A REGULATORY ROLE FOR *N*-ACYLETHANOLAMINE METABOLISM IN *Arabidopsis thaliana* SEEDS AND SEEDLINGS

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N-Acylethanolamines (NAEs) are bioactive acylamides that are present in a wide range of organisms. Because NAE levels in seeds decline during imbibition similar to ABA, a physiological role was predicted for these metabolites in *Arabidopsis thaliana* seed germination and seedling development. There is also a corresponding increase of AtFAAH (fatty acid amide hydrolase), transcript levels and activity, which metabolizes NAE to ethanolamine and free fatty acids. Based on whole genome microarray studies it was determined that a number of up-regulated genes that were responsive to NAE were also ABA responsive. NAE induced gene expression in these ABA responsive genes without elevating endogenous levels of ABA. It was also determined that many of these NAE/ABA responsive genes were associated with an ABA induced secondary growth arrest, including *ABI3*. ABI3 is a transcription factor that regulates the transition from embryo to seedling growth, the analysis of transcript levels in NAE treated seedlings revealed a dose dependent, inverse relationship between *ABI3* transcript levels and growth, high *ABI3* transcript levels were associated with growth inhibition. Similar to ABA, NAE negatively regulated seedling growth within a narrow window of early seedling establishment. When seedlings are exposed to NAE or ABA within the window of sensitivity, the induction of genes normally associated with the ungerminated desiccation tolerant state resumed. The NAE tolerant FAAH overexpressor and the NAE sensitive FAAH knockout both had a NAE/ABA sensitive window similar to the wild type *A. thaliana*. The *abi3-1* ABA insensitive mutant does not undergo growth arrest upon

exposure to ABA, but NAE did induce growth arrest when treated within the sensitivity window. This evidence showed that although NAE functions within an ABA dependent pathway, it also functions in an ABA independent signaling pathway. The FAAH overexpressor is tolerant to NAE through its ability to quickly metabolize NAE from the growth media, yet it is hypersensitive to ABA. The FAAH overexpressor also displayed hypersensitivity to GA, which improved its delayed germination in non-stratified seed, while the FAAH knock out showed GA insensitivity. Overall, these results showed that NAE functions as a negative regulator of germinating seed and seedling growth in ABA dependent and independent signaling pathways, and that altered NAE metabolism may interfere with ABA/GA perception in germinating seed.

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COMPREHENSIVE LIST OF ABBREVIATIONS

ABA	Abscisic acid
ABRE	ABA response element
BODIPY	4,4-difluoro-3a,4a-diaza-s-indacene
BSTFA	Bis (trimethylsilyl) trifluoroacetamide
CB	Cannabinoid
DMSO	Dimethyl sulfoxide
FAAH	Fatty acid amide hydrolase
FFA	Free fatty acid
GA	Gibberellic acid
GO	Gene ontogeny
GC-MS	Gas chromatography
HPLC	High performance liquid chromatography
KNO_3	Potassium nitrate
MAFP	Methyl arachidonyl fluorophosphonate
MS	Murashige and Skoog
NAE12:0	*N*-lauroylethanolamine
NAE14:0	*N*-myristoylethanolamine
NAE16:0	*N*-palmitoylethanolamine
NAE18:2	*N*-linoleoylethanoamine
NAE20:4	*N*-arahidonoylethanolamine
NAE	*N*-acylethanolamine
NAPE	*N*-acylphosphatidylethanolamine

NMR	Nuclear magnetic resonance
PE	Phosphatidylethanolamine
PLD	Phospholipase D
RT-PCR	Reverse transcriptase-polymerase chain reaction
SYBR	Synergy Brands, Inc.
TAG	Triacylglycerol
TAIR	The *Arabidopsis* information resource
TLC	Thin layer chromatography
WT	Wild type

CHAPTER 1

INTRODUCTION AND BACKGROUND

N-Acylethanolamines (NAEs) were first reported as constituents of soy lecithin and peanut meal in the 1950s (Kuehl et al., 1957). The formation of NAEs has been associated with tissue damage in mammals, but more recently also has been shown to be part of the endocannabinoid signaling system (Schmid and Berdyshev, 2002). This signaling system regulates an array of normal physiological functions, including neurotransmission (Wilson and Nicoll, 2002), immune responses (Berdyshev, 2000), embryo development (Paria and Dey, 2000) and cell proliferation (De Petrocellis et al., 2000). Despite the occurrence and tight control of NAE metabolism in plants its role in fundamental plant physiological processes remains unclear.

