N-ACYLETHANOLAMINE (NAE) PROFILES CHANGE DURING *Arabidopsis thaliana* SEED GERMINATION AND SEEDLING GROWTH

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An understanding of the potential roles as lipid mediators of a family of bioactive metabolites called *N*-acylethanolamines (NAEs) depends on their accurate identification and quantification. The levels of 18C unsaturated NAEs (e.g. NAE18:2, NAE 18:3, etc.) in wild-type seeds (about 2000 ng/g fw) generally decreased by about 80% during germination and post-germinative growth. In addition, results suggest NAE-degradative fatty acid amide hydrolase (*FAAH*) expression does not play a major role in normal NAE metabolism as previously thought. Seedlings germinated and grown in the presence of abscisic acid (ABA), an endogenous plant hormone, exhibited growth arrest and secondary dormancy, similar to the treatment of seedlings with exogenous *N*-lauroylethanolamine (NAE12:0). ABA-mediated growth arrest was associated with higher levels of unsaturated NAEs. Overall, these results are consistent with the concept that NAE metabolism is activated during seed germination and suggest that the reduction in unsaturated NAE levels is under strict temporal control and may be a requirement for normal seed germination and post-germinative growth.

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INTRODUCTION

N-Acylethanolamines (NAEs) were first reported as constituents of soy lecithin and peanut meal in the 1950s (Kuehl et al., 1957). Yet it was not until the discovery that membrane cannabinoid receptors in mammalian brain tissue bind to marijuana's principal psychoactive compound, Δ^9-tetrahydrocannabinol (THC), that interest in this class of lipids widely increased (Devane et al., 1992). *N*-arachidonylethanolamine (AEA), or anandamide (NAE20:4), an NAE type present at low concentrations in mammalian tissues, has now been shown to have potent cannabimimetric activities such as the induction of antinociception, hypothermia, hypomotility and catalepsy (Fride and Mechoulam, 1993; Crawley et al., 1993; Smith et al., 1994). AEA also was shown to activate vanilloid receptors and function as an endogenous analgesic (Pertwee, 2001). This led to the establishment of the endocannabinoid signaling pathway as a key regulator of important neurological processes in animals (De Petrocellis et al., 2004).

Mounting *in vitro* and *in vivo* data suggest that AEA, as well as some endogenous plant and synthetic cannabinoids, have neuroprotective effects following brain injury (Fowler, 2003). These cannabinoids inhibit glutamatergic synaptic transmission and reduce the production of tumor necrosis factor-α and reactive oxygen intermediates, which are factors in causing neuronal damage (Fontana, 1995). More recently, AEA concentrations were reported to increase in the brain in response to increased neuronal activity (Freund et 2003) as well as following traumatic brain injury in mice leading to ischemic conditions, such as transient middle cerebral artery occlusion (MCAo, Franklin

et al., 2003) or post-mortem (Schmid et al., 1995), or NMDA-induced excitotoxicity (Hansen et al., 2002). In addition to neuromodulatory action of NAE, there is growing support for the physiological role of AEA in immunomodulation (Buckley et al., 2000), prolonged hypotension, bradycardia and the possible peripheral regulation of vasodialation (Kunos et al., 2000), as well as several aspects of mammalian reproduction (Gerard et al., 1991; Varga et al., 1995). Although AEA is the most widely studied NAE in animals, other NAE types have emerged as regulators of important physiological processes such as embryo development, cell proliferation, immune responses and apoptosis.

The characterization of NAE metabolism and its physiological significance is best understood in mammalian systems. In plant systems, NAE occurrence, metabolism and physiological significance have remained, until recently, underappreciated. A combination of biochemical and biophysical approaches, in the mid 1960s, supported the existence of *N*-acylphosphatidylethanolamine (NAPE, NAE precursor) in higher plants as a minor, endogenous constituent of plant seeds (Bomstein, 1965; Dawson et al., 1969; Chapman and Moore, 1993; Sandoval et al., 1995). Radiolabeling experiments with [1,2-^{14}C]-ethanolamine *in vivo* revealed that a variety of plant cells and tissues had the ability to synthesize NAPE *de novo* via a phosphatidylethanolamine (PE) intermediate (Chapman and Moore, 1993). NAPE is synthesized from free fatty acids (FFAs) and PE (McAndrew and Chapman, 1998) both potentially bilayer-destabilizing lipids. NAPE is metabolized by phospholipase D (PLD), a phosphodiesterase, to yield phosphatidic acid (PA) and NAE (Chapman et al., 1995; Schmid et al., 1996; Chapman, 1998).

Concentrations of individual NAE molecular species (quantified by GC/MS) among seeds of several plant species and of several cultivars of cotton have been reported (Chapman et al., 1999). GC/MS quantification procedures have identified tissue-specific differences in types and quantities of NAEs in plant systems (Chapman et al., 1999). It is possible that different NAEs are accumulated at different developmental stages or in different tissues of plants for different purposes (Chapman et al., 1999). The specific NAE produced likely depends on a number of regulatory factors such as PLD specificity and the *N*-acyl composition of the precursor pool (Chapman et al., 2004).

Similar to animal systems, where the relatively minor NAE 20:4 (about 1% of the total NAE pool; Schmid et al., 2002) is believed to be responsible for many of the cannabinoid receptor-mediated physiological effects of NAE (Di Marzo et al., 2002), the minor NAE constituents (e.g. NAE14:0) found in plant tissues have potent biological activities and function in defense-related signaling in leaves (Tripathy et al., 1999). A signal-mediated release of NAE from NAPE was identified in elicitor-treated tobacco cell suspensions (Chapman et al., 1998) and leaves (Tripathy et al., 1999), and identified by gas chromatography mass spectrometry (GC/MS) as *N*-lauroyl- and *N*-myristoylethanolamine, raising the possibility that these molecules function in plant defense signaling. Although a gross over-simplification, one emerging theme is that in both plant and animal tissues, the largest proportion of NAEs are 16:0, 18:1 and 18:2 NAEs; however in terms of bioactivity, animal physiology is largely regulated by low concentrations of long-chain polyunsaturated NAEs (e.g., NAE 20:4), whereas, plants are particularly responsive to low concentrations of medium-chain NAEs, both of which constitute a relatively minor fraction of the total NAE pool (Chapman, 2004).

Much has been published on the biosynthesis of NAPE during cottonseed development, germination and seedling growth (Sandoval et al., 1995; Chapman and Sprinkle, 1996). Biochemical studies showed an increase in NAPE biosynthesis in cottonseed cotyledons upon imbibition raising interest in the occurrence and metabolic fate of NAEs in imbibed seeds and germinated seedlings (Chapman et al., 1999; Shrestha et al., 2003).

Seed germination involves the breaking of dormancy, which is triggered partly by imbibition, and the resumption of growth processes in the embryo. Early seedling growth is sustained by metabolizable substrates stored in the seed itself. Yet subsequent establishment of the plant as a self-sustaining organism requires a coordinated pattern of cell division and expansion that is regulated by a variety of external stimuli and endogenous factors (Esau 1977). Although there have been several endogenous compounds (e.g. plant hormones) and a variety of genes identified that regulate plant growth, the complexity of these processes makes it likely that other yet unidentified endogenous constituents regulate these important cellular processes (Blancaflor et al., 2003).

The occurrence of substantial amounts of NAEs in desiccated seeds and their rapid depletion during imbibition may suggest a possible endogenous physiological role for NAEs during seed germination (Chapman et al., 2004). While it is merely speculation, it is possible that NAE acts as an endogenous inhibitor that must be removed or degraded during imbibition for synchronous events associated with germination and post-germinative growth (Chapman et al., 1999). The observed decline in NAE concentration

during seed germination has been shown to occur by either oxidation and/or hydrolysis (Shrestha, 2002).

The formation of hydroperoxy NAEs and NAE-derived oxylipins has been reported in imbibed cottonseeds via a lipoxygenase (LOX)-mediated pathway (Fig. 1) with high specificity for polyunsaturated NAEs (Chapman et al., 1999). Cell fractionation studies indicated LOX was distributed in cytosol-enriched fractions and microsomes with the highest activity observed 8 h after imbibition (Shrestha et al., 2002). Purified 5-lipoxygenase from barley and tomato was shown to convert AEA, the mammalian neurotransmitter, into hydroperoxy NAE by (Van Zadelhoff et al., 1998). The physiological significance of these oxylipins and their potential role as lipid mediators is still unknown. This pathway of oxylipin formation was originally considered to be a minor route of NAE metabolism in imbibed seeds and seedlings, as it applies only to polyunsaturated NAE species (e.g., NAEs 18:2 and 18:3).

In animal systems, an amidase enzyme shown to hydrolyze NAEs, designated fatty acid amide hydrolase (FAAH; Fig. 1), has been characterized at the biochemical and molecular levels (Schmid et al., 1985; Cravatt et al., 1996). The degradation of AEA and other NAEs by FAAH is the mechanism by which AEA is inactivated in the mammalian brain following its selective uptake by neuronal cells (Cravatt et al., 1996; Beltramo et al., 1997) or postmortem (Patel et al., 2004).

In plants, homogenates of tobacco cell suspensions hydrolyzed NAEs to form FFAs and ethanolamine, providing evidence for an intracellular amidohydrolase activity capable of metabolizing NAEs *in planta* (Chapman et al., 1999). In imbibed cottonseeds, an active amidohydrolase activity that hydrolyzed NAE18:2 was identified and

characterized in cytosolic fractions (Chapman et al., 1999). Yet subsequent cell fractionation studies indicated the NAE amidohydrolase was associated mostly with microsomes with the highest specific activity observed 4 to 8 h after imbibition (Shrestha et al., 2002).

Evidence of conserved enzymatic machinery in plants for the formation and degradation of NAEs made clear the necessity to address NAE function in plants by forward and reverse genetics approaches (Shrestha et al., 2003). The expression of enzymes responsible for NAE metabolism could be manipulated to aid in the determination of the endogenous physiological role(s) of NAEs in plants. In animals, analysis of *FAAH* knockout mice revealed a 15-fold increase of AEA levels and supersensitivity to endogenous cannabinoid lipid mediators (Cravatt et al., 2001). The identification of an *Arabidopsis* cDNA clone encoding a functional NAE amidohydrolase re-enforces the similarities in NAE metabolism between plants and animal, but more importantly, provided a tool to alter endogenous NAE levels as a means to better understand the physiological significance and contribution of NAEs (Shrestha et al, 2003). Recently, the molecular identification of a functional homologue of the mammalian FAAH in *Arabidopsis* that converts NAEs to the corresponding FFAs and ethanolamine was reported. The cDNA from *Arabidopsis* was isolated, sequenced and predicted to encode a protein of 607 amino acids with 37% identity to rat FAAH within the amidase signature domain (18% over the entire length) with the complete conservation of the residues determined to be important for FAAH catalysis. A single transmembrane domain near the N terminus was predicted in the *Arabidopsis* protein sequence, similar to that of the rat FAAH protein (Shrestha et al., 2003). Kinetic parameters and inhibition data for

the recombinant *Arabidopsis* protein were consistent with these properties of the enzyme activity characterized previously in plant and animal systems (Shrestha et al., 2003). Functional homologues of the *Arabidopsis* FAAH (AtFAAH) also were identified in *Oryza sativa* (rice) and *Medicago truncatula* supporting a common mechanism for the regulation of NAE hydrolysis in diverse plant species (including dicot and monocot species; Shrestha et al., 2003).

Two AtFAAH T-DNA knockout lines with disruptions in the At5g64440 gene locus were identified among the publicly available T-DNA tagged *Arabidopsis* insertional mutant lines (Salk_095108 and Salk_118043; Shrestha, 2004). Transgenic lines overexpressing AtFAAH were generated by the Blancaflor laboratory (in the Columbia background) and were compared with T-DNA knockouts and wild type seeds and seedlings in terms of overall seedling growth (Shrestha, 2004). Seedlings of these overexpressor lines displayed less sensitivity toward exogenous-applied NAE12:0 compared to wild-type seedlings or vector controls, and this difference in their responses to elevated concentrations of NAE was most notable during extended exposures (e.g. >18 d; Blancaflor et al., 2003). Despite the high levels of exogenous NAE12:0, cotyledon and hypocotyl expansion of the overexpressors was not significantly inhibited. Yet, under these conditions, wild-type and vector control seedlings were severely stunted. The effect of NAE12:0 at concentrations at or above 30 μM caused a significant increase in radial expansion of the root and immunofluorescence microscopy revealed the occurrence of disorganized and fragmented microtubules in severely disrupted cell files close to the root tip with prolonged exposure to NAE12:0 (Blancaflor et al., 2003). In addition, results from electron-microscopic analysis and plants expressing modified green fluorescent protein

with an endoplasmic reticulum targeting sequence (mGFP5-ER) revealed abnormal ER dynamics, distorted and incomplete cell walls and the accumulation/aggregation of vesicles, suggesting the occurrence of defects in normal cell division (Blancaflor et al., 2003). It is noteworthy the pronounced dose-dependent reduction in root elongation rate was observed for seedlings grown in NAE12:0, but not for seedlings grown in either lauric acid (FFA 12:0) or *N*-palmitoylethanolamine (NAE16:0; Blancaflor et al., 2003). Also, the NAE treatment was not generally toxic to seedlings as, even at 100 µM NAE12:0, the rate of growth was not reduced to zero. Vital staining with propidium iodide indicated the cells remained viable. Moreover, roots reverted to normal growth rates when removed from NAE12:0 source (Blancaflor et al., 2003).

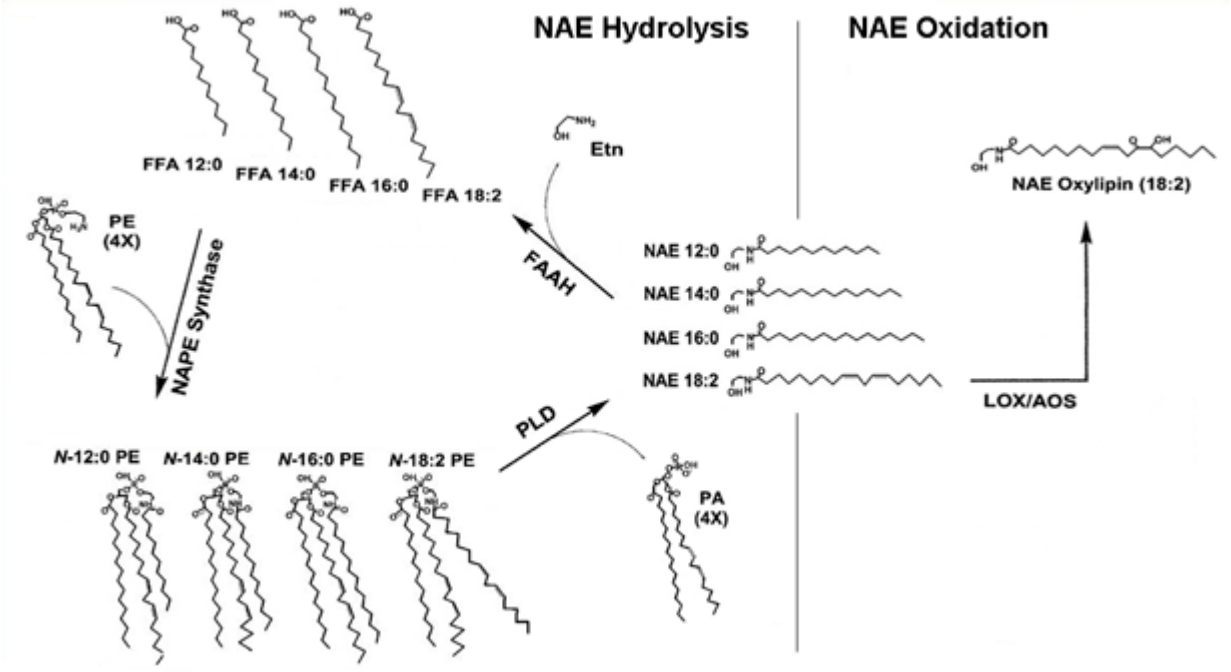

Figure 1. General scheme of NAE metabolism in plants. *N*-Acylated PEs (e.g. *N*-18:2 PE, etc.), the molecular precursor of the entire class of NAEs, are acted upon by a PLD-type phosphodiesterase, to create PA and a corresponding NAE. NAEs are hydrolyzed by AtFAAH to produce a FFA and ethanolamine. FFAs are utilized by NAPE synthase to

acylate the amino head group of PE and produce *N*-acyl PEs. A noteworthy competitive pathway to hydrolyzation by AtFAAH is the oxidation of NAEs containing two or more double bonds (e.g. NAEs 18:2, 18:3, etc.) by 13-lipoxygenase (LOX) to produce ethanolamide oxylipins (e.g. NAE oxylipin (18:2), etc.).

Abscisic acid (ABA), a plant stress hormone, is a sesquiterpene first described in the mid-1960s similar to other phytohormones with its influence on an array of both physiological and developmental events (Koornneef et al., 1998). In seeds, ABA is implicated in the regulation of protein and lipid reserve accumulation, the acquisition of desiccation tolerance, the inhibition of precocious germination and the induction of primary dormancy (Liotenberg, 1999). After embryonic pattern formation is complete, ABA concentrations rise while the embryo establishes dormancy and synthesizes storage reserves. In seedlings, ABA acts antagonistic to gibberillic acid, and its levels drop to allow for germination and post-germinative growth under normal circumstances. However if seedlings are exposed to osmotic stress, ABA levels increase and they induce growth arrest (Lopez-Molina et al., 2002). Endogenous ABA concentrations can rise and fall dramatically in response to several abiotic stresses such as drought, high salinity and low temperatures (Bonetta et al., 1998). Some of the ABA responses are long term and involve changes in gene expression while others are rapid and involve the modification of ion fluxes (Koornneef et al., 1998). For example, leaf ABA concentrations can increase 10- to 50-fold within a few hours of the onset of water deficiency and subsequent rewatering will return the concentrations to normal over the same time period (Zeevaart et al., 1988). So, in general, ABA is a negative regulator of germination and is activated by a variety of environmental stresses.

Various seedling growth defects similar to those described in ABA-treated seedlings were also seen in seeds germinated and grown in media containing *N*-lauroylethanolamine (NAE12:0). Relative to wild-type and empty-vector control seedlings, the AtFAAH overexpressors displayed less sensitivity and the knockout, without a functional AtFAAH enzyme, exhibited an increased sensitivity to exogenous NAE12:0 (Blancaflor et al., 2003). Root tips of wild-type and knockout seedlings germinated in a high concentration of exogenous NAE12:0 (35 μM) were shown to have irregular cell files and root cells with severe cytoskeletal disorganization resulting in short, bulbous root tips with impaired gravitropism (Blancaflor et al., 2003). Interestingly, exogenous ABA (0.25 nM) and NAE12:0 (35 μM), when added together, produced a substantially more severe effect on growth than either alone, suggesting a synergistic interaction between NAE and ABA (Motes, Cotter, Wang, Chapman and Blancaflor, poster). In addition, microarray analysis and quantitative, real-time (RT)-PCR of NAE-treated seedlings revealed elevated transcripts for a number of genes known to be regulated by ABA and increased levels of ABA after 7-8 days of growth (Teaster and Chapman et al., unpublished results). Collectively these results suggest the pathways of ABA and NAE signaling are strongly parallel and most likely interconnected.

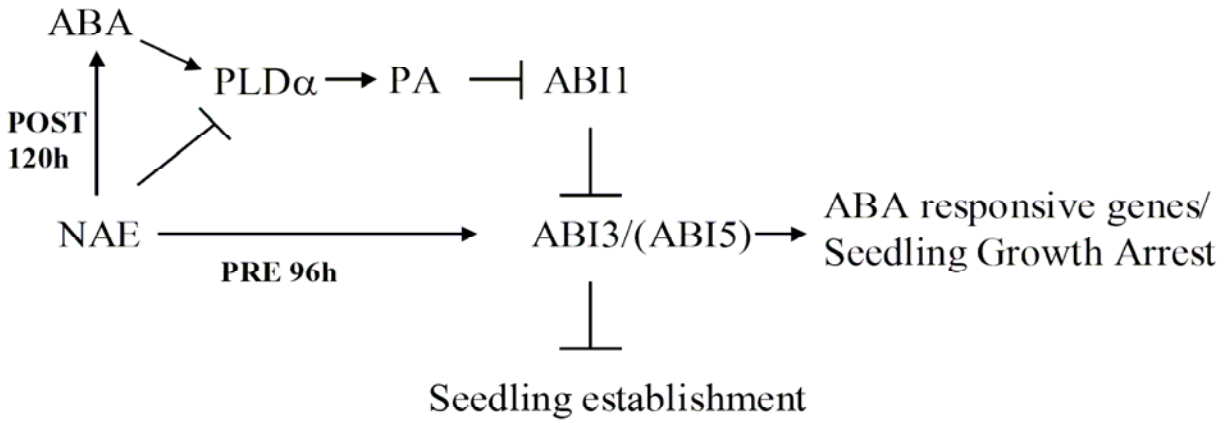

Figure 2. Working model of action of elevated ABA levels on seedling growth and development, and its relationship to NAEs. ABA regulates a PLDα/PA signaling pathway by inducing PLDα activity, which increases phosphatidic acid (PA). PA in turn reduces ABI1 (a phosphatase) activity, which maintains higher levels of ABI3/ABI5 (both transcription factors) and thus arrests growth. NAEs inhibit PLDα activity but increase the levels of ABI3 prior to germination (<96 h). It is possible that the effects of exogenously-treated NAE12:0 may influence a pathway parallel to ABA in early seed development and germination, but through ABA following germination and through post-germinative growth. Exogenously-treated NAE12:0 increases ABI3 expression prior to germination and leads to increased ABA concentrations after 120 h leading to a resumption of an embryo/dormancy program and seedling growth arrest in *Arabidopsis* seedlings reminiscent of the ABA-induced secondary dormancy program operable in young seedlings (Lopez-Molina et al., 2002).

