the *abi3-1* seedlings, would therefore also be expected to grow more slowly, possibly obscuring the insensitivity of ABI3 mutants to the combined NAE and ABA treatment. In order to remove these complications, the effects of NAE and ABA on *abi3-1* seedlings needs to be repeated with ABI3 knockout seeds in the Columbia background. This is expected to result in an increased observed tolerance to the combined effects of NAE and ABA compared to the WT (Col) seedlings. It should be noted, however, that previous work with ABI knockout seedlings in the WT (Col) background, *abi4-1*, did demonstrate tolerance to the combined NAE and ABA treatment (Teaster et al., 2007). It is therefore unlikely that ecotypic differences would account for the observed growth differences.

Although ecotype growth differences are unlikely to account for the changes in growth in NAE treatment, the elevated levels of NAE and ABA in the WT (L*er*) seedlings could be due to differences between the two ecotypes. NAE levels in WT (L*er*) seedlings treated with NAE and ABA combined are 10 times higher than in WT (Col) seedlings. ABA levels are 5 times higher than in WT (Col) (Figure 10). The Landsberg *erecta* ecotype is characterized by a mutation in ERECTA, a leucine-rich repeat receptor-like Ser/Thr kinase (Torii et al., 1996). ERECTA has been identified as a

pleiotropic regulator of developmental and physiological processes (van Zanten et al., 2009). It is possible that the elevated levels of NAE and ABA in the WT (L*er*) seedlings is related to the *ERECTA* mutation, and is not due to the growth reduction when seedlings are grown in NAE and ABA. One way to test this is to quantify the effects of exogenous NAE and ABA on endogenous levels in *ERECTA* mutants in the Columbia background. If the levels of NAE and ABA are also elevated in these mutants, it is likely that ERECTA itself is responsible for differences in NAE and ABA levels in WT (L*er*) seedlings. If levels do not increase, the differences are likely due to an increased sensitivity of the WT (L*er*) seedlings to exogenous NAE and ABA.

Future experiments should focus on understanding exactly how NAE interacts with ABA. The first step should be to complete work to identify how FAAH interacts with ABA. Because NAE and ABA levels do not change dramatically in NAE or ABA treatment, the interaction is likely to take place downstream of the production of NAE and ABA. Because of this, the mutants for the ABA receptor, especially PYL1 (Miyazono et al., 2009), should be tested for NAE sensitivity. If NAE functions through this receptor (upstream of the ABI genes), there should be no reduction in growth when treated with high levels of NAE. There is also some evidence that FAAH itself can directly interact with ABA signaling, independent of its catalytic activity (Kim et al., 2009). This interaction could explain why *FAAH* overexpressors are hypersensitive to ABA and the combined NAE and ABA treatment, even though ABA does not alter FAAH amidohydrolase activity (Figure 11). The mechanism of this interaction between ABA and FAAH is also not yet known and needs to be further characterized.

In addition to working on how NAE interacts with the ABA signaling pathway, the ABA-independent mechanism of NAE-induced growth arrest needs to be investigated more thoroughly. It is possible NAE functions independently of ABA signaling by modulating levels of secondary messengers or other signaling pathways. The effects of NAE on secondary messenger levels, especially changes to calcium levels and reactive oxygen species, should be studied. ABA has been shown to induce both of these (Bright et al., 2006; Chai et al., 2006), so if NAE also induces one or more of these secondary messengers the combined levels could greatly impact growth. NAE could also function to inhibit seedling growth by modulating any of the many MAP kinase pathways found in *Arabidopsis* (Hua et al., 2006; Fiil et al., 2009; Andreasson and Ellis, 2010). These various MAPK pathways play important roles in seedling development and response to stress. Microarray data on NAE 12:0 treated seedlings did not identify any known MAPK genes with significantly altered expression compared to DMSO treated seedlings (Teaster et al., 2007). However, this does not necessarily indicate that NAE 12:0 does not interact with any MAPKs. Several genes that do interact strongly with different MAPKs were up-regulated in NAE treatment, including *RD29B*. NAE could be interacting with these other signaling mechanisms to induce growth arrest. Understanding these interactions would likely help not only in determining how ABA-dependent NAE-induced growth occurs, but also in identifying how NAE can induce growth arrest independent of ABA.

In summary, NAE can induce seedling growth arrest through ABA-signaling pathway-dependent and -independent mechanisms. The strong reduction in growth in seedlings treated with both NAE 12:0 and ABA suggests that NAEs, which are relatively

minor lipids in *Arabidopsis*, may play a more significant role in early seedling development than previously thought. In addition, this interaction with an important phytohormone, ABA, illustrates the complex connections between components in plant development which still need to be identified and characterized. Overall, identifying the mechanism of NAE-induced growth arrest could help shed light on other general mechanisms of arresting seedling growth in the early stages of development as a physiological response to unfavorable environmental conditions.