NAE Metabolism

NAEs are fatty acid amides that are derived from an *N*-acyl phosphatidylethanolamine (NAPE) precursor, a minor membrane lipid component of plant and animal cells. In mammals, NAEs are derived from the hydrolysis of NAPE by a Ca^{2+}-stimulated phospholipase D (PLD) (Schmid et al., 1996; Schmid et al., 2002). The mammalian NAPE-PLD was cloned recently, and it belongs to the zinc metallohydrolase family exhibiting activity toward NAPE but not to other phospholipid classes (Okamoto et al., 2005). The overexpression of NAPE-PLD in COS-7 cells resulted in decreased NAPE levels and increased NAE levels (Okamoto et al., 2005), and this supports the notion that this enzyme hydrolyzes NAPE to form NAEs. No obvious homologs of the mammalian NAPE-PLD have been identified in plants. Nonetheless, radiolabeling

experiments indicated that NAPE was the precursor for NAEs in plant cells and that the NAE types produced by plants were a reflection of the *N*-acyl composition of the NAPE precursor (Chapman, 2000). Five PLD isoforms (encoded by 12 genes) have been identified in *Arabidopsis thaliana* (Wang, 2004), and two isoforms PLDβ and PLDγ, were capable of hydrolyzing NAPE to NAE in vitro (Pappan et al., 1998). PLDα did not hydrolyze NAPE in vitro (Pappan et al., 1998), nor did PLDδ (Chapman and McHugh, unpublished observations). PLDζ showed strict specificity for phosphatidylcholine (PC) in vitro (Wang, 2004), but NAPE was not tested as a potential substrate for this family member. Collectively then, the data suggest that PLDβ and PLDγ isoforms may catalyze the formation of NAEs in vivo, and these enzymes indeed hydrolyze multiple phospholipid substrates in a polyphosphoinositide-dependent manner (Wang, 2004). However, there are two PLDβ genes and three PLDγ genes in *A. thaliana*, so finding the PLD isoform(s) in plants responsible for NAE formation in vivo may prove difficult.

The types of NAEs that are formed usually are a reflection of the acyl groups present in the NAPE precursor pool, (Schmid et al., 1996). Where it has been tested, NAPE-PLDs did not seem to show any selectivity for NAPE molecular species (Okamoto et al., 2004; Okamoto et al., 2006). In plants, NAE profiles in cotton seeds were a direct reflection of the *N*-acyl composition of NAPE (Chapman et al., 1999). So the profile of available NAEs may be determined by the synthesis of NAPE precursors, thus affecting the signaling pathways by the NAE species they contain.

The synthesis of NAPE in animal systems proceeds by an ATP-independent transacylation reaction whereby the *sn*-1-*O*-acyl moiety of PC is transferred to the *N*-position of phosphatidylethanolamine (PE) without a free fatty acid (FFA) intermediate

(Schmid and Berdyshev, 2002). This proposed pathway involves the incorporation of fatty acids into the *N*-position of NAPE by the coordinate action of acyltransferase and transcylase activities.

In plants, NAPE synthase, a membrane bound enzyme, directly synthesizes NAPE from FFA (Chapman, 2000). The product of NAPE synthase is a bilayer stabilizing lipid that is constructed from two membrane bilayer destabilizing lipid substrates. This may be a method for scavenging FFAs and protecting membrane integrity during plant cell stress (Sandoval et al., 1995; Chapman, 2004). FFA and PE are utilized in both plants and animals by different methods to synthesize NAPE. This difference may reflect the alternative mechanisms of overall regulation of NAE metabolism.

Tight control of NAEs in the signaling pathway depends on their timely degradation. Fatty acid amide hydrolase (FAAH) is responsible for the enzymatic degradation of NAEs in animal cells (McKinney and Cravatt, 2005). The cloning of rat *FAAH* (Cravatt et al., 1996) led to the identification of *FAAH* orthologs in other mammalian species. The mammalian FAAH enzymes are 579 amino acids in length and belong to a large group of proteins containing conserved amidase signature (AS) sequences. FAAH is distinct from other enzymes belonging to the AS family in that it carries an *N*-terminal transmembrane domain that predicts it to localize to cellular membranes (Cravatt et al., 1996; McKinney and Cravatt, 2005). The recent identification of an *A. thaliana FAAH* homolog (At5g64440) was based upon the occurrence of the AS domain and conservation of several key catalytic residues (Shrestha et al., 2003). The *A. thaliana FAAH* was predicted to encode a protein of 607

amino acids and the AS domain was nearly 60% identical to that of the mammalian FAAH, and the *A. thaliana* FAAH possessed a putative transmembrane domain near the *N*-terminus (Shrestha et al., 2003). The recombinant *A. thaliana* FAAH was shown by biochemical analysis to hydrolyze a variety of plant NAEs and its activity was strongly inhibited by methylarchidonyl fluorophosphonate (MAFP), an active site directed inhibitor of mammalian FAAH (Shrestha et al., 2003) Interestingly, there was only 18.5% amino acid sequence identity over their entire lengths between mammalian and *A. thaliana* FAAH, and yet molecular and biochemical evidence indicated that the *A. thaliana* At5g64440 gene encodes a functionally conserved NAE amidohydrolase. Moreover these results suggest that hydrolysis is at least part of the molecular mechanism in plants responsible for regulating NAE levels. Additional plant homologues of *FAAH* have been cloned and expressed confirming the widespread occurrence of this enzyme in plants (Shrestha et al., 2006).