In our working model (Fig. 2), ABA regulates a PLDα/PA signaling pathway (Wang, 2005). Results indicated that PLD plays multiple regulatory roles in diverse plant processes including ABA signaling, root growth, root hair patterning, freezing tolerance and other stress responses (Wang, 2005). Elevated ABA concentrations induce PLDα activity to increase PA levels. PA reduces ABI1 activity, a negative regulator of ABI3/ABI5-dependent gene expression. In seedlings, the activation of ABA-responsive gene expression leads to growth arrest (Foster et al., 1999). NAEs inhibit PLDα activity (Austin-Brown et al., 2002) but increase ABI3 levels in imbibed seeds (Teaster and Chapman, unpublished results). Preliminary results suggest exogenously-treated

NAE12:0 may influence a parallel pathway to ABA in pre-germinated seedlings and interact directly with the ABA pathway following germination and post-germinative growth. Exogenously-applied NAE12:0 increases ABI3 expression in seeds prior to germination and leads to increased ABA concentrations in seedlings after 120 h (Teaster and Chapman, unpublished results). These events likely promote a resumption of an embryo/dormancy program and seedling growth arrest reminiscent of the ABA-induced secondary dormancy program which is operable in young seedlings (Lopez-Molina et al., 2002). The validity of our model depends on the quantification of NAE profiles during seed germination and growth and ABA-induced arrest.

NAE metabolism is emerging as a central signaling pathway in animal (Schmid et al., 1996) and plant (Chapman, 1998) systems. It is likely that the regulation of NAE levels is important for normal plant growth and development and certain stress responses. While it is speculation, it is possible that NAEs act as endogenous inhibitors that must be removed or degraded during imbibition (Chapman et al., 1999) to allow for normal germination and development. Understanding the pathways involved in NAE metabolism and the characteristics of enzymes in these pathways is important to unraveling the physiological functions of these lipid metabolites in plants (Shrestha, 2004). There presently exists a strong demand to measure NAE levels in plant tissues during seed germination and seedling establishment in a variety of environments to investigate an ever-growing range of conditions associated with NAE metabolism. Therefore, the quantitative work described here is critical to understand the metabolism and function of NAEs *in planta*.

MATERIALS AND METHODS

Plant Materials and Chemicals

Arabidopsis M_3 seeds of *FAAH* insertional knockouts were provided by the Arabidopsis Biological Resource Stock Center, Ohio State University (Columbus OH). Seeds of AtFAAH overexpressor lines provided by E. Blancaflor of Samuel Roberts Noble Foundation (Ardmore OK). *Arabidopsis* growth supplies purchased from Arasystem (Gent Belgium) for soil culture and seed propagation. For liquid culture, Murashige-Skoog (MS) 10X basal salt macro- and micronutrient solutions (50 mL/L each), MES (500 mg/L), myo-inositol (100 mg/L) and sucrose (10 g/L), as well as vitamins nicotinic acid (0.5 mg/L), pyridoxine-HCl (0.5 mg/L) and thiamine (1 mg/L) were purchased from Fisher Scientific (Springfield NJ) and prepared accordingly (Murashige and Skoog, 1962). ABA was purchased from Sigma (St. Louis MO). Deuterated (d_4) NAEs used as internal standards were synthesized using deuterated ethanolamine from New England Nuclear (Boston MA) and acyl chlorides from Nu-Check Prep (Elysian MN). Glass tissue homogenizers used for extraction were purchased from Kontes (Vineland NJ). Millipore 13 mm Durapore™ polyvinylidene fluoride (PVDF) filters, with 0.22 µm pore size (Millipore Corp., Bedford MA, www.millipore.com), were purchased from Fisher Scientific (Springfield NJ). Bis(trimethylsilyl)trifluoroacetamide (BSTFA; derivatization agent) and vials, caps and glass LV self-centering inserts purchased from Alltech (Deerfield IL).

Seed Germination and Seedling Growth

Arabidopsis thaliana (ecotype: Columbia) seeds were surface-sterilized in 95% ethanol and 20% bleach followed by extensive washing with sterile, deionized water (MilliQ® UF plus, Millipore Corp., Bedford MA, www.millipore.com). Sterilized seeds were stratified (imbibed in the dark at 4°C) for 24 hours in sterile water. The low temperature during stratification allows for seed imbibition to occur but restricts germination or further development (Zhong et al., 1998). In imbibition experiments, wild-type seeds were processed for NAE quantification following 4, 12 and 24 hours of imbibition in the dark at 4°C (stratification), along with desiccated and surface sterilized seeds for direct comparison. Otherwise, seeds that were first surface sterilized and stratified were incubated in sealed flasks containing sterile liquid MS growth media (Blancaflor et al., 2003) on an orbital shaker (70-75 rpm) at 22°C under long day conditions (16 h of 50-70 μmol/m^2/s light). For germination and post-germinative growth experiments, wild-type seeds and/or seedlings were analyzed for NAE content in triplicate sets after 1, 2, 4 and 8 days of incubation in MS growth media. The exogenous ABA experiments were performed as above yet involved AtFAAH transgenic, mutant and wild-type seeds incubated in sealed flasks and extracted after 4 and 8 days in MS media under similar long day conditions. Images were captured on a Canon EOS™ 10D digital camera (Canon U.S.A., Inc., Lake Success NY, http://www.usa.canon.com/html/canonindex.html) using a Canon 50 mm compact-macro lens EF. Seed and seedling tissue was harvested by vacuum filtration, repeatedly pressed between filter paper to remove excess moisture and weighed gravimetrically for fresh weight determination.

Lipid Extraction

Our previous method for the extraction and identification/quantification by GC/MS has been described in previous studies (Chapman et al., 1999, 2003; Tripathy et al., 1999). However, the method described here relies on deuterated (d_4) NAEs as internal standards (Venables et al., 2005). Individual d_4 NAEs were synthesized by reacting d_4 ethanolamine with specific acyl chlorides (e.g., 12:0, 18:0 and 20:4; Giuffrida and Piomelli, 1999). Purity and concentration was confirmed by GC/MS. Concentrated stocks of three combined d_4 NAEs (12:0, 18:0 and 20:4) were diluted to 2.5 ppm prior to extraction and verified by GC/MS (Venables et al., 2005).

After fresh weight determination, 50-200 mg tissue samples were homogenized in glass tissue grinders containing 1 mL of 2-propanol heated to 70°C, to inhibit endogenous phospholipase activity (Chapman et al., 1998). To each extract, 100 µL of the d_4 internal standards at 2.5 ppm was added. NAE were extracted using a modified Bligh and Dyer (1959) lipid extraction method, but instead with a final solvent ratio of 2 mL: 1 mL: 0.45 mL [2-propanol: $CHCl_3$: H_2O] was maintained to ensure thorough penetration and extraction of soluble lipids in the monophasic solution. Following overnight extraction at 4°C, samples were centrifuged at 2000 rpm for >5 min to pellet fine particulate material. Supernatants were decanted and partitioned into 2 phases with the addition of 1 mL of $CHCl_3$. The aqueous (upper) phase was removed by aspiration and the organic layer was washed with 2 mL of KCl (1 M), mixed by vortexing, and centrifuged at 1000 rpm for >5 min. The aqueous layer was aspirated and the organic layer was washed two additional times as above. Total lipid extracts were evaporated to dryness under stream of N_2 gas, suspended in <1 mL of $CHCl_3$ and passed through a

syringe-fitted 0.22 µm PVDF filter. After again being evaporated to dryness under N_2 for gravimetric lipid mass determination, lipids were suspended in 160-180 µL of $CHCl_3$ for high-pressure liquid chromatography (HPLC).

NAE Chromatography and Quantification

Lipid extracts were subjected to normal-phase HPLC (Gilson 712; Middleton WI), using an Alltech 250 mm x 10 mm x 10µm semi-preparative Econosphere™ silica column (Deerfield IL, http://www.alltech-bio.com/index.cfm). The percentage of 2-propanol in the mobile phase (initially 100% hexane) was increased linearly to 10% by 5 min, and remained at 10% until minute 15 when it began its final increase to 50 %, to flush the column. An NAE elution window was collected (approx. 10-15 min.) and evaporated to dryness under N_2 stream. Residues were incubated in 50 µL of bis(trimethylsilyl)trifluoroacetamide (BSTFA) at 55°C for 30 min, to form NAE trimethylsilyl (TMS)-ether derivatives. After removal of BSTFA under N_2 stream, suspended residues were analyzed by gas chromatography mass spectrometry (GC/MS) with an Agilent (Palo Alto CA) 6890N gas chromatograph equipped with an Alltech Econocap™ EC-5 30 m x 250 µm x 0.25 mm capillary column (Deerfield IL, http://www.alltech-bio.com/index.cfm), coupled to an Agilent (Palo Alto CA) 5973 mass selective detector using an Agilent (Palo Alto CA) 7683 Series automated injector. The inlet temperature was 265°C at 7.1 psi and was operated in the pulsed splitless mode. Helium was the carrier gas and flowed at a constant flow rate of 1 mL/min. Following solvent delay, the oven temperature increased at 50°C/min to 220°C in 4.6 min, then slowed to 5°C /min until 285°C at minute 17.6. Following ionization, each d_4 and endogenous NAE yielded characteristic ions and

several confirming ions, in selective ion mode, that when detected unequivocally identified each NAE species (Table 1). Analytes were quantified against linear regression curves, prepared by injection of 5.0 ng of each internal standard with target NAEs ranging from 0.01-10 ng, using Environmental ChemStation™ D.01.02.15 software by Agilent (Palo Alto CA, www.home.agilent.com/). Original NAE concentrations were calculated on a fresh weight basis and on a per individual basis (Table 2).

RESULTS

NAE Profiles Through Imbibition and Post-Germinative Growth

Endogenous NAEs were quantified relative to three deuterated (d_4) NAEs, with acyl chains ranging from saturated 12C to polyunsaturated 18C chains. Only the saturated d_4 NAE12:0 and d_4 NAE18:0 were used for quantification purposes to avoid issues of unequal standard degradation and its distortional effect on quantification. These d_4 NAEs used as internal standards yielded primary quantitative ions 4 m/*z* units greater than their endogenous counterparts (Table 1) because of the four deuterium atoms bound to the ethanolamine head group.

The dynamic fluctuations in NAE content in *Arabidopsis* seeds through imbibition and post-germinative growth can be expressed in a variety of ways. Final NAE content was calculated on a fresh weight, per number of individuals and on a lipid weight basis. The total quantity of NAEs per individual plant largely increases in post-germinative growth (Table 2, Fig. 4) as new biomass is accumulated. The total concentration of NAEs, on a lipid weight basis (Table 2), also increases at germination and into post-germinative growth. Whereas, the total concentration of NAEs, on a fresh weight basis, decrease at germination and again in post-germinative growth (Table 2, Fig. 4).

The overall total NAE content per individual did not change during imbibition (Fig. 5A); however, this did not reflect the dynamic changes that occurred in the saturated and unsaturated NAE pools (Figs. 5C & 5D). Not until 24 h of stratification was there a significant ($p < 0.055$) reduction in total NAE content per individual (Fig. 5A). Examination of the individual molecular species profiles revealed that the quantity of the saturated NAEs more than doubled immediately upon imbibition (Fig. 5C). The saturated NAE

content remained higher than in desiccated seeds throughout the stratification period. Conversely, the total content of the unsaturated NAEs decreased following sterilization and into stratification (Fig. 5D). After 24 h of stratification, the total unsaturated NAE content was half that of desiccated seeds.

Molecular species profiles indicated that the pattern of unsaturated NAE concentrations in *Arabidopsis* seeds through imbibition and post-germinative growth (Fig. 6D) closely mirrored that of the total NAE concentrations (Fig. 6A) since saturated NAEs made up a small proportion of the total. Upon initiation of imbibition, the concentration of total NAEs (Fig. 6A), and unsaturated NAEs (Fig. 6D), rapidly dropped to a third of the levels in desiccated seeds. Concentrations remained at these reduced levels, or perhaps slightly increased, until the period of post-germinative growth (192 h), which coincided with another decrease in total and unsaturated NAE concentrations. Little or no change was observed in total saturated NAE concentrations through post-germinative growth (Fig. 6C).

Impact of *FAAH* Expression on NAE Profiles

Arabidopsis transgenic and mutant lines with altered *FAAH* expression were analyzed for NAE content relative to wild type (Fig. 7). Surprisingly, regardless of the overexpression of AtFAAH cDNA or knockout of the endogenous *FAAH* gene, by 96 h all genotypes tested had reduced their total NAE concentrations to levels identical to wild type (Fig. 7A). Total NAE concentrations also were similar in all genotypes as post-germinative growth progressed (192 h). By contrast, desiccated seeds revealed altered total NAE concentrations depending on the expression of *FAAH*. Seeds with an

insertional disruption in the gene locus encoding AtFAAH (At5g64440), shown to hydrolyze NAEs *in vitro* (Shrestha et al., 2003), had total NAE concentrations greater (25%) than wild type desiccated seeds (Compare Figs. 7A & 7B). Conversely, the constitutive overexpression of *FAAH* resulted in desiccated seeds with total NAE concentrations nearly 50% lower than wild type seeds (Compare Figs. 7C & 7D). Similarly, the quantity of total NAEs on a per individual basis in desiccated seed varied depending on the expression of *FAAH* (Fig. 8). The desiccated seeds of the *FAAH* knockout (SK095) had more NAEs per individual than the desiccated seeds of wild type (Compare Figs. 8A & 8B). The overexpression of *FAAH* resulted in seeds with less total NAE per individual than wild type (Compare Figs. 8A, 8C & 8D). Following 96 h, all genotypes tested had total NAE quantities indistinguishable from wild type seedlings.

Effect of ABA Treatment on NAE Profiles

The growth of *Arabidopsis* seedlings in exogenous NAE12:0 (35 μM) was severely reduced in a dose-dependent manner (Blancaflor et al., 2003; Motes et al., 2005) and these seedlings contained elevated levels of ABA and several ABA-regulated genes. (Teaster, Wiant, Cotter, Blancaflor and Chapman, poster). Moreover, low levels of ABA and NAE12:0 together produced a substantially more severe effect on growth than either alone, suggesting a synergistic interaction between NAE and ABA (Motes, Cotter, Wang, Chapman and Blancaflor, poster). Consequently, we wanted to test whether ABA treatment might lead to an increase in NAE content.

Germinating and growing seeds in liquid media containing a low concentration of ABA (0.25 μM) resulted in a dose-dependent reduction in growth (Figs. 9 & 10). At 96 h,

treated wild-type seeds appeared to be developmentally arrested and unable to progress beyond radical emergence (Figs. 9C-D). This phenotype was also seen in the different transgenic lines at 96 h (Figs. 9H, 9L & 9P) yet not seen in any of the untreated seedlings (Figs. 9B, 9F, 9J & 9N). At 192 h, the difference between the phenotypes of untreated and ABA-treated seedlings was much larger (Fig. 10). The development of treated wild-type seedlings had progressed to the onset of post-germinative growth, showing the accumulation of photosynthetic pigments (Fig. 10D), but remained substantially behind the development of untreated seedlings, also at 192 h (Fig 10B). The KO (SK095) displayed a phenotype similar to wild type (Fig 10H). Conversely, the *FAAH* overexpressors, principally OE 11A, showed a heightened sensitivity to exogenous ABA treatment (Figs. 10L & 10P). At 192 h, seeds of OE 11A looked no different than 96 h-old treated OE 11A seeds. None of the untreated *FAAH* transgenic seedlings showed any substantial difference in growth and maturation relative to wild-type seedlings (Figs. 10B, 10F, 10J & 10N).

The manipulation of endogenous *FAAH* expression had no effect on the total NAE concentration in 96 h- and 192 h-old mutant and transgenic seedlings grown in the presence of ABA (Fig. 11). On the other hand, ABA-treated wild-type seedlings had total NAE concentrations nearly 50% higher than in untreated seedlings of the same age. All transgenic lines showed similar total NAE concentrations when treated or untreated, relative to each other.

The total NAE content on a per individual basis was affected in ABA-treated seedlings (Fig. 12). Wild-type seedlings at 96 h had total NAE quantities over 100% larger than untreated wild-type seedlings. More striking, is the lack of accumulation of

NAEs by 192 h in all treated genotypes that was observed in untreated seedlings of all genotypes tested. Instead, the amount of total NAEs on a per individual basis dropped from 96 to 192 h in seedlings of all treated genotypes.

Growing the *FAAH* mutant lines in low concentrations of ABA (0.25 μM) had little or no effect on the total concentration of saturated NAEs (Fig. 13). Only at 192 h did seedlings of OE 11A exhibit a substantial increase in total saturated NAE concentration with ABA treatment, relative to other genotypes (Fig. 13B). The total saturated NAE content on a per individual basis was unaltered by ABA treatment in all genotypes tested at 96 h (Fig. 14B). However, at 192 h, none of the ABA-treated seedlings of any genotype appeared to accumulate saturated NAEs in contrast to untreated seedlings, and therefore remained an order of magnitude below the levels observed in untreated seedlings on a per individual basis (Fig. 14A). This suggests the synthesis of saturated NAEs during post-germinative growth may play a substantial role in the metabolic regulation of saturated NAE species in plant tissues.

Little effect of ABA treatment was seen in unsaturated NAE concentrations at either 96 or 192 h-old seedlings of any genotype (Fig. 13). The concentrations of total unsaturated NAEs in ABA-treated wild-type seedlings at 96 and 192 h (Fig. 13D) were ~2 times greater than untreated wild-type seedlings (Fig. 13C), whereas the *FAAH* knockout and overexpressor lines showed no substantial adjustment of their total unsaturated NAE concentrations with ABA treatment at either time point (Compare Figs. 13C and D).

The profiles of total unsaturated NAE content per individual depended on whether tissue was grown in the presence or absence of 0.25 μM ABA (Fig. 14). At 96 h, treated wild-type and KO SK095 seedlings (Fig. 14D) contained 4 and 2 times more unsaturated

NAEs on a per individual basis, respectively, than untreated seeds (Fig. 14C). From 96 to 192 h, the total unsaturated NAE content per individual decreased by more than half in all genotypes treated with ABA (Fig. 14D), opposite to the situation with untreated seedlings where unsaturated NAE types increased with seedling growth on a per individual basis.

Concentrations of NAEs 12:0 (Fig. 15), 14:0 (Fig. 17) and 18:0 (Fig. 21) in wild-type seedlings, as well as all transgenics, were unaffected by ABA treatment at either 96 or 192 h. One exception was OE 11A, which increased its concentration of NAE14:0 by 192 h, following ABA treatment (Fig. 17B). Despite the accumulation of the saturated NAEs 12:0 (Fig. 16A), 14:0 (Fig. 18A) and 18:0 (Fig. 22A) on a per individual basis seen in untreated seedlings, the amount of NAEs 12:0 (Fig. 16B), 14:0 (Fig. 18B) and 18:0 (Fig. 22B) on a per individual basis in ABA-treated seedlings did not substantially change. This is consistent with the trend observed for total saturated NAE (Fig. 12), suggesting that the coordinated regulation of saturated NAE accumulation/degradation is important for normal growth and development and that ABA treatment negatively affects the accumulation of saturated NAEs on a per individual basis.

ABA treatment had no effect on NAE16:0 concentrations (Fig. 19B) or NAE16:0 quantity on a per individual basis (Fig. 20B) at 96 h. Whereas, at 192 h, both *FAAH* overexpressor lines treated with ABA had metabolized endogenous NAE16:0 to below detectable limits (Figs. 19B & 20B). Also at 192 h, neither wild type nor KO SK095 seedlings showed any alteration in NAE16:0 concentrations or quantity per individual with ABA treatment.