REFERENCES

Andreasson, E., and Ellis, B. (2010). Convergence and specificity in the Arabidopsis MAPK nexus. Trends Plant Sci **15,** 106-113.

Beemster, G.T.S., De Vusser, K., De Tavernier, E., De Bock, K., and Inze, D. (2002). Variation in growth rate between Arabidopsis ecotypes is correlated with cell division and A-type cyclin-dependent kinase activity. Plant Physiol. **129,** 854-864.

Blancaflor, E.B., Hou, G., and Chapman, K.D. (2003). Elevated levels of N-lauroylethanolamine, an endogenous constituent of desiccated seeds, disrupt normal root development in Arabidopsis thaliana seedlings. Planta **217,** 206-217.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72,** 248-254.

Bright, J., Desikan, R., Hancock, J.T., Weir, I.S., and Neill, S.J. (2006). ABA-induced NO generation and stomatal closure in Arabidopsis are dependent on H2O2 synthesis. Plant J **45,** 113-122.

Chai, M.-F., Wei, P.-C., Chen, Q.-J., An, R., Chen, J., Yang, S., and Wang, X.-C. (2006). NADK3, a novel cytoplasmic source of NADPH, is required under conditions of oxidative stress and modulates abscisic acid responses in Arabidopsis. Plant J **47,** 665-674.

Chapman, K.D. (2004). Occurrence, metabolism, and prospective functions of N-acylethanolamines in plants. Prog Lipid Res **43,** 302-327.

Chapman, K.D., Venables, B., Markovic, R., Blair Jr, R.W., and Bettinger, C. (1999). N-Acylethanolamines in seeds. Quantification of molecular species and their degradation upon imbibition. Plant Physiol **120,** 1157-1164.

Colby, S.R. (1967). Calculating synergistic and antagonistic responses of herbicide combinations. Weeds **15,** 20-22.

Coursol, S., Fan, L.-M., Le Stunff, H., Spiegel, S., Gilroy, S., and Assmann, S.M. (2003). Sphingolipid signalling in Arabidopsis guard cells involves heterotrimeric G proteins. Nature (London) **423,** 651-654.

Cutler, S.R., Rodriguez, P.L., Finkelstein, R.R., and Abrams, S.R. (2009). Abscisic acid: Emergence of a core signaling network. Annu Rev Plant Physiol Plant Mol Biol.

Devane, W.A., Hanus, L., Breuer, A., Pertwee, R.G., Stevenson, L.A., Griffin, G., Gibson, D., Mandelbaum, A., Etinger, A., and Mechoulam, R. (1992). Isolation and structure of a brain constituent that binds to the cannabinoid receptor. Science **258,** 1946-1949.

Fiil, B.K., Petersen, K., Petersen, M., and Mundy, J. (2009). Gene regulation by MAP kinase cascades. Curr Opin Plant Biol **12,** 615-621.

Finkelstein, R.R., Gampala, S.S.L., and Rock, C.D. (2002). Abscisic acid signaling in seeds and seedlings. Plant Cell **14,** S15.

Hua, Z.-M., Yang, X., and Fromm, M.E. (2006). Activation of the NaCl- and drought-induced RD29A and RD29B promoters by constitutively active Arabidopsis MAPKK or MAPK proteins. Plant Cell Environ **29,** 1761-1770.

Keereetaweep, J., Kilaru, A., Feussner, I., Venables, B.J., and Chapman, K.D. (2010). Lauroylethanolamide is a potent competitive inhibitor of lipoxygenase activity. FEBS Lett **In Press, Corrected Proof**.

Kilaru, A., Blancaflor, F.B., Venables, B.J., Tripathy, S., Mysore, K.S., and Chapman, K.D. (2007). The N-acylethanolamine-mediated regulatory pathway in plants. Chem Biodivers **4,** 1933-1955.

Kim, S.-C., Kang, L., Nagaraj, S., Blancaflor, E.B., Mysore, K.S., and 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. J Biol Chem **284,** 34065-34074.

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

Lois, L.M., Lima, C.D., and Chua, N.-H. (2003). Small ubiquitin-like modifier modulates abscisic acid signaling in Arabidopsis. Plant Cell **15,** 1347-1359.

Lopez-Molina, L., Mongrand, S., McLachlin, D.T., Chait, B.T., and Chua, N.H. (2002). ABI5 acts downstream of ABI3 to execute an ABA-dependent growth arrest during germination. Plant J **32,** 317-328.

Mehra, M. (1996). RNA isolation from cells and tissues. (Wiley-Liss, Inc.; Wiley-Liss, Ltd.).