The concentration of unsaturated NAEs 18:1 (Fig. 23B), 18:2 (Fig. 25B) and 18:3 (Fig. 27B) were higher in ABA-treated seedlings of the wild type and OE 10A at 96 h

compared to untreated seedlings, while the KO SK095 and OE 11A had unsaturated NAE concentrations no different from untreated seedlings at 96 h. The amount of unsaturated NAEs 18:1 (Fig. 24B), 18:2 (Fig. 26B) and 18:3 (Fig. 28B) on a per individual basis in 96 h-old seedlings was unchanged in the KO SK095 and OE 11A with ABA treatment. Similarly, ABA treatment had little or no effect on NAE18:1, NAE18:2 and NAE18:3 concentrations in 192 h-old seedlings. Whereas, with ABA treatment, wild-type seedlings had roughly 5 times more and OE 10A seedlings had 2 times more NAE18:1, NAE18:2 and NAE18:3 on a per individual basis at 96 h. In untreated seedlings, by 192 h, all genotypes had either maintained or increased the amount of NAE18:1 (Fig. 24A), NAE18:2 (Fig. 26A) and NAE18:3 (Fig. 28A) on a per individual basis. Yet, with ABA treatment, every genotype had reduced the amount of the unsaturated NAEs by more than 2 times, relative to untreated seedlings, suggesting ABA treatment may impair the ability of seedlings to accumulate unsaturated NAEs, and maintain similar NAE concentrations, coinciding with the increase in mass associated with growth.

The composition of NAE species in seeds and seedlings changed dramatically during post-germinative growth (Fig. 29). In desiccated seeds, nearly 90% of the total NAEs were unsaturated 18C species. In 192 h-old seedlings, the proportions of unsaturated 18C NAEs were 50% or less – knockouts (50%) having the largest percentage, then wild-type (40%) seedlings, followed by both overexpressors (25-30%). By contrast, the relative proportion of the total NAE pool for saturated NAE types (e.g. NAEs 12:0, 14:0, 16:0, 18:0) in 96 h-old seedlings was generally much higher than in desiccated seeds or 192 h-old seedlings. The knockout seedlings had the lowest percentage of saturated NAE (50%) followed by wild type (60%), followed by

overexpressors (70-75%). In other words, both NAE content and NAE composition changed during the course of seed germination and seedling growth, and this change in composition may be genotype dependent.

Treatment of seedlings with ABA altered this pattern of change in seedling NAE composition (Fig. 30). In wild-type seedlings treated with ABA, not only were there more total NAEs compared to untreated seedlings, the proportion of 18C unsaturated species in these seedlings was dramatically higher (80% in ABA vs. 40% without). *FAAH* knockout seedlings treated with ABA were similar to wild-type seedlings in that the percentage of 18C unsaturates was elevated. Overexpressors, which appeared hypersensitive to ABA treatment, reduced their 18C unsaturates to proportions similar to untreated seedlings. Here, however, there seemed to be some substantial differences in medium to long chain saturated NAEs. In ABA, *FAAH* overexpressing seedlings had no detectable NAE16:0, and in OE 11A, NAE14:0 made up 50% of the total NAE pool. Overall these results suggest that the metabolism of unsaturated NAEs in ABA-treated seedlings, at rates similar to untreated seedlings, result in a decreased ability to continue growth.

Table 1. NAEs and their respective quantitative ions. Selective ions (m/*z*) of d_4 and endogenous NAEs detected under selected ion monitoring. The quantitative ions corresponding to the d_4 internal standards (d_4 NAE12:0 and d_4 NAE18:0) are 4 m/*z* units greater than their endogenous counterparts (NAE12:0 and NAE18:0).

NAEs	Quantitative Ion (m/*z*)	Confirming Ion (m/*z*)	Retention Time (minutes)
d_4 12:0	304	228	8.06
12:0	300	272	8.07
14:0	328	300	9.73
d_4 18:0	388	312	14.01
16:0	356	328	11.84
18:0	384	309	14.03
18:1	382	397	13.73
18:2	380	395	13.69
18:3	378	303	13.79
d_4 20:4	408	332	15.03

Figure 3. Stages of *Arabidopsis* seed imbibition, germination, and post-germinative growth and development. Numbers refer to hours spent in liquid MS media at 22°C under long day conditions. All seeds, other than desiccated (Des), were surface sterilized and stratified in H_2O at 4°C for 24 h. Bar represents 5 mm.

Table 2. Total NAE content in seeds and seedlings on a per g fresh weight, per individual and per g lipid basis. Identical data set of NAEs quantified from 3 extractions of wild-type *Arabidopsis* normalized to either original fresh weight (FW), number of individuals, or lipid weight (LW).

	Per g FW (ng NAE/ g FW)	Per # of Indiv. (ng NAE/ 2500 individuals)	Per g LW (ng NAE/ g LW)
Desiccated	1805.8	93.4	6618.2
Stratified	526.4	76.9	5010.6
96 h	776.9	231.2	17191.8
192 h	323.0	736.4	20372.2

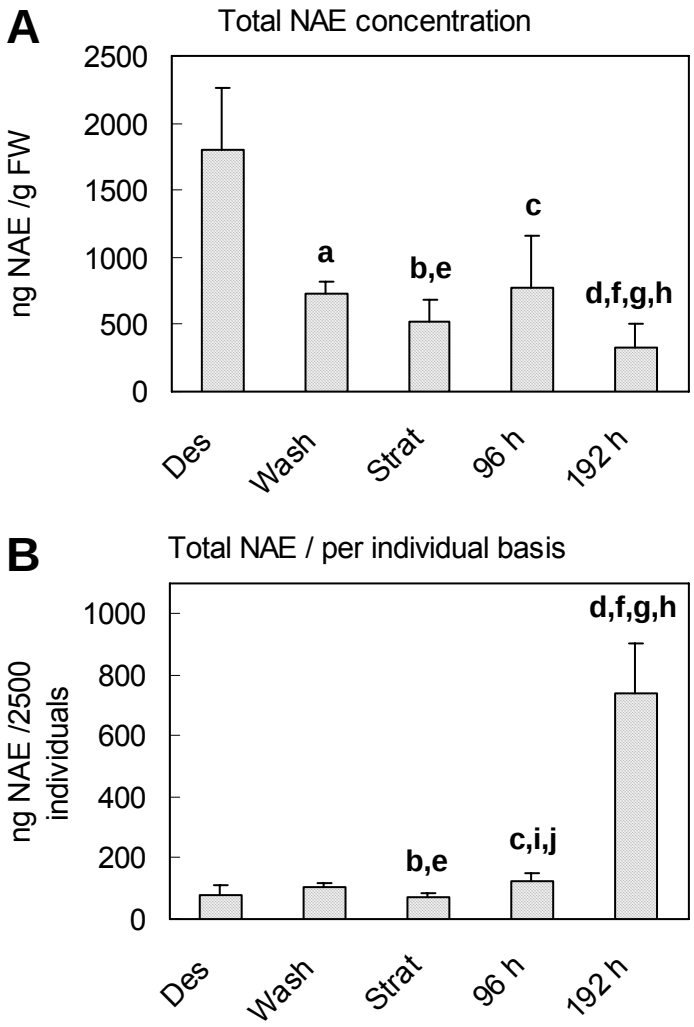

Figure 4. NAE quantification in desiccated and stratified seeds. Identical data set of total NAE content in desiccated (Des), sterilized (Wash), stratified (Strat, imbibed in H_2O at 4°C for 24 h), and germinated seeds expressed as nanograms per gram FW (A) or as nanograms per number of individuals (B, 2500 seeds ≈ 50 mg). NAE totals summed from individual molecular species profiles. Values compared by t-test. Only those comparisons considered to be significant ($p \leq 0.055$) are listed below. Error bars represent ± SD of three to sixteen replicate extractions.

Total NAE values per g FW:
(a) Des vs. Wash: $p < 0.02$
(b) Des vs. Strat: $p < 0.00025$
(c) Des vs. 96 h: $p < 0.0006$
(d) Des vs. 192 h: $p < 0.00000075$
(e) Wash vs. Strat: $p < 0.035$
(f) Wash vs. 192 h: $p < 0.0006$
(g) Strat vs. 192 h: $p < 0.0055$
(h) 96 h vs. 192 h: $p < 0.035$

Total NAE values per # of individuals:
(b) Des vs. Strat: $p < 0.045$
(c) Des vs. 96 h: $p < 0.003$
(d) Des vs. 192 h: $p < 0.000003$
(e) Wash vs. Strat: $p < 0.003$
(i) Wash vs. 96 h: $p < 0.04$
(f) Wash vs. 192 h: $p < 0.0008$
(j) Strat vs. 96 h: $p < 0.00085$
(g) Strat vs. 192 h: $p < 0.000002$
(h) 96 h vs. 192 h: $p < 0.005$

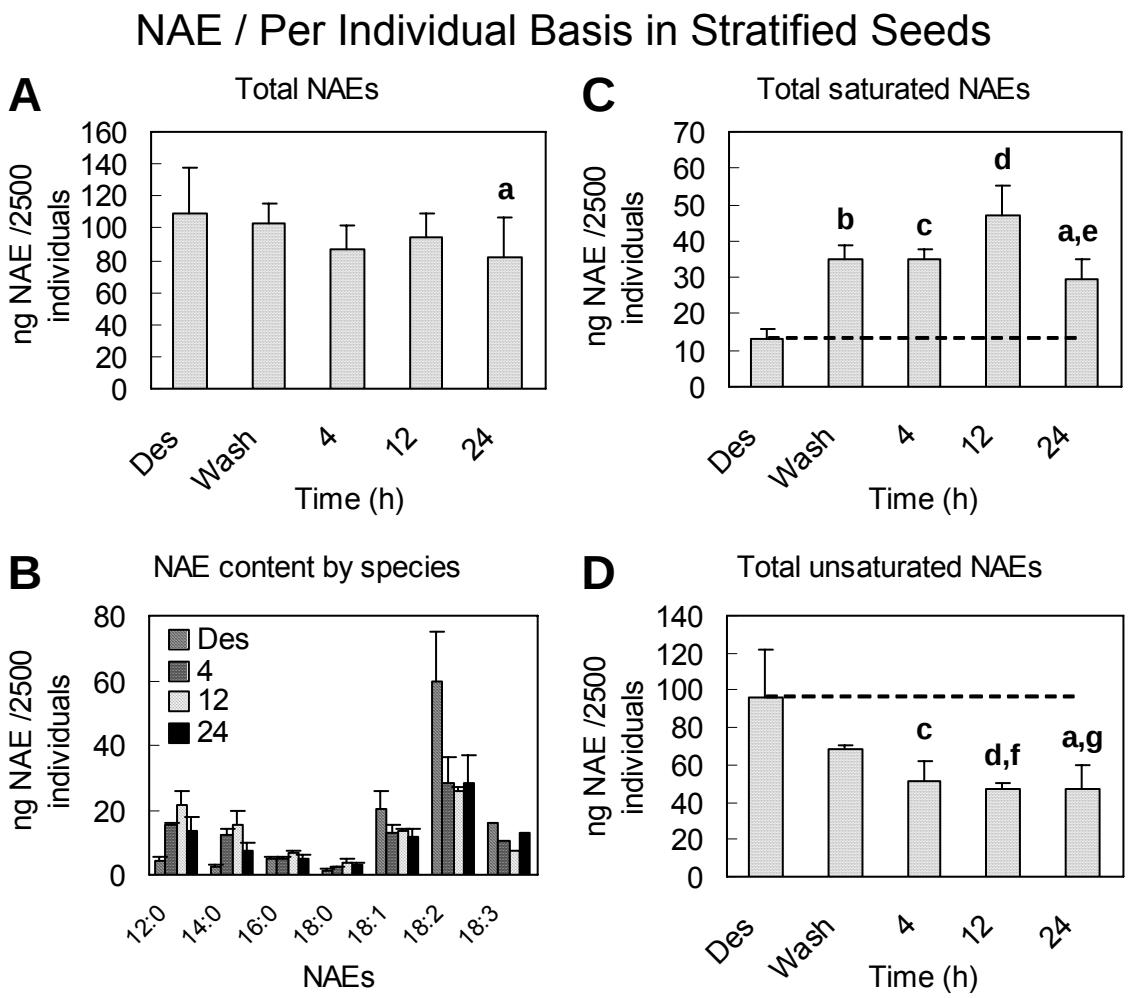

Figure 5. NAE quantity in seeds during stratification. NAE totals expressed as nanograms per number of individuals (2500 seeds ≈ 50 mg) in stratified (imbibed in H_2O at 4°C for 24 h) *Arabidopsis* (Col.) seeds. Desiccated (Des) and sterilized (Wash; ~30 min.) seed samples were immediately analyzed and others stratified. Total NAE content (A) summed from individual molecular species profiles (B). Total saturated NAE [12:0, 14:0, 16:0, 18:0] content (C) and 18C unsaturated NAE [18:1, 18:2, 18:3] content (D) summed from individual species profiles. Values compared by t-test. Only those comparisons considered to be significant ($p \leq 0.055$) are listed below. Error bars represent ± SD of three to six replicate extractions.

Total NAE values:
(a) Des vs. 24 h: $p < 0.03$

Total saturated NAE values:
(b) Des vs. Wash: $p < 0.00002$
(c) Des vs. 4 h: $p < 0.000015$
(d) Des vs. 12 h: $p < 0.00003$
(a) Des vs. 24 h: $p < 0.00007$
(e) 12 h vs. 24 h: $p < 0.0055$

Total unsaturated NAE values:
(c) Des vs. 4 h: $p < 0.03$
(d) Des vs. 12 h: $p < 0.02$
(a) Des vs. 24 h: $p < 0.002$
(f) Wash vs. 12 h: $p < 0.001$
(g) Wash vs. 24 h: $p < 0.03$

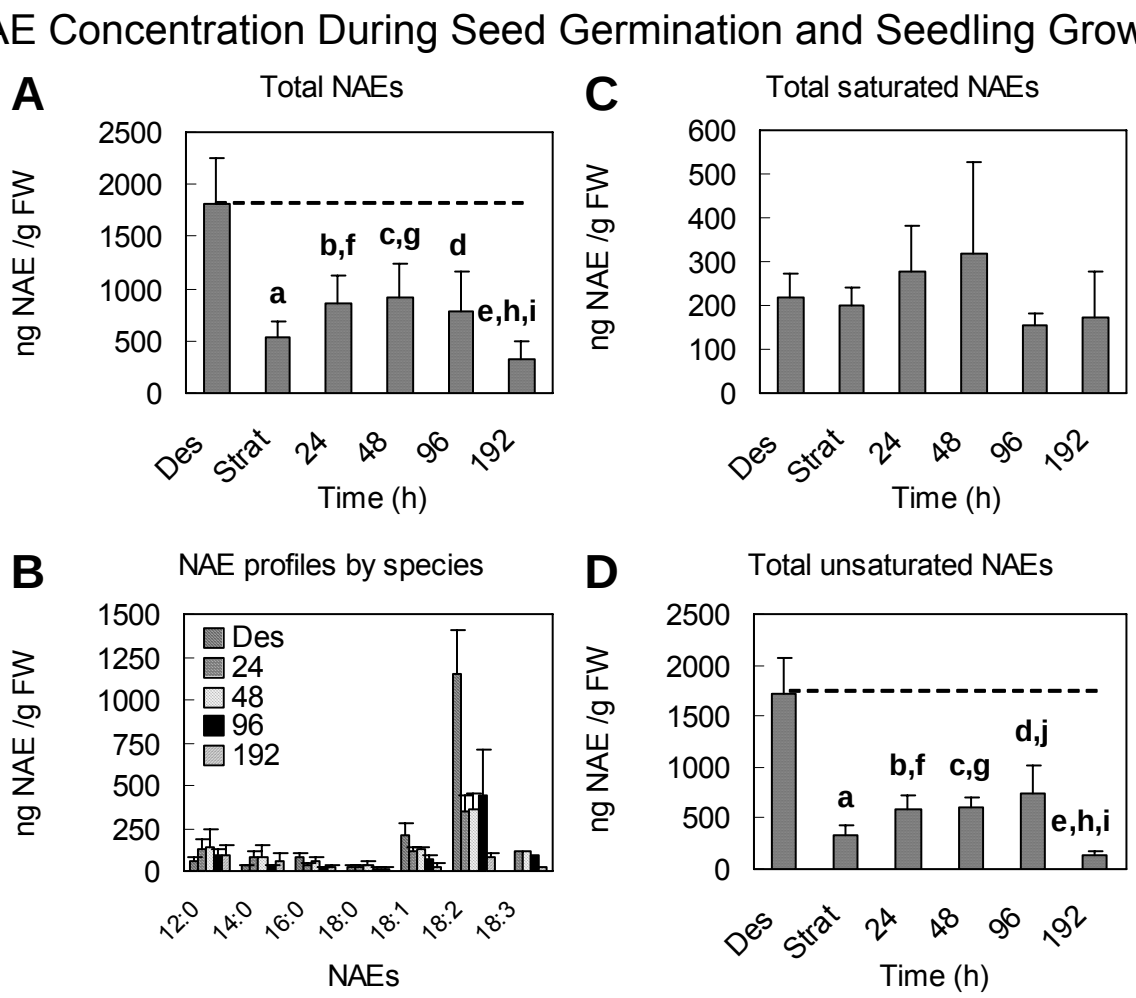

Figure 6. NAE concentration in seeds and seedlings during germination and post-germinative growth. Total NAE content expressed as nanograms per gram FW. Desiccated (Des) and 24 h stratified (Strat) seed samples were immediately analyzed and others germinated in liquid MS media, following stratification in H_2O at 4°C for 24 h. Totals (A) summed from individual molecular species profiles (B). Total saturated NAE [12:0, 14:0, 16:0, 18:0] content (C) and 18C unsaturated NAE [18:1, 18:2, 18:3] content (D) summed from individual species profiles. Values compared by t-test. Only those comparisons considered to be significant ($p \leq 0.055$) are listed below. Error bars represent ± SD of three to sixteen replicate extractions.

Total NAE values:
(a) Des vs. Strat: $p < 0.00025$
(b) Des vs. 24 h: $p < 0.04$
(c) Des vs. 48 h: $p < 0.055$
(d) Des vs. 96 h: $p < 0.0006$
(e) Des vs. 192 h: $p < 0.00000075$
(f) Strat vs. 24 h: $p < 0.015$
(g) Strat vs. 48 h: $p < 0.0045151788$
(h) Strat vs. 192 h: $p < 0.0055$
(i) 96 h vs. 192 h: $p < 0.035$

Total unsaturated NAE values:
(a) Des vs. Strat: $p < 0.00000005$
(b) Des vs. 24 h: $p < 0.00015$
(c) Des vs. 48 h: $p < 0.00015$
(d) Des vs. 96 h: $p < 0.0006$
(e) Des vs. 192 h: $p < 0.0000000015$
(f) Strat vs. 24 h: $p < 0.015$
(g) Strat vs. 48 h: $p < 0.006$
(j) Strat vs. 96 h: $p < 0.01$
(h) Strat vs. 192 h: $p < 0.0015$
(i) 96 h vs. 192 h: $p < 0.0003$

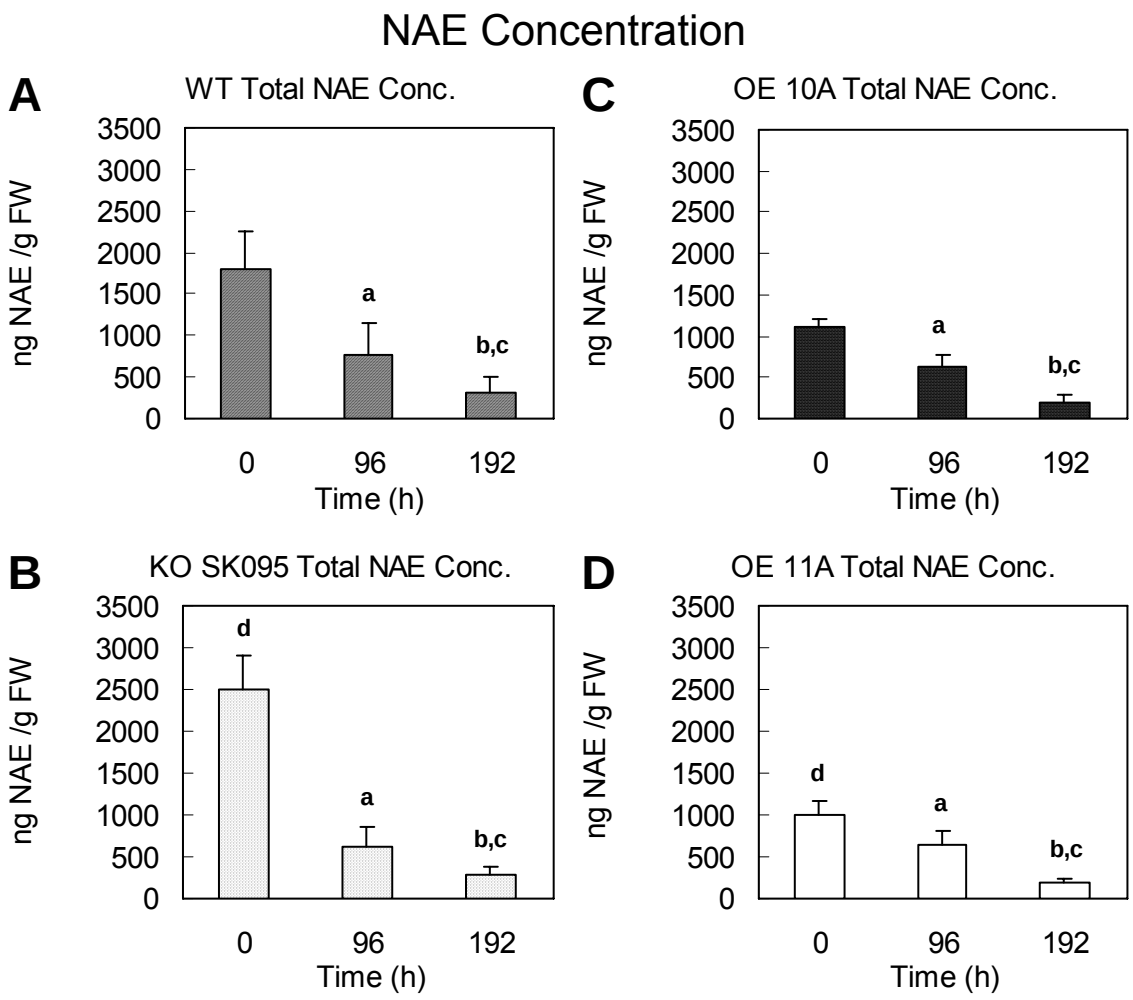

Figure 7. Total NAE concentration in desiccated, germinating and germinated seedlings. Seeds of wild type (A) and the *FAAH* mutants KO SK095 (B) OE 10A (C) OE 11A (D) were grown for 96 and 192 h in liquid MS media at 22°C under long day conditions, following sterilization and stratification in H_2O at 4°C for 24 h. Total NAE content, expressed in nanograms per gram FW, summed from molecular species profiles. Values compared by t-test. Only those comparisons considered significant ($p \leq 0.055$) are listed below. Bars represent ± SD of three to sixteen replicate extractions.

WT:
(a) 0 h vs 96 h: $p < 0.0006$
(b) 0 h vs 192 h: $p < 0.0000000015$
(c) 96 h vs 192 h: $p < 0.0003$

KO SK095:
(a) 0 h vs 96 h: $p < 0.0008$
(b) 0 h vs 192 h: $p < 0.000000065$
(c) 96 h vs 192 h: $p < 0.007$
(d) WT 0 h vs KO 0 h: $p < 0.0085$

OE 10A:
(a) 0 h vs 96 h: $p < 0.03$
(b) 0 h vs 192 h: $p < 0.000004$
(c) 96 h vs 192 h: $p < 0.0015$

OE 11A:
(a) 0 h vs 96 h: $p < 0.0015$
(b) 0 h vs 192 h: $p < 0.0000002$
(c) 96 h vs 192 h: $p < 0.00006$
(d) WT 0 h vs OE11 0 h: $p < 0.025$

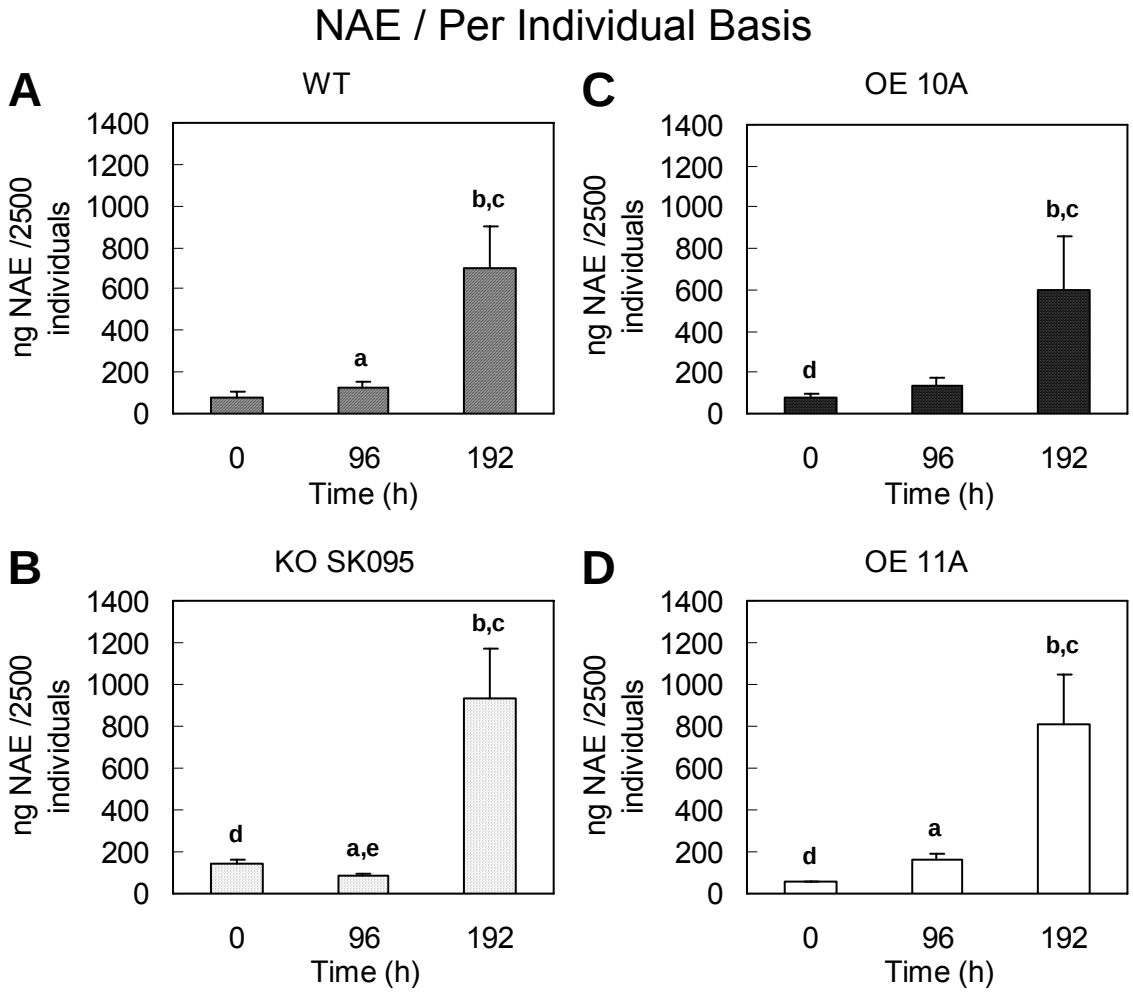

Figure 8. Total NAE quantity in desiccated, germinating and germinated seedlings. Seeds of wild type (A) and the *FAAH* mutants KO SK095 (B) OE 10A (C) OE 11A (D) were grown for 96 and 192 h in liquid MS media at 22°C under long day conditions, following sterilization and stratification in H_2O at 4°C for 24 h. Total NAE content, expressed in nanograms per 2500 individuals, summed from molecular species profiles. Values compared by t-test. Only those comparisons considered to be significant ($p \leq 0.055$) are listed below. Bars represent ± SD of three to sixteen replicate extractions.

WT:
(a) 0 h vs 96 h: p< 0.0034512616
(b) 0 h vs 192 h: p< 0.0000005267
(c) 96 h vs 192 h: p< 0.0007528182

KO SK095:
(a) 0 h vs 96 h: p< 0.0000105585
(b) 0 h vs 192 h: p< 0.0000032564
(c) 96 h vs 192 h: p< 0.0001081153
(d) WT 0 h vs KO 0 h: p< 0.0000028161
(e) WT 96 h vs KO 96 h: p< 0.0059430869

OE 10A:
(b) 0 h vs 192 h: p< 0.0003743955
(c) 96 h vs 192 h: p< 0.0040978106
(d) WT 0 h vs OE10 0 h: p< 0.0238407924

OE 11A:
(a) 0 h vs 96 h: p< 0.0000001000
(b) 0 h vs 192 h: p< 0.0001206036
(c) 96 h vs 192 h: p< 0.0204410463
(d) WT 0 h vs OE11 0 h: p< 0.0000043501

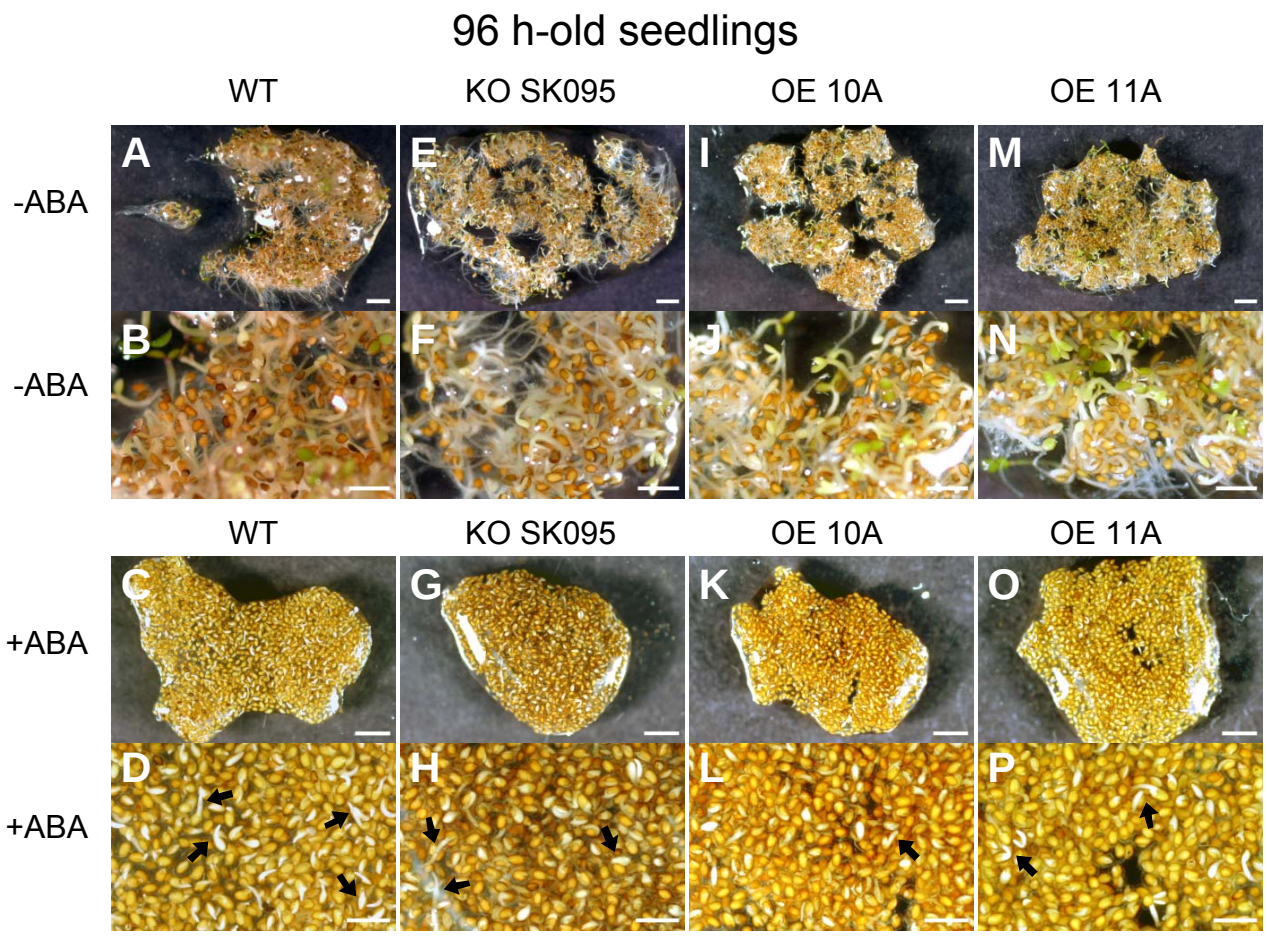

Figure 9. Effects of ABA on 96 h-old *Arabidopsis* mutants with altered *FAAH* activity. Wild type (A-D), KO SK095 (E-H), OE 10A (I-L) and OE 11A (M-P) seedlings grown in liquid MS media containing either solvent control (A-B, E-F, I-J, M-N) or 0.25 µM ABA (C-D, G-H, K-L, O-P). Each seed set (~30 mg of dry seed) was sterilized, stratified and grown under long day conditions, 16 h light for 96 h at 22°C. Arrows indicate radicles of ABA-treated seedlings. Bars on lower magnification images (A, E, I, M, C, G, K & O) represent 10 mm. Bars on higher magnification images (B, F, J, N, D, H, L & P) represent 5 mm.

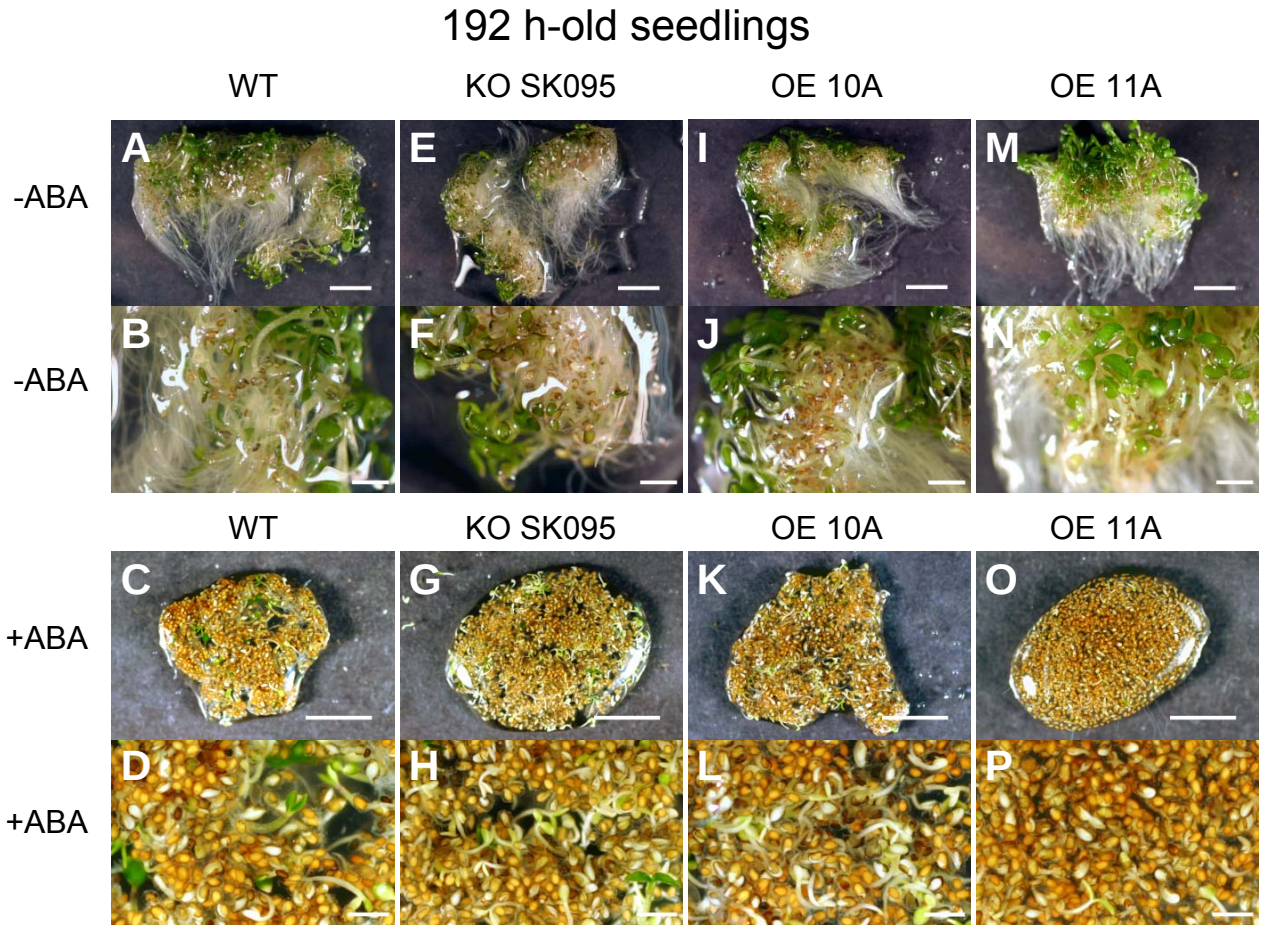

Figure 10. Effects of ABA on 192 h-old *Arabidopsis* mutants with altered *FAAH* activity. Wild type (A-D), KO SK095 (E-H), OE 10A (I-L) and OE 11A (M-P) seedlings grown in liquid MS media containing either solvent control (A-B, E-F, I-J & M-N) or 0.25 µM ABA (C-D, G-H, K-L & O-P). Each seed set (~30 mg of dry seed) was sterilized, stratified and grown under long day conditions, 16 h light for 192 h at 22°C. Bars on lower magnification images (A, E, I, M, C, G, K & O) represent 10 mm. Bars on higher magnification images (B, F, J, N, D, H, L & P) represent 5 mm.

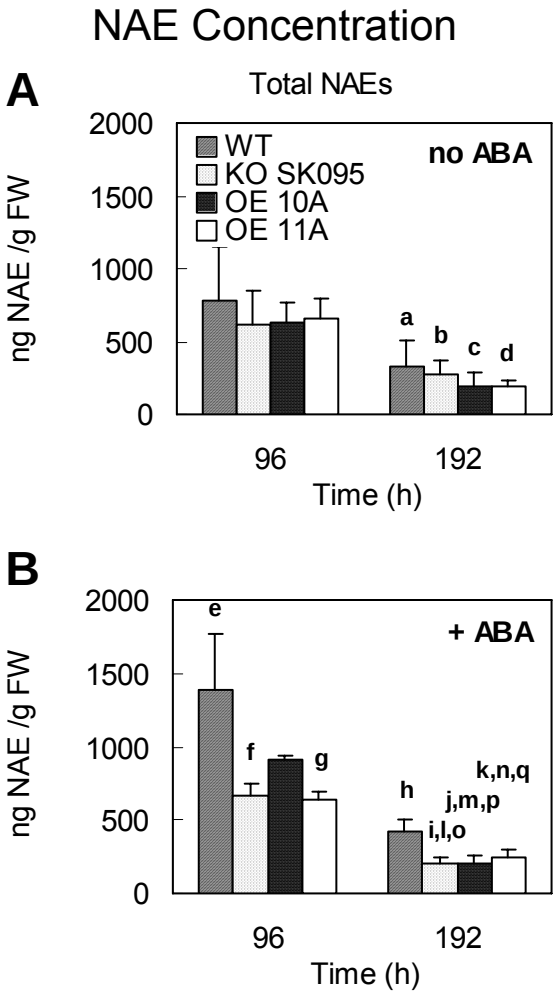

Figure 11. Impact of ABA treatment on NAE concentration in germinated seedlings. Seeds grown for 96 and 192 h in liquid MS media containing either solvent control (A) or 0.25 μM ABA (B) at 22°C under long day conditions, following sterilization and stratification in H_2O at 4°C for 24 h. Totals summed from molecular species profiles and expressed in nanograms per gram FW. Values compared by t-test. Only those comparisons considered to be significant ($p \leq 0.055$) are listed below. Error bars represent ± SD of three to nine individual extractions.

96 h vs. 192 h:
(a) WT 96 h vs. WT 192 h: $p < 0.05$
(b) KO 96 h vs. KO 192 h: $p < 0.015$
(c) OE10 96 h vs. OE10 192 h: $p < 0.0015$
(d) OE11 96 h vs. OE11 192 h: $p < 0.0015$

96 h vs. 96 h +ABA:
(e) WT 96 h vs. WT 96 h +ABA: $p < 0.030$

96 h +ABA vs. 192 h +ABA:
(f) WT 96 h +ABA vs. KO 96 h +ABA: $p < 0.055$
(g) WT 96 h +ABA vs. OE11 96 h +ABA: $p < 0.055$
(h) WT 96 h +ABA vs. WT 192 h +ABA: $p < 0.025$
(i) KO 96 h +ABA vs. KO 192 h +ABA: $p < 0.00006$
(j) OE10 96 h +ABA vs. OE10 192 h +ABA: $p < 0.02$
(k) OE11 96 h +ABA vs. OE11 192 h +ABA: $p < 0.015$
(l) WT 192 h +ABA vs. KO 192 h +ABA: $p < 0.0015$
(m) WT 192 h +ABA vs. OE10 192 h +ABA: $p < 0.00025$
(n) WT 192 h +ABA vs. OE11 192 h +ABA: $p < 0.015$

192 h vs. 192 h +ABA:
(o) KO 192 h vs. KO 192 h +ABA: $p < 0.025$
(p) OE10 192 h vs. OE10 192 h +ABA: $p < 0.05$
(q) OE11 192 h vs. OE11 192 h +ABA: $p < 0.035$

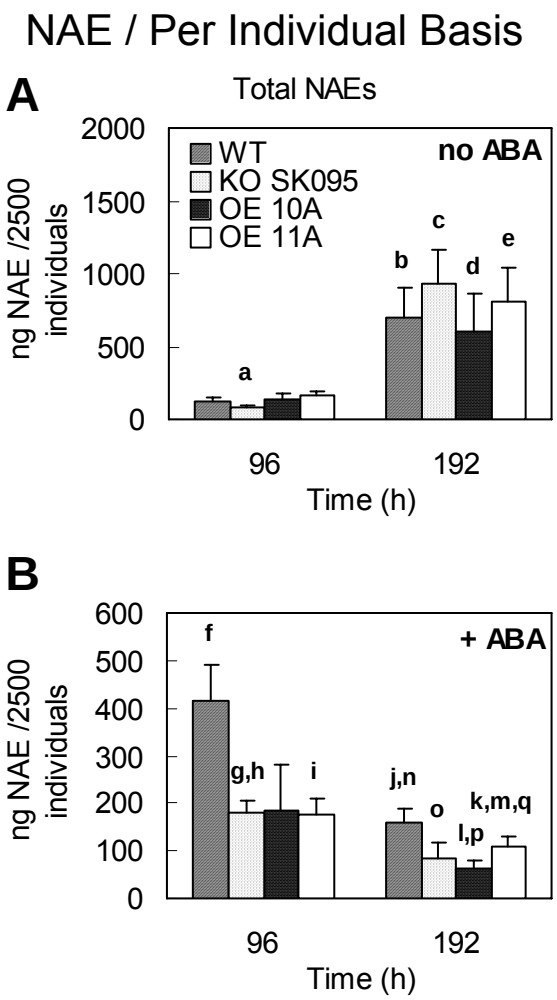

Figure 12. Impact of ABA treatment on NAE quantity in germinated seedlings. Seeds grown for 96 and 192 h in liquid MS media containing either solvent control (A) or 0.25 µM ABA (B) at 22°C under long day conditions, following sterilization and stratification in H_2O at 4°C for 24 h. Totals summed from molecular species profiles and expressed in nanograms per 2500 individuals. Values compared by t-test. Only those comparisons considered to be significant ($p \leq 0.055$) are listed below. Error bars represent ± SD of three to nine individual extractions.