Melcher, K., Ng, L.-M., Zhou, X.E., Soon, F.-F., Xu, Y., Suino-Powell, K.M., Park, S.-Y., Weiner, J.J., Fujii, H., Chinnusamy, V., Kovach, A., Li, J., Wang, Y., Li, J., Peterson, F.C., Jensen, D.R., Yong, E.-L., Volkman, B.F., Cutler, S.R., Zhu,

J.-K., and Xu, H.E. (2009). A gate-latch-lock mechanism for hormone signalling by abscisic acid receptors. Nature **462,** 602-608.

Mishra, G., Zhang, W.H., Deng, F., Zhao, J., and Wang, X.M. (2006). A bifurcating pathway directs abscisic acid effects on stomatal closure and opening in Arabidopsis. Science **312,** 264-266.

Miyazono, K.-i., Miyakawa, T., Sawano, Y., Kubota, K., Kang, H.-J., Asano, A., Miyauchi, Y., Takahashi, M., Zhi, Y., Fujita, Y., Yoshida, T., Kodaira, K.-S., Yamaguchi-Shinozaki, K., and Tanokura, M. (2009). Structural basis of abscisic acid signalling. Nature **462,** 609-614.

Motes, C.M., Cotter, M.Q., Wang, Y.S., Chapman, K.D., and Blancaflor, E.B. (2006). Synergistic effects of N-acylethanolamine (NAE) and abscisic acid (ABA) on Arabidopsis seed germination and seeedling growth. In SS-ASPB Annual Meeting 2006 (Daytona Beach, FL).

Motes, C.M., Pechter, P., Yoo, C.M., Wang, Y.-S., Chapman, K.D., and Blancaflor, E.B. (2005). Differential effects of two phospholipase D inhibitors, 1-butanol and N-acylethanolamine, on in vivo cytoskeletal organization and Arabidopsis seedling growth. Protoplasma **226,** 109.

Nakashima, K., Fujita, Y., Katsura, K., Maruyama, K., Narusaka, Y., Seki, M., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2006). Transcriptional regulation of ABI3-and ABA-responsive genes including RD29B and RD29A in seeds, germinating embryos, and seedlings of Arabidopsis. Plant Mol Biol **60,** 51-68.

Rider, S.D., Jr., Henderson, J.T., Jerome, R.E., Edenberg, H.J., Romero-Severson, J., and Ogas, J. (2003). Coordinate repression of regulators of embryonic identity by PICKLE during germination in Arabidopsis. Plant J **35,** 33-43.

Rock, C.D. (2000). Tansley Review No. 120. Pathways to abscisic acid-regulated gene expression. New Phytol **148,** 357-396.

Shrestha, R., Dixon, R.A., and Chapman, K.D. (2003). Molecular identification of a functional homologue of the mammalian fatty acid amide hydrolase in Arabidopsis thaliana. J Biol Chem **278,** 34990-34997.

Shrestha, R., Noordermeer, M.A., van der Stelt, M., Veldink, G.A., and Chapman, K.D. (2002). N-Acylethanolamines are metabolized by lipoxygenase and amidohydrolase in competing pathways during cottonseed imbibition. Plant Physiol **130,** 391-401.

Teaster, N.D. (2009). A regulatory role for N-acylethanolamine metabolism in Arabidopsis thaliana seeds and seedlings. In Biological Sciences (Denton: University of North Texas).

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., and Chapman, K.D. (2007). N-Acylethanolamine metabolism interacts with abscisic acid signaling in Arabidopsis thaliana seedlings. Plant Cell **19,** 2454-2469.

Torii, K.U., Mitsukawa, N., Oosumi, T., Matsuura, Y., Yokayama, R., Whittier, R.F., and Komeda, Y. (1996). The Arabidopsis ERECTA gene encodes a putative receptor protein kinase with extracellular leucine-rich repeats. Plant Cell **8,** 735-746.

van Zanten, M., Snoek, L.B., Proveniers, M.C.G., and Peeters, A.J.M. (2009). The many functions of ERECTA. Trends Plant Sci **14,** 214-218.

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

Wang, Y., Mopper, S., and Hasenstein, K.H. (2001). Effects of salinity on endogenous aba, iaa, ja, and a in Iris hexagona. J Chem Ecol **27,** 327.

Wang, Y.S., Shrestha, R., Kilaru, A., Wiant, W., Venables, B.J., Chapman, K.D., and Blancaflor, E.B. (2006). Manipulation of Arabidopsis fatty acid amide hydrolase expression modifies plant growth and sensitivity to N-acylethanolamines. Proc Natl Acad Sci U S A **103,** 12197-12202.

Webb, A.A.R., Larman, M.G., Montgomery, L.T., Taylor, J.E., and Hetherington, A.M. (2001). The role of calcium in ABA-induced gene expression and stomatal movements. Plant J **26,** 351-362.