96 h vs. 192 h:
(a) WT 96 h vs. KO 96 h: $p < 0.006$
(b) WT 96 h vs. WT 192 h: $p < 0.0008$
(c) KO 96 h vs. KO 192 h: $p < 0.00015$
(d) OE10 96 h vs. OE10 192 h: $p < 0.0045$
(e) OE11 96 h vs. OE11 192 h: $p < 0.025$

96 h vs. 96 h +ABA:
(f) WT 96 h vs. WT 96 h +ABA: $p < 0.002$
(g) KO 96 h vs. KO 96 h +ABA: $p < 0.00007$

96 h +ABA vs. 192 h +ABA:
(h) WT 96 h +ABA vs. KO 96 h +ABA: $p < 0.0027179103$
(i) WT 96 h +ABA vs. OE11 96 h +ABA: $p < 0.005$
(j) WT 96 h +ABA vs. WT 192 h +ABA: $p < 0.003$
(k) OE11 96 h +ABA vs. OE11 192 h +ABA: $p < 0.0055$
(l) WT 192 h +ABA vs. OE10 192 h +ABA: $p < 0.0035$
(m) WT 192 h +ABA vs. OE11 192 h +ABA: $p < 0.0025$

192 h vs. 192 h +ABA:
(n) WT 192 h vs. WT 192 h +ABA: $p < 0.0015$
(o) KO 192 h vs. KO 192 h +ABA: $p < 0.00015$
(p) OE10 192 h vs. OE10 192 h +ABA: $p < 0.00035$
(q) OE11 192 h vs. OE11 192 h +ABA: $p < 0.0035$

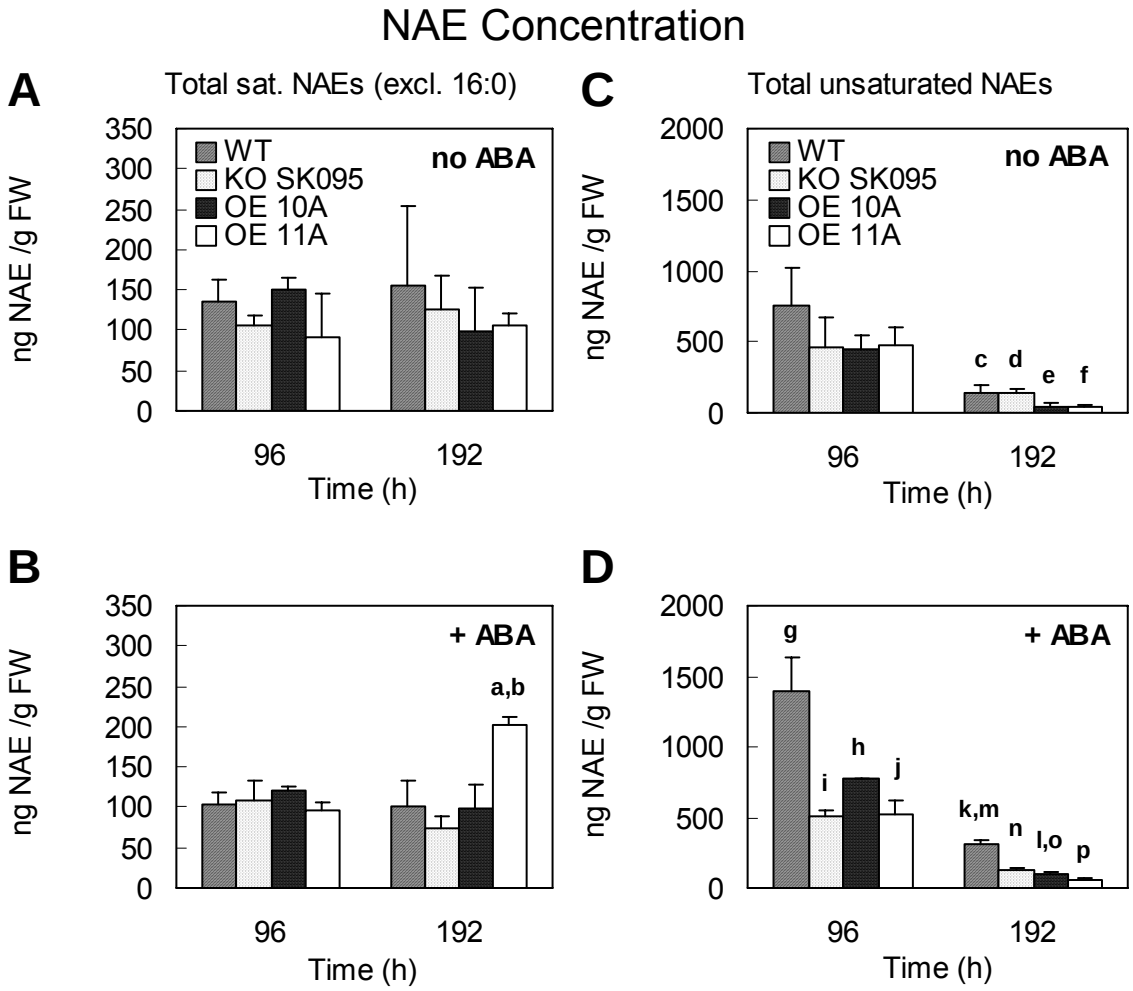

Figure 13. Effects of ABA treatment on total saturated and unsaturated NAE concentrations in germinated seedlings. Total saturated NAE [12:0, 14:0, 18:0, excluding 16:0] content (A-B) and 18C unsaturated NAE [18:1, 18:2, 18:3] content (C-D), expressed as nanograms per gram FW, summed from individual species profiles. Seeds grown for 96 and 192 h in liquid MS media containing either solvent control (A & C) or 0.25 µM ABA (B & D) at 22°C under long day conditions, following sterilization and stratification in H_2O at 4°C for 24 h. Values compared by t-test. Only those comparisons considered to be significant ($p \leq 0.055$) are listed below. Error bars represent ± SD of three to nine individual extractions.

Total saturated NAE values when treated:
(a) OE11 192 h vs. OE11 192 h +ABA: p<0.0035
(b) OE11 96 h +ABA vs. OE11 192 h +ABA: p<0.0015

Total unsaturated NAE values:
(c) WT 96 h vs. WT 192 h: p< 0.0003
(d) KO 96 h vs. KO 192 h: p< 0.005
(e) OE10 96 h vs. OE10 192 h: p< 0.00035
(f) OE11 96 h vs. OE11 192 h: p< 0.0002

Total unsaturated NAE values when treated:
(g) WT 96 h vs. WT 96 h +ABA: p< 0.03
(h) OE10 96 h vs. OE10 96 h +ABA: p< 0.02
(i) WT 96 h +ABA vs. KO 96 h +ABA: p< 0.055
(j) WT 96 h +ABA vs. OE11 96 h +ABA: p< 0.055
(k) WT 192 h vs. WT 192 h +ABA: p< 0.001
(l) OE10 192 h vs. OE10 192 h +ABA: p< 0.0015
(m) WT 96 h +ABA vs. WT 192 h +ABA: p< 0.0035
(n) KO 96 h +ABA vs. KO 192 h +ABA: p< 0.000015
(o) OE10 96 h +ABA vs. OE10 192 h +ABA: p< 0.0000000008
(p) OE11 96 h +ABA vs. OE11 192 h +ABA: p< 0.0015

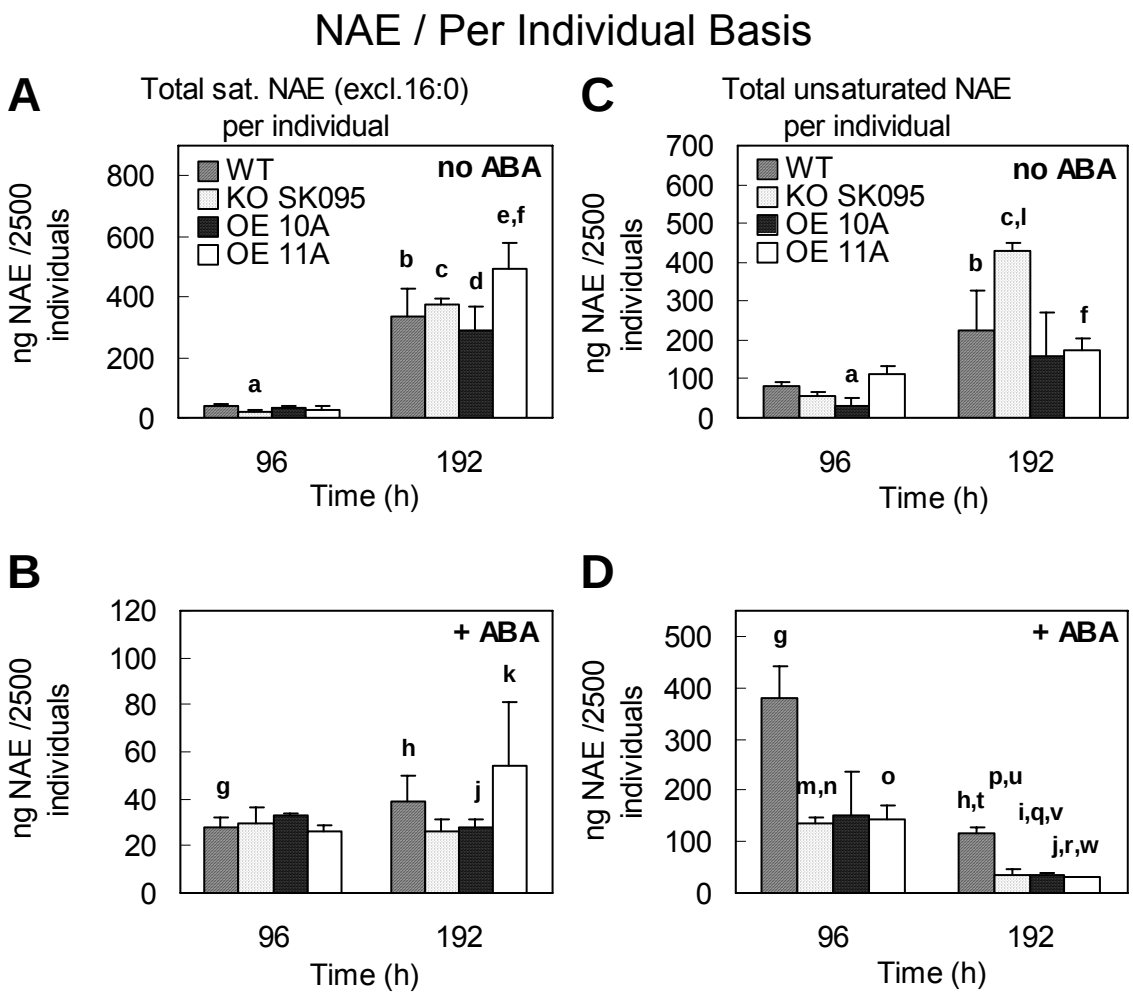

Figure 14. Impact of ABA treatment on total saturated and unsaturated NAE quantities in germinated seedlings. Total saturated NAE [12:0, 14:0, 18:0, excluding 16:0] content (A-B) and 18C unsaturated NAE [18:1, 18:2, 18:3] content (C-D), expressed as nanograms per 2500 individuals, summed from individual species profiles. Seeds grown for 96 and 192 h in liquid MS media containing solvent control (A & C) or 0.25 µM ABA (B & D) at 22°C under long day conditions, following sterilization and stratification in H_2O at 4°C for 24 h. Values compared by t-test. Only those comparisons considered to be significant ($p \leq 0.055$) are listed below. Error bars represent ± SD of three to nine individual extractions.

Total saturated NAE values:
(a) WT 96 h vs. KO 96 h: p< 0.006
(b) WT 96 h vs. WT 192 h: p< 0.0001
(c) KO 96 h vs. KO 192 h: p< 0.000015
(d) OE10 96 h vs. OE10 192 h: p< 0.00045
(e) OE11 96 h vs. OE11 192 h: p< 0.005
(f) WT 192 h vs. OE11 192 h: p< 0.05
Total saturated NAE values when treated:
(g) WT 96 h vs. WT 96 h +ABA: p< 0.05
(h) WT 192 h vs. WT 192 h +ABA: p< 0.002
(i) KO 192 h vs. KO 192 h +ABA: p< 0.000015
(j) OE10 192 h vs. OE10 192 h +ABA: p< 0.00075
(k) OE11 192 h vs. OE11 192 h +ABA: p< 0.001
Total unsaturated NAE values:
(b) WT 96 h vs. WT 192 h: p< 0.000015
(c) KO 96 h vs. KO 192 h: p< 0.00000055
(l) WT 192 h vs. KO 192 h: p< 0.000025
(f) WT 192 h vs. OE11 192 h: p< 0.009
Total unsaturated NAE values when treated:
(g) WT 96 h vs. WT 96 h +ABA: p< 0.00055
(m) KO 96 h vs. KO 96 h +ABA: p< 0.00085
(n) WT 96 h +ABA vs. KO 96 h +ABA: p< 0.006
(o) WT 96 h +ABA vs. OE11 96 h +ABA: p< 0.009
(p) WT 192 h +ABA vs. KO 192 h +ABA: p< 0.00025
(q) WT 192 h +ABA vs. OE10 192 h +ABA: p< 0.00001
(r) WT 192 h +ABA vs. OE11 192 h +ABA: p< 0.003
(h) WT 192 h vs. WT 192 h +ABA: p< 0.00035
(s) KO 192 h vs. KO 192 h +ABA: p< 0.00000002
(i) OE10 192 h vs. OE10 192 h +ABA: p< 0.0000000004
(j) OE11 192 h vs. OE11 192 h +ABA: p< 0.0075
(t) WT 96 h +ABA vs. WT 192 h +ABA: p< 0.005
(u) KO 96 h +ABA vs. KO 192 h +ABA: p< 0.000065
(v) OE10 96 h +ABA vs. OE10 192 h +ABA: p< 0.003
(w) OE11 96 h +ABA vs. OE11 192 h +ABA: p< 0.015

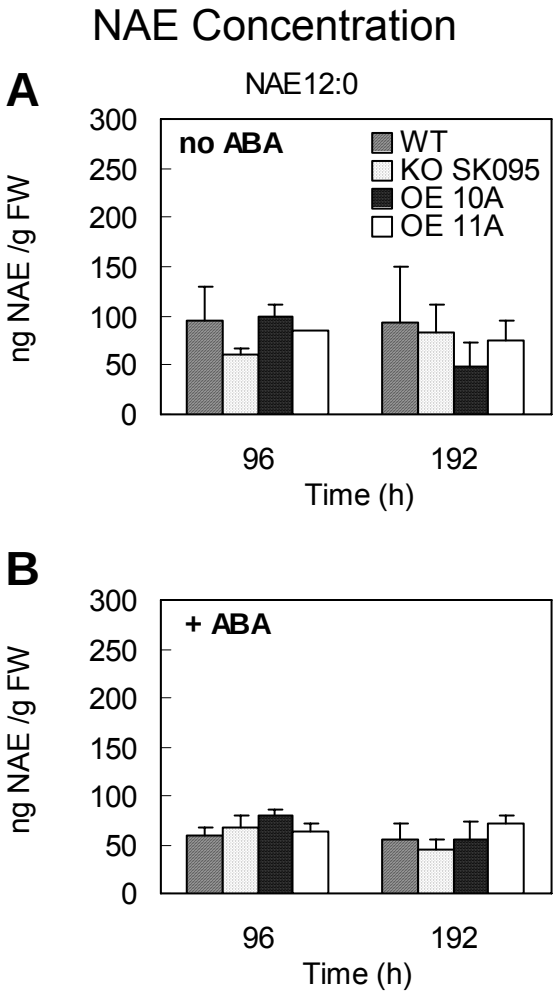

Figure 15. Effects of ABA treatment on NAE12:0 concentrations in germinated seedlings. Seeds grown for 96 and 192 h in liquid MS media containing either solvent control (A) or 0.25 μM ABA (B) at 22°C under long day conditions, following sterilization and stratification in H_2O at 4°C for 24 h. Values expressed as nanograms per gram FW. Values compared by t-test. Only those comparisons considered to be significant ($p \leq 0.055$) are listed below. Error bars represent ± SD of three to nine individual extractions.

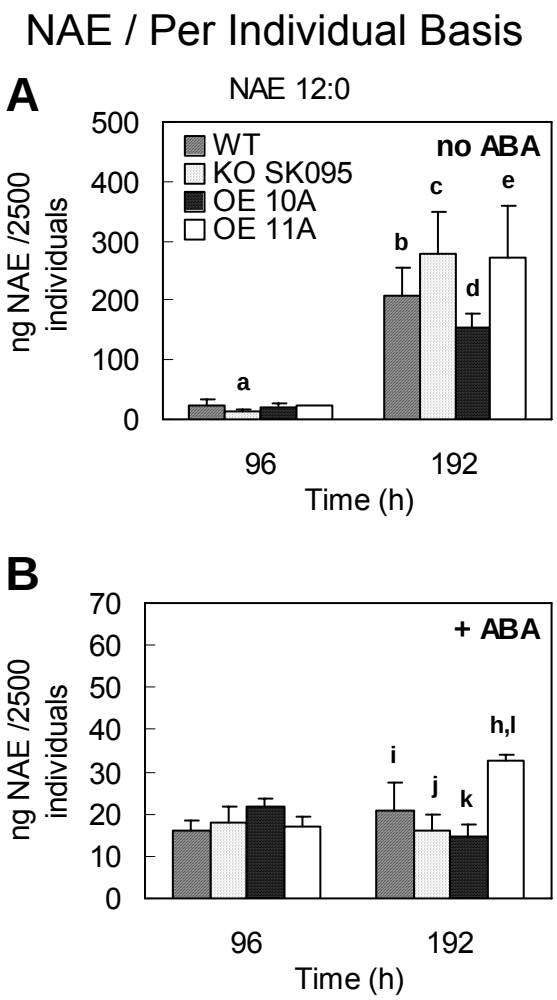

Figure 16. Effects of ABA treatment on NAE12:0 quantities in germinated seedlings. Seeds grown for 96 and 192 h in liquid MS media containing either solvent control (A) or 0.25 µM ABA (B) at 22°C under long day conditions, following sterilization and stratification in H_2O at 4°C for 24 h. Values expressed as nanograms per 2500 individuals. Values compared by t-test. Only those comparisons considered to be significant ($p \leq 0.055$) are listed below. Error bars represent ± SD of three to nine individual extractions.

96 h vs. 192 h:
(a) WT 96 vs. KO 96 h: $p < 0.03$
(b) WT 96 h vs. WT 192 h: $p < 0.00000045$
(c) KO 96 h vs. KO 192 h: $p < 0.0015$
(d) OE10 96 h vs. OE10 192 h: $p < 0.0001$
(e) OE11 96 h vs. OE11 192 h: $p < 0.035$

96 h vs. 96 h +ABA:

96 h +ABA vs. 192 h +ABA:
(h) OE11 96 h +ABA vs. OE11 192 h +ABA: $p < 0.0035$

192 h vs. 192 h +ABA:
(i) WT 192 h vs. WT 192 h +ABA: $p < 0.00025$
(j) KO 192 h vs. KO 192 h +ABA: $p < 0.0015$
(k) OE10 192 h vs. OE10 192 h +ABA: $p < 0.0005$
(l) OE11 192 h vs. OE11 192 h +ABA: $p < 0.00035$

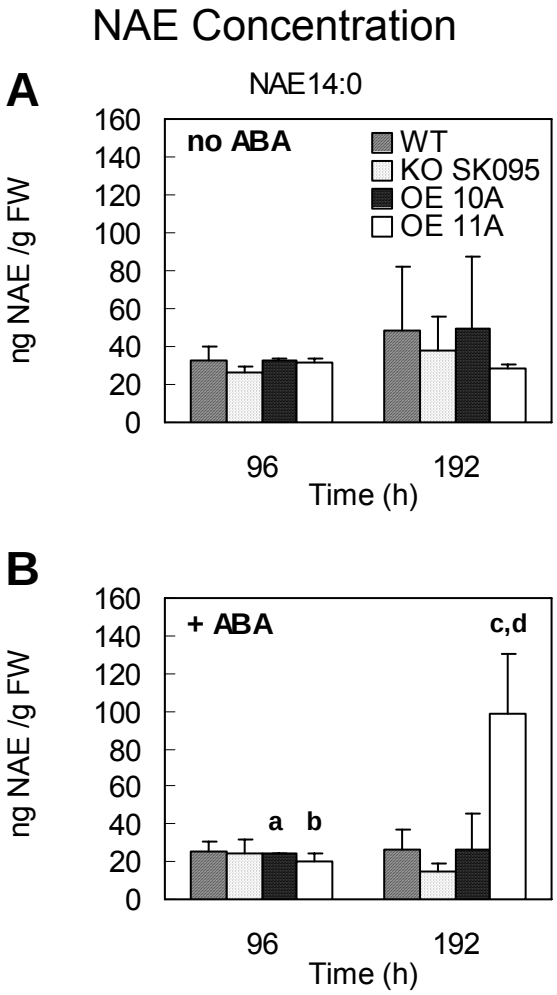

Figure 17. Effects of ABA treatment on NAE14:0 concentrations in germinated seedlings. Seeds grown for 96 and 192 h in liquid MS media containing either solvent control (A) or 0.25 μM ABA (B) at 22°C under long day conditions, following sterilization and stratification in H_2O at 4°C for 24 h. Values expressed as nanograms per gram FW. Values compared by t-test. Only those comparisons considered to be significant ($p \leq 0.055$) are listed below. Error bars represent ± SD of three to nine individual extractions.

96 h vs. 192 h:

96 h vs. 96 h +ABA:
(a) OE10 96 h vs. OE10 96 h +ABA: $p < 0.00035$
(b) OE11 96 h vs. OE11 96 h +ABA: $p < 0.03$

96 h +ABA vs. 192 h +ABA:
(c) OE11 96 h +ABA vs. OE11 192 h +ABA: $p < 0.015$
(d) WT 192 h +ABA vs. OE11 192 h +ABA: $p < 0.025$

192 h vs. 192 h +ABA:

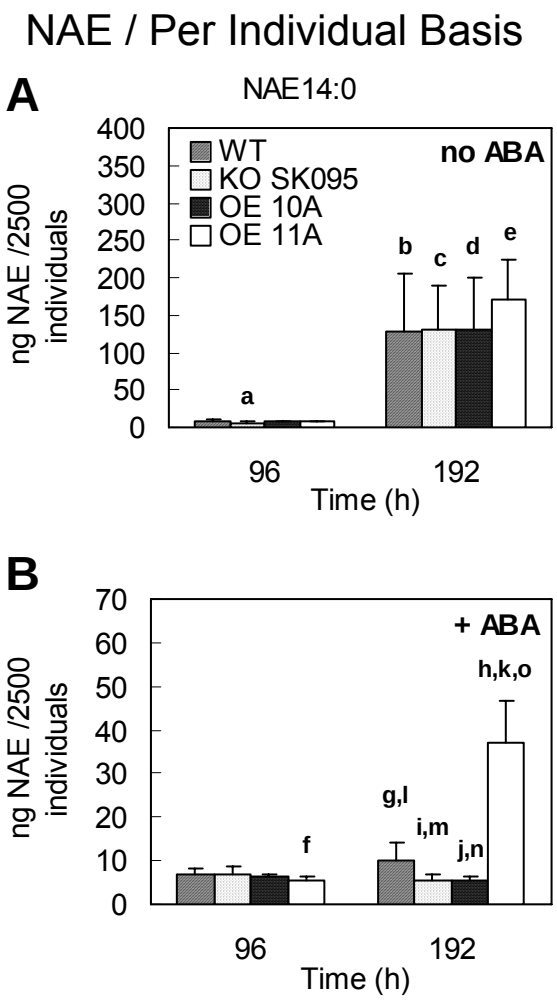

Figure 18. Impact of ABA treatment on NAE14:0 quantities in germinated seedlings. Seeds grown for 96 and 192 h in liquid MS media containing either solvent control (A) or 0.25 µM ABA (B) at 22°C under long day conditions, following sterilization and stratification in H_2O at 4°C for 24 h. Values expressed as nanograms per 2500 individuals. Values compared by t-test. Only those comparisons considered to be significant ($p \leq 0.055$) are listed below. Error bars represent ± SD of three to nine individual extractions.

96 h vs. 192 h:
(a) WT 96 vs. KO 96 h: $p < 0.055$
(b) WT 96 h vs. WT 192 h: $p < 0.0075$
(c) KO 96 h vs. KO 192 h: $p < 0.025$
(d) OE10 96 h vs. OE10 192 h: $p < 0.0055$
(e) OE11 96 h vs. OE11 192 h: $p < 0.025$

96 h vs. 96 h +ABA:
(f) OE11 96 h vs. OE11 96 h +ABA: $p < 0.03$

96 h +ABA vs. 192 h +ABA:
(g) WT 96 h +ABA vs. WT 192 h +ABA: $p < 0.055$
(h) OE11 96 h +ABA vs. OE11 192 h +ABA: $p < 0.0085$
(i) WT 192 h +ABA vs. KO 192 h +ABA: $p < 0.035$
(j) WT 192 h +ABA vs. OE10 192 h +ABA: $p < 0.025$
(k) WT 192 h +ABA vs. OE11 192 h +ABA: $p < 0.0075$

192 h vs. 192 h +ABA:
(l) WT 192 h vs. WT 192 h +ABA: $p < 0.004$
(m) KO 192 h vs. KO 192 h +ABA: $p < 0.0025$
(n) OE10 192 h vs. OE10 192 h +ABA: $p < 0.003$
(o) OE11 192 h vs. OE11 192 h +ABA: $p < 0.015$

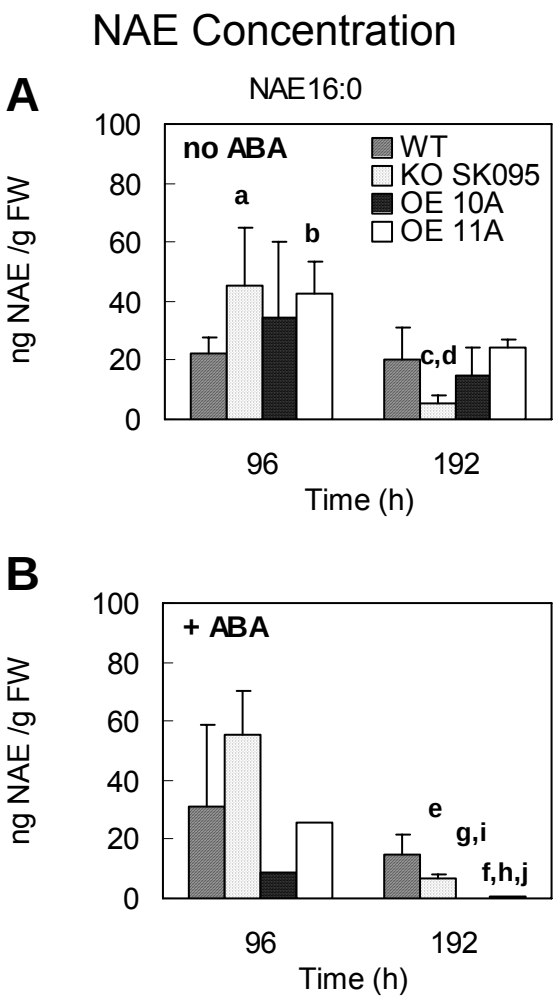

Figure 19. Impact of ABA treatment on NAE16:0 concentrations in germinated seedlings. Seeds grown for 96 and 192 h in liquid MS media containing either solvent control (A) or 0.25 µM ABA (B) at 22°C under long day conditions, following sterilization and stratification in H_2O at 4°C for 24 h. Values expressed as nanograms per gram FW. Values compared by t-test. Only those comparisons considered to be significant ($p \leq 0.055$) are listed below. Error bars represent ± SD of three to nine individual extractions.

96 h vs. 192 h:
(a) WT 96 h vs. KO 96 h: $p < 0.04$
(b) WT 96 h vs. OE11 96 h: $p < 0.02$
(c) KO 96 h vs. KO 192 h: $p < 0.035$
(d) WT 192 h vs. KO 192 h: $p < 0.05$

96 h vs. 96 h +ABA:

96 h +ABA vs. 192 h +ABA:
(e) KO 96 +ABA vs. KO 192 h +ABA: $p < 0.045$
(f) OE11 96 h +ABA vs. OE11 192 h +ABA: $p < 0.03$
(g) WT 192 h +ABA vs. OE10 192 h +ABA: $p < 0.00055$
(h) WT 192 h +ABA vs. OE11 192 h +ABA: $p < 0.025$

192 h vs. 192 h +ABA:
(i) OE10 192 h vs. OE10 192 h +ABA: $p < 0.0035$
(j) OE11 192 h vs. OE11 192 h +ABA: $p < 0.00045$

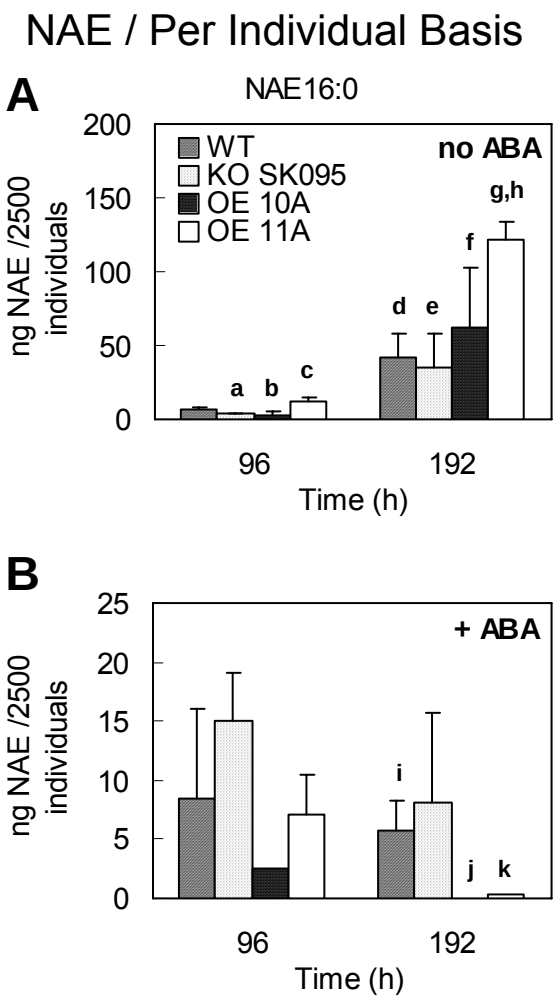

Figure 20. Effects of ABA treatment on NAE16:0 quantities in germinated seedlings. Seeds grown for 96 and 192 h in liquid MS media containing either solvent control (A) or 0.25 µM ABA (B) at 22°C under long day conditions, following sterilization and stratification in H_2O at 4°C for 24 h. Values expressed as nanograms per 2500 individuals. Values compared by t-test. Only those comparisons considered to be significant ($p \leq 0.055$) are listed below. Error bars represent ± SD of three to nine individual extractions.

96 h vs. 192 h:
(a) WT 96 h vs. KO 96 h: $p < 0.03$
(b) WT 96 h vs. OE10 96 h: $p < 0.035$
(c) WT 96 h vs. OE11 96 h: $p < 0.025$
(d) WT 96 h vs. WT 192 h: $p < 0.0015$
(e) KO 96 h vs. KO 192 h: $p < 0.002$
(f) OE10 96 h vs. OE10 192 h: $p < 0.025$
(g) OE11 96 h vs. OE11 192 h: $p < 0.0075$
(h) WT 192 h vs. OE11 192 h: $p < 0.004$

96 h vs. 96 h +ABA:

96 h +ABA vs. 192 h +ABA:

192 h vs. 192 h +ABA:
(i) WT 192 h vs. WT 192 h +ABA: $p < 0.04$
(j) OE10 192 h vs. OE10 192 h +ABA: $p < 0.01$
(k) OE11 192 h vs. OE11 192 h +ABA: $p < 0.001$

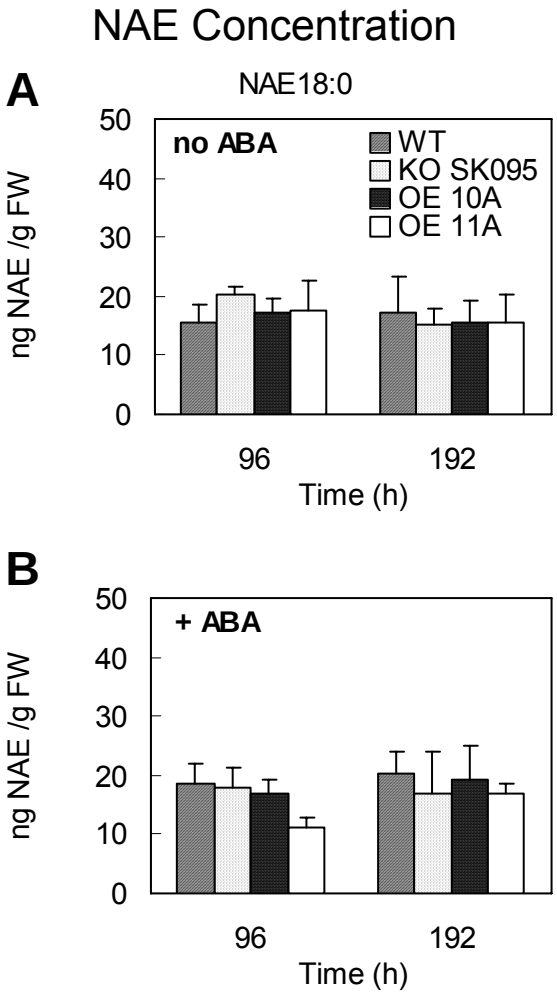

Figure 21. Impact of ABA treatment on NAE18:0 concentrations in germinated seedlings. Seeds grown for 96 and 192 h in liquid MS media containing either solvent control (A) or 0.25 µM ABA (B) at 22°C under long day conditions, following sterilization and stratification in H_2O at 4°C for 24 h. Values expressed as nanograms per gram FW. Values compared by t-test. Only those comparisons considered to be significant ($p \leq 0.055$) are listed below. Error bars represent ± SD of three to nine individual extractions.

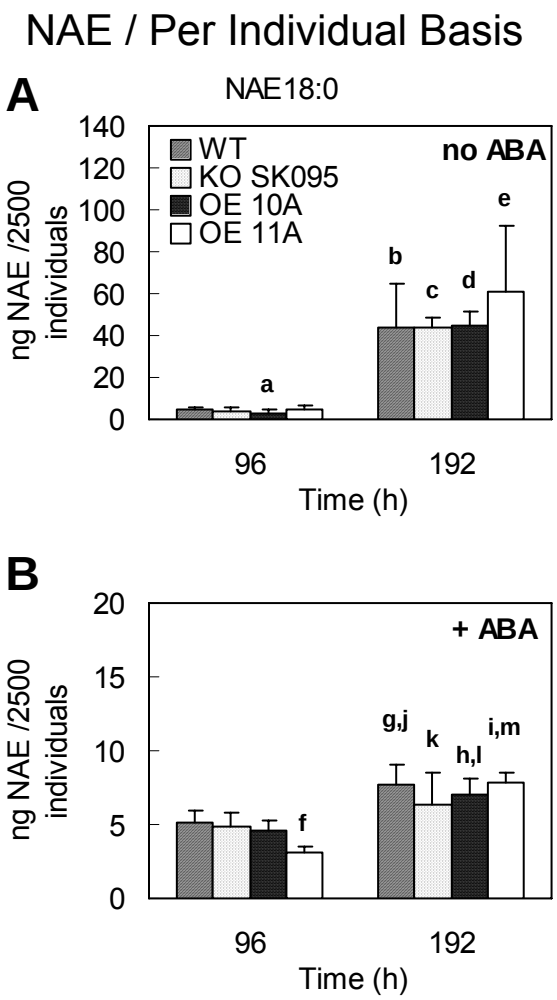

Figure 22. Effects of ABA treatment on NAE18:0 quantities in germinated seedlings. Seeds grown for 96 and 192 h in liquid MS media containing either solvent control (A) or 0.25 µM ABA (B) at 22°C under long day conditions, following sterilization and stratification in H_2O at 4°C for 24 h. Values expressed as nanograms per 2500 individuals. Values compared by t-test. Only those comparisons considered to be significant ($p \leq 0.055$) are listed below. Error bars represent ± SD of three to nine individual extractions.

96 h vs. 192 h:
(a) WT 96 h vs. OE10 96 h: $p < 0.015$
(b) WT 96 h vs. WT 192 h: $p < 0.000015$
(c) KO 96 h vs. KO 192 h: $p < 0.00000015$
(d) OE10 96 h vs. OE10 192 h: $p < 0.0000008$
(e) OE11 96 h vs. OE11 192 h: $p < 0.04$

96 h vs. 96 h +ABA:

96 h +ABA vs. 192 h +ABA:
(f) WT 96 h +ABA vs. OE11 96 h +ABA: $p < 0.025$
(g) WT 96 h +ABA vs. WT 192 h +ABA: $p < 0.055$
(h) OE10 96 h +ABA vs. OE10 192 h +ABA: $p < 0.045$
(i) OE11 96 h +ABA vs. OE11 192 h +ABA: $p < 0.0006$

192 h vs. 192 h +ABA:
(j) WT 192 h vs. WT 192 h +ABA: $p < 0.0015$
(k) KO 192 h vs. KO 192 h +ABA: $p < 0.00000025$
(l) OE10 192 h vs. OE10 192 h +ABA: $p < 0.000015$
(m) OE11 192 h vs. OE11 192 h +ABA: $p < 0.015$

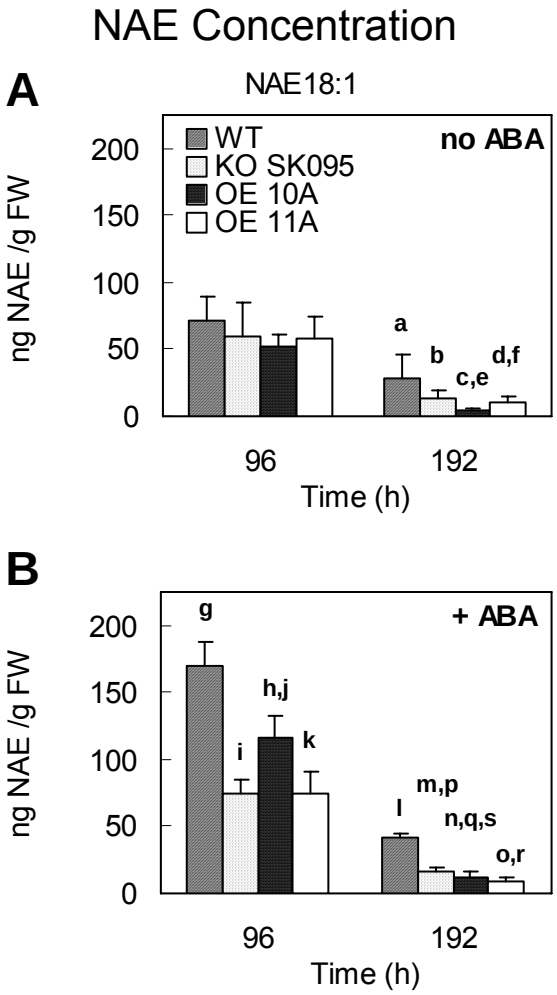

Figure 23. Impact of ABA treatment on NAE18:1 concentrations in germinated seedlings. Seeds grown for 96 and 192 h in liquid MS media containing either solvent control (A) or 0.25 µM ABA (B) at 22°C under long day conditions, following sterilization and stratification in H_2O at 4°C for 24 h. Values expressed as nanograms per gram FW. Values compared by t-test. Only those comparisons considered to be significant ($p \leq 0.055$) are listed below. Error bars represent ± SD of three to nine individual extractions.

96 h vs. 192 h:
(a) WT 96 h vs. WT 192 h: p< 0.002
(b) KO 96 h vs. KO 192 h: p< 0.0025
(c) OE10 96 h vs. OE10 192 h: p< 0.00015
(d) OE11 96 h vs. OE11 192 h: p< 0.0006
(e) WT 192 h vs. OE10 192 h: p< 0.03
(f) WT 192 h vs. OE11 192 h: p< 0.05

96 h vs. 96 h +ABA:
(g) WT 96 h vs. WT 96 h +ABA: p< 0.0015
(h) OE10 96 h vs. OE10 96 h +ABA: p< 0.01

96 h +ABA vs. 192 h +ABA:
(i) WT 96 h +ABA vs. KO 96 h +ABA: p< 0.005
(j) WT 96 h +ABA vs. OE10 96 h +ABA: p< 0.055
(k) WT 96 h +ABA vs. OE11 96 h +ABA: p< 0.0085
(l) WT 96 h +ABA vs. WT 192 h +ABA: p< 0.001
(m) KO 96 h +ABA vs. KO 192 h +ABA: p< 0.00003
(n) OE10 96 h +ABA vs. OE10 192 h +ABA: p< 0.00003
(o) OE11 96 h +ABA vs. OE11 192 h +ABA: p< 0.003
(p) WT 192 h +ABA vs. KO 192 h +ABA: p< 0.00001
(q) WT 192 h +ABA vs. OE10 192 h +ABA: p< 0.00006
(r) WT 192 h +ABA vs. OE11 192 h +ABA: p< 0.000085

192 h vs. 192 h +ABA:
(s) OE10 192 h vs. OE10 192 h +ABA: p< 0.035

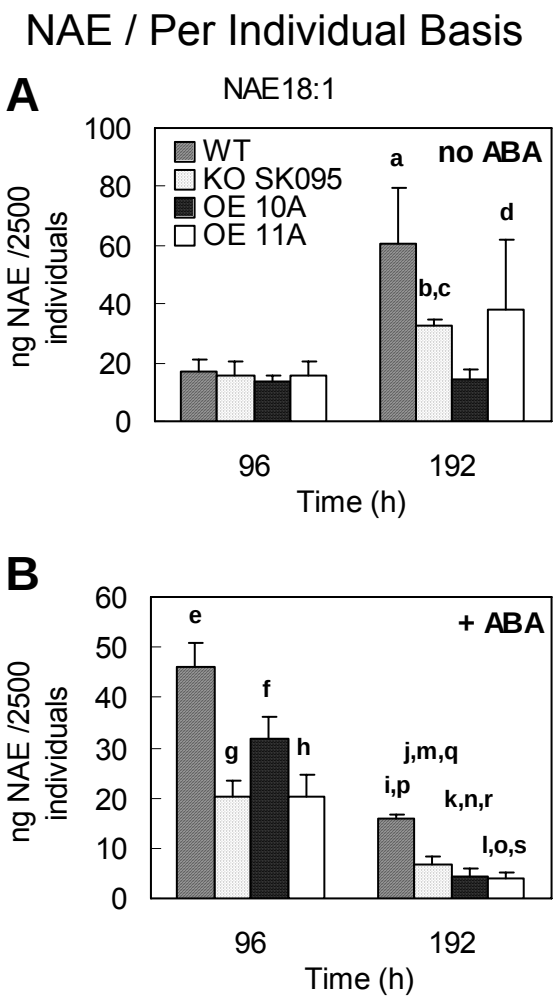

Figure 24. Impact of ABA treatment on NAE18:1 quantities in germinated seedlings. Seeds grown for 96 and 192 h in liquid MS media containing either solvent control (A) or 0.25 µM ABA (B) at 22°C under long day conditions, following sterilization and stratification in H_2O at 4°C for 24 h. Values expressed as nanograms per 2500 individuals. Values compared by t-test. Only those comparisons considered to be significant ($p \leq 0.055$) are listed below. Error bars represent ± SD of three to nine individual extractions.

96 h vs. 192 h:
(a) WT 96 h vs. WT 192 h: p< 0.00065
(b) KO 96 h vs. KO 192 h: p< 0.0001
(c) WT 192 h vs. KO 192 h: p< 0.009
(d) WT 192 h vs. OE10 192 h: p< 0.0015

96 h vs. 96 h +ABA:
(e) WT 96 h vs. WT 96 h +ABA: p< 0.0006
(f) OE10 96 h vs. OE10 96 h +ABA: p< 0.0025

96 h +ABA vs. 192 h +ABA:
(g) WT 96 h +ABA vs. KO 96 h +ABA: p< 0.005
(h) WT 96 h +ABA vs. OE11 96 h +ABA: p< 0.009
(i) WT 96 h +ABA vs. WT 192 h +ABA: p< 0.0015
(j) KO 96 h +ABA vs. KO 192 h +ABA: p< 0.000055
(k) OE10 96 h +ABA vs. OE10 192 h +ABA: p< 0.000045
(l) OE11 96 h +ABA vs. OE11 192 h +ABA: p< 0.0035
(m) WT 192 h +ABA vs. KO 192 h +ABA: p< 0.000055
(n) WT 192 h +ABA vs. OE10 192 h +ABA: p< 0.000025
(o) WT 192 h +ABA vs. OE11 192 h +ABA: p< 0.0001

192 h vs. 192 h +ABA:
(p) WT 192 h vs. WT 192 h +ABA: p< 0.005
(q) KO 192 h vs. KO 192 h +ABA: p< 0.0000000025
(r) OE10 192 h vs. OE10 192 h +ABA: p< 0.00085
(s) OE11 192 h vs. OE11 192 h +ABA: p< 0.055

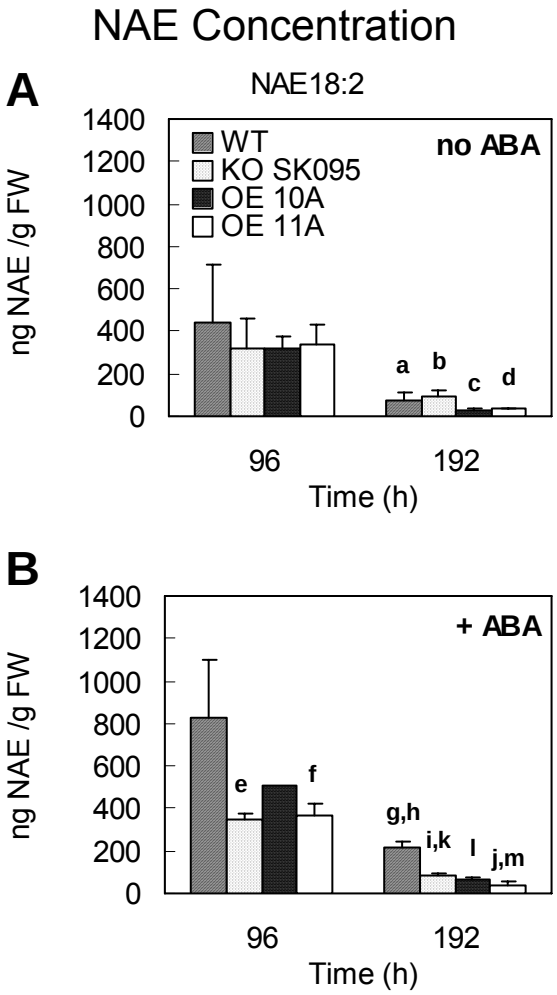

Figure 25. Effects of ABA treatment on NAE18:2 concentrations in germinated seedlings. Seeds grown for 96 and 192 h in liquid MS media containing either solvent control (A) or 0.25 µM ABA (B) at 22°C under long day conditions, following sterilization and stratification in H_2O at 4°C for 24 h. Values expressed as nanograms per gram FW and compared by t-test. Only those comparisons considered to be significant ($p \leq 0.055$) are listed below. Error bars represent ± SD of three to nine individual extractions.

96 h vs. 192 h:
(a) WT 96 h vs. WT 192 h: $p < 0.0015$
(b) KO 96 h vs. KO 192 h: $p < 0.005$
(c) OE10 96 h vs. OE10 192 h: $p < 0.00015$
(d) OE11 96 h vs. OE11 192 h: $p < 0.0004$

96 h vs. 96 h +ABA:

96 h +ABA vs. 192 h +ABA:
(e) WT 96 h +ABA vs. KO 96 h +ABA: $p < 0.045$
(f) WT 96 h +ABA vs. OE11 96 h +ABA: $p < 0.05$
(h) WT 96 h +ABA vs. WT 192 h +ABA: $p < 0.02$
(i) KO 96 h +ABA vs. KO 192 h +ABA: $p < 0.00001$
(j) OE11 96 h +ABA vs. OE11 192 h +ABA: $p < 0.00085$
(k) WT 192 h +ABA vs. KO 192 h +ABA: $p < 0.0004$
(l) WT 192 h +ABA vs. OE10 192 h +ABA: $p < 0.000005$
(m) WT 192 h +ABA vs. OE11 192 h +ABA: $p < 0.00085$

192 h vs. 192 h +ABA:
(g) WT 192 h vs. WT 192 h +ABA: $p < 0.000055$

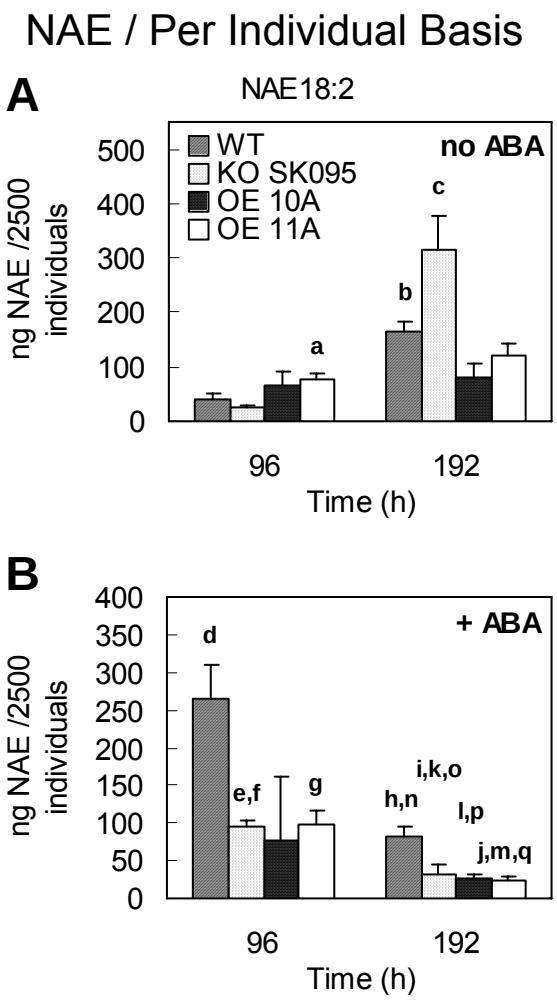

Figure 26. Impact of ABA treatment on NAE18:2 quantities in germinated seedlings. Seeds grown for 96 and 192 h in liquid MS media containing either solvent control (A) or 0.25 µM ABA (B) at 22°C under long day conditions, following sterilization and stratification in H_2O at 4°C for 24 h. Values expressed as nanograms per 2500 individuals and compared by t-test. Only those comparisons considered to be significant ($p \leq 0.055$) are listed below. Error bars represent ± SD of three to nine individual extractions.

96 h vs. 192 h:
(a) WT 96 h vs. OE11 96 h: $p < 0.015$
(b) WT 96 h vs. WT 192 h: $p < 0.0000007$
(c) KO 96 h vs. KO 192 h: $p < 0.0003$

96 h vs. 96 h +ABA:
(d) WT 96 h vs. WT 96 h +ABA: $p < 0.00045$
(e) KO 96 h vs. KO 96 h +ABA: $p < 0.0002$

96 h +ABA vs. 192 h +ABA:
(f) WT 96 h +ABA vs. KO 96 h +ABA: $p < 0.0065$
(g) WT 96 h +ABA vs. OE11 96 h +ABA: $p < 0.0085$
(h) WT 96 h +ABA vs. WT 192 h +ABA: $p < 0.006$
(i) KO 96 h +ABA vs. KO 192 h +ABA: $p < 0.00055$
(j) OE11 96 h +ABA vs. OE11 192 h +ABA: $p < 0.01$
(k) WT 192 h +ABA vs. KO 192 h +ABA: $p < 0.0025$
(l) WT 192 h +ABA vs. OE10 192 h +ABA: $p < 0.00002$
(m) WT 192 h +ABA vs. OE11 192 h +ABA: $p < 0.007$

192 h vs. 192 h +ABA:
(n) WT 192 h vs. WT 192 h +ABA: $p < 0.00015$
(o) KO 192 h vs. KO 192 h +ABA: $p < 0.00006$
(p) OE10 192 h vs. OE10 192 h +ABA: $p < 0.0009$
(q) OE11 192 h vs. OE11 192 h +ABA: $p < 0.02$

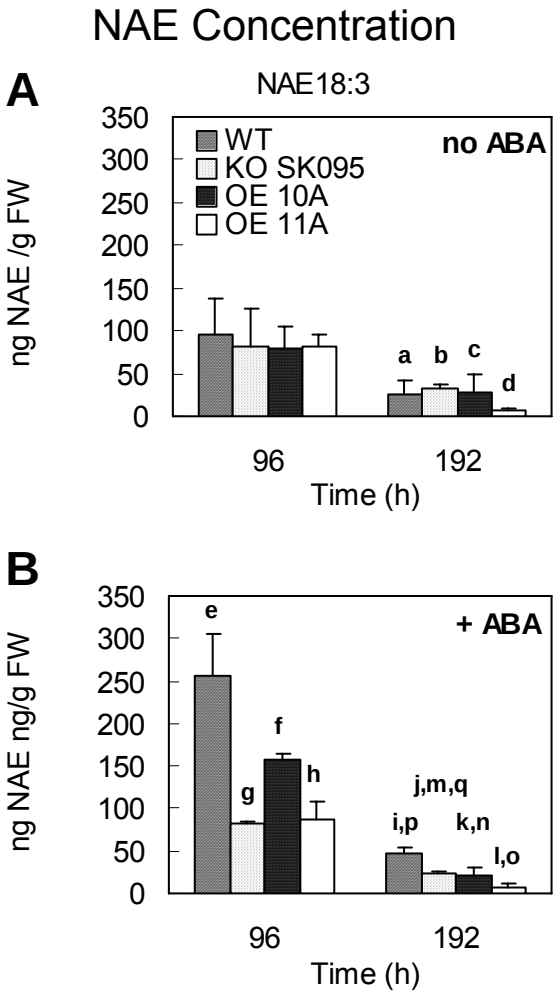

Figure 27. Impact of ABA treatment on NAE18:3 concentrations in germinated seedlings. Seeds grown for 96 and 192 h in liquid MS media containing either solvent control (A) or 0.25 µM ABA (B) at 22°C under long day conditions, following sterilization and stratification in H_2O at 4°C for 24 h. Values expressed as nanograms per gram FW and compared by t-test. Only those comparisons considered to be significant ($p \leq 0.055$) are listed below. Error bars represent ± SD of three to nine individual extractions.

96 h vs. 192 h:
(a) WT 96 h vs. WT 192 h: $p < 0.00065$
(b) KO 96 h vs. KO 192 h: $p < 0.02$
(c) OE10 96 h vs. OE10 192 h: $p < 0.05$
(d) OE11 96 h vs. OE11 192 h: $p < 0.00085$

96 h vs. 96 h +ABA:
(e) WT 96 h vs. WT 96 h +ABA: $p < 0.004$
(f) OE10 96 h vs. OE10 96 h +ABA: $p < 0.03$

96 h +ABA vs. 192 h +ABA:
(g) WT 96 h +ABA vs. KO 96 h +ABA: $p < 0.007$
(h) WT 96 h +ABA vs. OE11 96 h +ABA: $p < 0.015$
(i) WT 96 h +ABA vs. WT 192 h +ABA: $p < 0.0045$
(j) KO 96 h +ABA vs. KO 192 h +ABA: $p < 0.00000055$
(k) OE10 96 h +ABA vs. OE10 192 h +ABA: $p < 0.000002$
(l) OE11 96 h +ABA vs. OE11 192 h +ABA: $p < 0.0035$
(m) WT 192 h +ABA vs. KO 192 h +ABA: $p < 0.00055$
(n) WT 192 h +ABA vs. OE10 192 h +ABA: $p < 0.005$
(o) WT 192 h +ABA vs. OE11 192 h +ABA: $p < 0.0015$

192 h vs. 192 h +ABA:
(p) WT 192 h vs. WT 192 h +ABA: $p < 0.05$
(q) KO 192 h vs. KO 192 h +ABA: $p < 0.045$

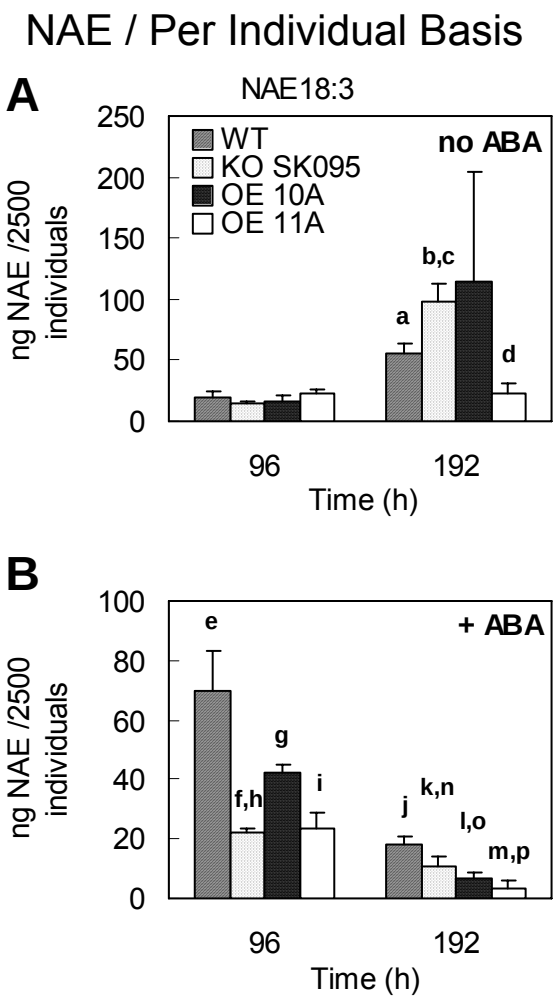

Figure 28. Effects of ABA treatment on NAE18:3 quantities in germinated seedlings. Seeds grown for 96 and 192 h in liquid MS media containing either solvent control (A) or 0.25 µM ABA (B) at 22°C under long day conditions, following sterilization and stratification in H_2O at 4°C for 24 h. Values expressed as nanograms per 2500 individuals and compared by t-test. Only those comparisons considered to be significant ($p \leq 0.055$) are listed below. Error bars represent ± SD of three to nine individual extractions.

96 h vs. 192 h:
(a) WT 96 h vs. WT 192 h: $p < 0.0002$
(b) KO 96 h vs. KO 192 h: $p < 0.00003$
(c) WT 192 h vs. KO 192 h: $p < 0.001$
(d) WT 192 h vs. OE11 192 h: $p < 0.0025$

96 h vs. 96 h +ABA:
(e) WT 96 h vs. WT 96 h +ABA: $p < 0.002$
(f) KO 96 h vs. KO 96 h +ABA: $p < 0.0015$
(g) OE10 96 h vs. OE10 96 h +ABA: $p < 0.003$

96 h +ABA vs. 192 h +ABA:
(h) WT 96 h +ABA vs. KO 96 h +ABA: $p < 0.007$
(i) WT 96 h +ABA vs. OE11 96 h +ABA: $p < 0.015$
(j) WT 96 h +ABA vs. WT 192 h +ABA: $p < 0.006$
(k) KO 96 h +ABA vs. KO 192 h +ABA: $p < 0.0015$
(l) OE10 96 h +ABA vs. OE10 192 h +ABA: $p < 0.000007$
(m) OE11 96 h +ABA vs. OE11 192 h +ABA: $p < 0.0055$
(n) WT 192 h +ABA vs. KO 192 h +ABA: $p < 0.02$
(o) WT 192 h +ABA vs. OE10 192 h +ABA: $p < 0.0007$
(p) WT 192 h +ABA vs. OE11 192 h +ABA: $p < 0.0025$

192 h vs. 192 h +ABA:
(q) WT 192 h vs. WT 192 h +ABA: $p < 0.00045$
(r) KO 192 h vs. KO 192 h +ABA: $p < 0.0000035$
(s) OE10 192 h vs. OE10 192 h +ABA: $p < 0.035$
(t) OE11 192 h vs. OE11 192 h +ABA: $p < 0.02$

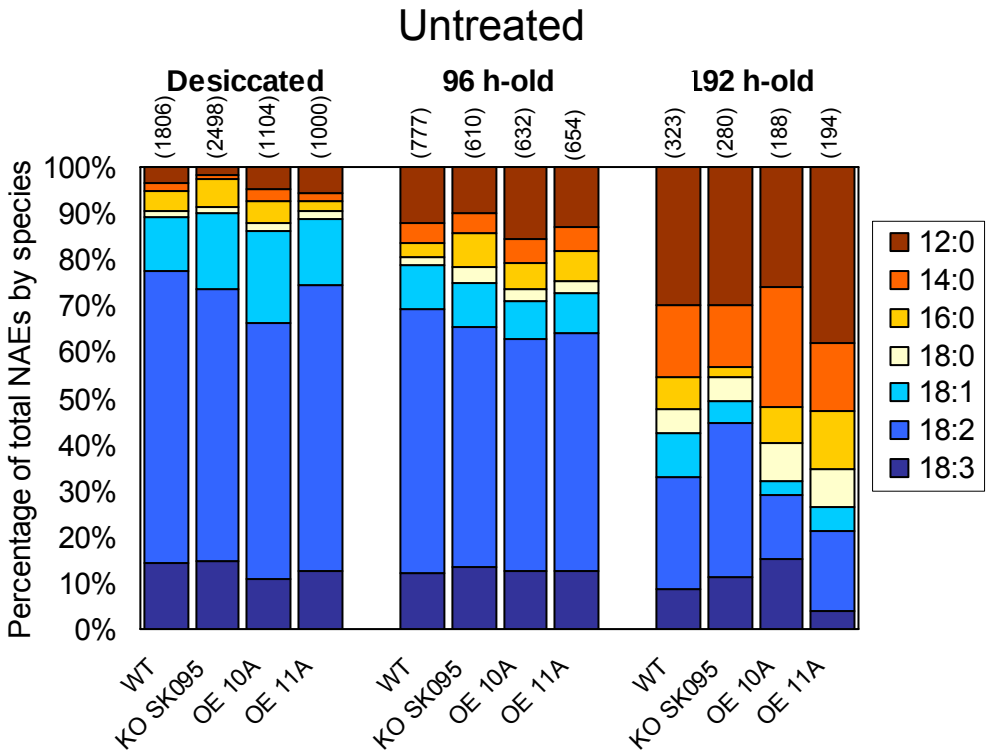

Figure 29. Proportion of individual NAE species in desiccated seeds and germinated seedlings. Seeds were grown for 96 and 192 h in liquid MS media containing solvent control at 22°C under long day conditions, following sterilization and stratification in H_2O at 4°C for 24 h. NAE compositions are presented as % of total. NAE totals summed from species profiles, expressed in nanograms per gram FW in parentheses.

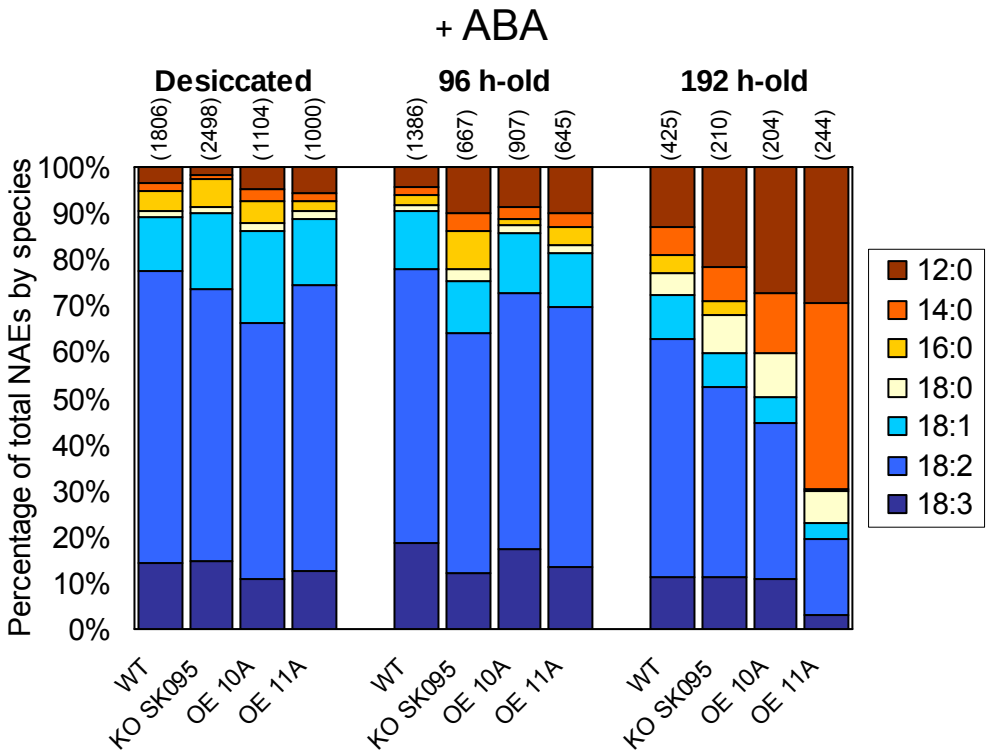

Figure 30. Proportion of individual NAE species in desiccated seeds and germinated seedlings treated with ABA. Seeds were grown for 96 and 192 h in liquid MS media containing 0.25 µM ABA at 22°C under long day conditions, following sterilization and stratification in H_2O at 4°C for 24 h. NAE compositions are presented as % of total. Total NAE content, in parentheses, summed from species profiles, expressed in nanograms per gram FW.

DISCUSSION

There is increasing interest in the accurate quantification of NAEs in plant tissues to investigate an ever-growing range of conditions associated with NAE metabolism (e.g. signaling, defense, etc.). The highly-refined isotope-dilution mass spectrometric method used here is a powerful tool for the unequivocal identification and quantification of NAE molecular species in total lipid extracts during seed imbibition and germination and seedling post-germinative growth. This tool will aid us in our general goal of understanding the role of NAE metabolism in *Arabidopsis* seed/seedling development.

The most likely modes of NAE signaling *in planta* are dynamic fluxes in concentration of one or more NAE species and accurate measurements of NAE concentrations in tissues is an important metric of NAE metabolism. In addition, an evaluation of the amount or quantity of metabolite present per individual, or group of individuals, allows for a means of comparison uncomplicated by issues of unequal growth and development (e.g. desiccated, imbibed and germinated seeds, etc.). Since this value is irrespective of the changing mass of an individual, it is useful when comparing plants in varying stages of growth and development as weight attributed to water can differentially distort quantification measurements based on fresh weight. This is especially the case with desiccated seeds compared with imbibed seeds. Hence, data were presented in two formats–NAEs on a per gram fresh weight basis and NAEs on a per individual basis–to provide as much information about overall NAE metabolism as possible.

The regulation of endogenous NAE pools is under temporal control as they are rapidly metabolized at precise stages of development that coincide with large physiological changes such as imbibition and germination. At the onset of imbibition (~30

m), an increase in saturated NAE content (Fig. 5C) was concurrent with a decrease in unsaturated NAE content, on a per individual basis (Fig. 5D). However, these dynamic changes in saturated and unsaturated NAE quantities resulted in a profile of total NAE content per individual that showed no overall change throughout the 24 h stratification procedure (Fig. 5A; imbibition in the dark for 24 h at 4°C).

NAE concentrations quantified during the period of post-germinative growth (96-192 h) revealed a drop in unsaturated NAEs by 192 h (Fig. 6D) while saturated NAE concentrations remained essentially unchanged, at trace levels, in desiccated and imbibed seeds as well as germinated seedlings (Fig. 6C). Despite the difference in dynamics of saturated and unsaturated NAE pools during normal seed imbibition and germination, the concentration of unsaturated NAEs by 192 h was ultimately lowered to levels very similar to that of the saturated NAEs (Figs. 6C and 6D). In other words, from these quantitative studies, it appears the most significant metabolism of NAEs during seed germination was in the depletion of the 18C unsaturated NAE species. It is worth noting that the polyunsaturated NAE20:4 (anandamide, AEA) was not detected in *Arabidopsis* seeds or seedlings at any stage of imbibition, germination or post-germinative growth (≤192 h). This is not surprising as arachidonic acid (20:4) is not normally considered to be a constituent of higher plant tissues.

In mammalian brain tissue, fatty acid amide hydrolase (FAAH) is responsible for the termination of AEA signaling via hydrolysis to free fatty acid and ethanolamine (Cravatt et al., 1996). In cottonseeds, NAEs are reported to be metabolized by two competing pathways during imbibition- a hydrolytic pathway similar to mammalian FAAH and an oxidative pathway via lipoxygenase (Fig. 1; Shrestha et al., 2002). The

Arabidopsis homolog of the FAAH enzyme (AtFAAH) was shown to have broad specificity for the various NAE molecular species *in vitro*. The lipoxygenase (LOX) pathway, the oxidative pathway leading to NAE depletion, has strict specificity for polyunsaturated NAE species (e.g. NAE18:2, NAE18:3, etc.) and was regarded until now, as a minor pathway for NAE metabolism.

To help elucidate the role of NAEs in normal seed imbibition and germination we compared transgenic lines with altered expression of the *Arabidopsis FAAH* gene shown to encode an enzyme capable of hydrolyzing all plant NAE species. It appears that manipulating AtFAAH expression can indeed lead to predictable changes in NAE content, at least in desiccated seeds. However, all genotypes appeared to metabolize NAEs to similar levels during germination and seedling growth (Fig. 7). Seedlings of the AtFAAH knockout (Salk_095108), despite the absence of a functional AtFAAH protein and higher total NAE concentrations in desiccated seeds, had managed to metabolize total NAE concentrations to levels nearly identical to wild-type seeds by 96 h, and again at 192 h (Compare Figs. 7A & 7B). Likewise, the overexpressors with a constitutively-active 35S-*FAAH* gene, which contained lower total NAE concentrations in desiccated seeds than wild-type seeds, had metabolized total NAE concentrations to wild-type levels by 96 h (Compare Figs. 7A, 7C & 7D). Therefore, the decrease in the concentration of unsaturated NAEs during imbibition and germination is likely a requirement for normal seedling establishment, or, at the very least, a metabolic obstacle to overcome. And it is likely that a pathway that is not AtFAAH-mediated is responsible for this depletion of unsaturated NAEs, perhaps via LOX.

The strict regulation of NAE metabolism is almost certainly important for normal seed germination and further seedling growth and development. This is supported by experiments where *Arabidopsis thaliana* (ecotype: Col.) seeds were germinated and grown in the continual presence of *N*-lauroylethanolamine (NAE12:0). Blancaflor et al., (2003) noted several morphological abnormalities evident in roots of NAE-treated seedlings that were specific to NAE12:0, since similar levels of lauric acid (12C FFA) had no visible effect on growth or development (e.g. germination). Generally, NAE12:0 treatment resulted in a concentration-dependent reduction in primary root length. In the presence of 50 μM NAE12:0, roots were devoid of root hairs and the tips of primary and lateral roots appeared swollen after several days of growth. A closer examination of root tips at the cellular level indicated the swollen root tips were comprised of disorganized cell files, and cells in the elongation zone were considerably swollen and irregular in shape, yet generally remained viable (Blancaflor et al., 2003). Moreover, the profound physiological effects of NAE12:0 on root growth and development were entirely reversible. When *Arabidopsis* seedlings with short and swollen root tips (germinated and grown in 50 μM NAE12:0) were transferred to NAE-free media, primary root growth rate resumed to normal after a 1 day lag period. Moreover, normal patterning and development of root hairs recovered while on NAE-free media with the emergence of root hairs in regions that were previously barren (Blancaflor et al., 2003). Therefore, elevated exogenous NAE12:0 concentrations (>35 μM) act as a negative regulator of growth, resulting in distorted root development and cellular organization, that must be removed to allow for the resumption of normal cell division and expansion.

The fact unsaturated NAEs changed most dramatically during seed germination even in the AtFAAH knockout seedlings suggests that *FAAH* expression and/or AtFAAH activity, most likely is not the major pathway for NAE depletion in normal seed/seedling growth as was previously assumed or that an alternative pathway can compensate in the absence of a hydrolytic pathway. Examples of metabolic compensation/redundancy have been noted in other areas of plant metabolism (Asai et al., 2004; Goodwin et al., 2005). Moreover, increases in lipoxygenase activity during germination have been reported for a number of plant species, including soybean, wheat, pea, barley and rice (Melan et al., 1994; Shrestha et al., 2002). Recently *Arabidopsis* lipoxygenase activity assays indicated that NAE-oxylipin formation, most likely the result of LOX activity, was increased at the time of germination (96 h), relative to both desiccated and 192 h-old seedlings (Kilaru and Chapman, unpublished results). This increased LOX activity could explain how seeds of the knockout line, verified to be without a functional *FAAH* gene or AtFAAH activity, were able to rapidly decrease their unsaturated NAE concentrations at the time of germination similar to the overexpressors. Interestingly, Melan et al. (1993) reported *LOX1* gene induction by bacterial pathogen attack, methyl jasmonate and, more importantly, ABA.

The observed decline in total NAE levels during seed germination and seedling growth is similar to the kinetics of ABA depletion. ABA, a plant stress hormone, influences an array of both physiological and developmental events (Koornneef et al., 1998). Fluxes in ABA concentrations are implicated in the regulation of protein and lipid reserve accumulation in seeds, as well as the acquisition of desiccation tolerance, the inhibition of precocious germination and the induction of primary (Liotenberg, 1999) and secondary (Foster et al., 1999) dormancy.

The regular progression of development appeared delayed and/or arrested in seeds/seedlings incubated in ABA-containing (0.25 μM) liquid growth media (Figs. 9 & 10). At 96 and 192 h, wild-type seedlings appeared to be least affected by ABA treatment while OE 11A showed the highest sensitivity. The majority of wild-type seeds had germinated by 96 h with the visible protrusion of the radicle (Figs. 9C-D). Although untreated seedlings of the same age had clearly defined roots, hypocotyls and cotyledons, not a single seedling treated with ABA had progressed further than the emergence of the radicle. Similar to exogenous NAE12:0, ABA treatment acted as a negative regulator of seedling growth in *Arabidopsis* seeds/seedlings.

Treated wild-type seedlings, which appeared least affected by the continual presence of exogenous ABA, had unsaturated NAE concentrations 2-3 times higher than in untreated seedlings at 96 and 192 h (Figs. 13D, 23B, 25B & 27B). Oddly, ABA-treated overexpressor 10A seedlings also had elevated unsaturated NAE concentrations (Figs. 13D, 23B, 25B & 27B) yet showed a higher sensitivity to ABA than wild type. The knockout and overexpressor 11A lines, which both showed a higher sensitivity to ABA similar to overexpressor 10A, did not show any significant change in unsaturated NAE concentrations with ABA treatment. At 192 h, however, OE 11A did have 3 times the NAE14:0 concentration seen in untreated seedlings (Fig. 17B). The significance or consequence of elevated NAE14:0 concentrations are unknown, yet apparently this NAE profile did not afford the 192 h-old seedlings any growth advantage in the presence of exogenous ABA (Fig. 10O-P).

What is likely to be more significant is the absence, or near absence, of NAE16:0 in both of the ABA-hypersensitive AtFAAH overexpressors. Although it is known that

NAE16:0 alone can not elicit such gross morphological changes (Blancaflor et al, 2003), the direct effect of a seedling's profile lacking NAE16:0 has not been investigated at this point. Interestingly, NAE12:0, on the other hand, recently was found to inhibit the endogenous NAE-LOX pathway in wild-type *Arabidopsis* seedlings (Kilaru and Chapman, unpublished results). More studies are required to determine if NAE12:0 plays a role in the regulation of the NAE-LOX pathway *in planta*.

The change in proportions of NAE species from desiccated seeds to germinated seedlings is most likely to be very important in the function of NAE. Desiccated seeds of all genotypes contained about 90% unsaturated NAEs and only 10% saturated NAEs (Fig. 29). The concentration of unsaturated NAEs decreased as untreated seeds germinated and grew, which means saturated NAEs made up an increasingly larger proportion of NAE species in established seedlings. Saturated NAE proportions reached 20% by 96 h and 55% at 192 h, although the concentration of saturated NAEs remained consistent throughout these time points. This shift in unsaturated versus saturated NAEs also was seen in all genotypes at 96 h (Fig. 29). At 192 h, both overexpressors had the lowest proportion of unsaturated NAEs. This may be because the overexpressed AtFAAH worked in addition to the unaltered LOX pathway to cumulatively metabolize more NAEs. This is supported by the fact both the overexpressors had total NAE concentrations at 191 (± 3) ng/g FW, compared to wild type seedlings with 323 ng/g FW (Fig. 29).

Seedlings treated with ABA contained higher proportions of unsaturated NAEs, and subsequently lower proportions of saturated NAEs, than untreated seedlings (Fig. 30). This was much more evident in 192 h-old seedlings. Wild type seedlings, which showed the lowest sensitivity to ABA, had altered their NAE proportions the most, relative

to untreated seedlings, and were more similar to younger, less developed seedlings. Wild-type seedlings also had the highest concentration of total NAEs at 425 ng/g FW (Fig. 30). Whereas the overexpressors contained proportions of NAEs most similar to untreated seedlings and displayed the highest sensitivity to ABA treatment. The sustained concentration and/or proportion of unsaturated NAEs is most likely involved with the ability of the wild-type seedlings to better handle ABA treatment, relative to all other *FAAH* genotypes.

Interestingly, several groups have noted that although the polyunsaturated NAE20:4 (anandamide, AEA) and its precursor are present in the mammalian brain at very low levels, their concentrations increase post-mortem or upon injury (Hansen et al., 1998; Hansen et al., 2000; Schmid et al., 1995; Mechoulam, 2002). In fact, there has been mounting experimental evidence for a cytoprotective role of plant-derived-, synthetic- and endogenous cannabinoids (Fowler, 2003) since it was reported in the late 1980s that such compounds were formed in canine heart tissue following ischemia (Mechoulam, 2002). It has been speculated that because endocannabinoids are rapidly inactivated by hydrolysis (Fowler, et al., 2001; Giuffrida et al., 2001), enhancement of their neuroprotective activity could possibly be achieved by impairment of their inactivation (Mechoulam, 2002). In other words, a mammal that metabolizes its endocannabinoids slower than the normal rate following brain injury (e.g. hypoxia, ischemia, etc.) would likely have an advantage over others that metabolized their endocannabinoids at the normal rate. Given the similarities between mammalian and plant NAE metabolism, it is reasonable to speculate that a decreased rate of NAE

metabolism in seedlings may be advantageous during instances of unfavorable germination and/or growth conditions.

However, under normal conditions, it may be that the efficiency of seed germination and seedling growth is directly related to the efficiency of the seed/seedling to metabolize its NAEs. This is consistent with the seeds of the AtFAAH overexpressors which contained the lowest concentration of NAEs in desiccated seeds allowing them to metabolize the NAE concentrations to low levels more rapidly than wild-type or KO (SK095) seeds, both of which contained higher starting concentrations of NAEs in desiccated seeds (Fig. 7). Indeed, the overexpressors grew quicker, had larger rosettes and bolted sooner than either the wild-type or KO (SK095) plants (Wang et al., 2006). Yet this growth advantage is compromised under stress conditions (e.g. ABA, drought, heavy metal or UV exposure, etc.; Gonzales, Kim, Blancaflor and Chapman, poster), suggesting that NAE metabolism may be important for the proper balance of growth and stress responses.

Here, the *Arabidopsis* AtFAAH overexpressors had lower concentrations of NAEs in desiccated seeds, relative to wild type (Fig. 7), and when imbibed and/or germinated exhibited a higher sensitivity to the presence of ABA (Figs. 9 & 10). This heightened sensitivity is possibly the result of rapid unsaturated NAE depletion upon germination seen in both overexpressor lines, the result of the constitutively-overexpressed AtFAAH cDNA. On the other hand, wild-type seedlings, which contained the highest concentration of NAEs at both 96 and 192 h and also exhibited the lowest degree of growth reduction by exogenous ABA, relative to other genotypes, appeared to be reducing the unsaturated NAE species at a slower rate than either the overexpressors or knockout. Perhaps the

slowing or delaying of NAE metabolism in seedlings when growth conditions are unfavorable affords the seedlings the ability to better withstand the unfavorable environment. Could elevated NAE concentrations provide increased seedling resiliency?

Perhaps this regulated NAE depletion in wild-type seedlings reflects a balance between growth and the ability to respond to ABA and other stresses (Foster et al., 1999) and perturbation of NAE metabolism during this period of seedling establishment disrupts this balance. More work is needed to test this concept. In particular, a plant line that is unable to metabolize NAEs in an efficient manner would be predicted to grow slowly and respond to stress in a very rapid manner. The AtFAAH knockout did show somewhat reduced seedling growth (Wang et al. 2006), however since NAE metabolism was not disrupted dramatically, it was reasonable to expect that the growth reduction would not be dramatic either (which was indeed observed). If the LOX pathway is indeed important for NAE metabolism during seed germination and seedling growth, then disruption of both AtFAAH and AtLOX pathways may be necessary in order to see dramatic seedling growth phenotypes. Future studies to elevate endogenous NAE content or manipulate NAE profiles will help to clarify the role(s) of NAE metabolism on plant growth and development, and the quantitative procedures incorporated here will be important to obtain a comprehensive model.

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