Oxylipin oxidation products are derived from the catabolism of fatty acids and comprise an important class of signaling molecules. The oxidation products of polyunsaturated NAEs in animals, include eicosanoid ethanolamides, prostaglandins and leukotrienes that participate in diverse physiological processes (De Petrocellis et al., 2004). Polyunsaturated NAEs found in plants such as NAE18:2 and NAE18:3 can serve as substrates for enzymatic oxidation by lipoxygenase (LOX) pathway to produce novel plant oxylipins (Van Der Stelt et al., 2000). In plants, NAE action could be accomplished, in part, through the formation of oxylipins (Shrestha et al., 2002). Jasmonic acid and its methyl esters represent a class of plant oxylipins that function as signaling compounds during plant development (Turner et al., 2002), so perhaps NAE

oxylipins will have important cellular function(s). In any case, plants appear to be capable of modulating NAE levels via hydrolysis and/or oxidation, although evidence for the latter *in planta* is lacking.

NAE Metabolism in Plant Defense Signaling

The observation that plants accumulate NAEs in response to stress (Chapman et al., 1998; Rawyler and Braendle, 2001) suggests that plants and animals may share a common pathway. The fungal elicitor xylanse stimulated tobacco suspension cells to release shorter chain NAEs, 12:0 and 14:0 (Chapman et al., 1998). This response to fungal elicitation is different from mammalian cells which release mostly long chain saturated and unsaturated acyl chain NAEs (16:0 and 20:4; (Berger et al., 2004). This difference could reflect a difference in targets or a difference in physiological effects of NAE types between plants and animals (Chapman, 2004). Tobacco cell cultures exhibit a short term alkalinization of the culture medium upon exposure to xylanse (Felix et al., 1993). NAE 14:0 added to the culture did not affect the alkalinization response, but when xylanase and NAE 14:0 are added the elicitor induced alkalinization process was inhibited. This process was inhibited by most plant NAEs and the mammalian NAE anandamide, and was dependent on concentration as well as the timing of addition (Tripathy et al., 1999).

The induction of phenylalanine ammonia lyase (PAL), an enzyme in the phenylpropanoid pathway, typically accompanies pathogen attack (Dixon et al., 2002). NAE 14:0 induced PAL2 gene expression in the same manner as the elicitors elevated PAL2. In elicitor treated tobacco leaves there was a 10-50 fold increase in NAE 14:0

levels, and these concentrations were capable of activating PAL2 gene expression. NAE formation after elicitor treatment may have a role in plant defense responses by participating in signal transduction, and the tight regulation of NAE levels are likely to be important in this process.

NAEs in vertebrates are perceived by transmembrane CB receptors at the cell surface (Wilson and Nicoll, 2002). NAE perception in plants may use a similar mechanism for signaling during plant pathogen elicitor perception. The recent identification of a high affinity NAE binding protein in cell suspensions and microsomal membranes from multiple plant sources supports this assumption (Tripathy et al., 2003). NAE 14:0 specific binding was reduced when CB receptor antagonists were included in assays. These antagonists also reduced NAE 14:0 induced PAL2 expression in tobacco leaves and the inhibition of short term elicitor induce alkalinization response (Tripathy et al., 2003). These results support the concept of a CB receptor-like NAE binding protein participating in defense signal transduction in plants.

NAE Metabolism in Seed Germination and Seedling Growth

Seed germination involves the breaking of dormancy and the resumption of growth processes in the embryo which is triggered partly by imbibition (Bewley, 1997). Desiccated seeds of a variety of plant species contain *N*-acylethanolamines (NAEs) with chain lengths of 12C to 18C (summarized in (Chapman, 2004). Because NAE levels in seeds decline after a few hours of imbibition (Chapman et al., 1999) a physiological role was predicted for these metabolites in seed germination and seedling development. *A. thaliana* seedlings grown in the presence of NAE 12:0 (*N*-lauroylethanolamine) have

provided evidence of the potent biological activities of NAEs on seedling growth and root cell development (Blancaflor et al., 2003). This raises the possibility that the rapid metabolism of NAE's during imbibition and germination is requisite to the establishment of normal seedling growth and development.

During imbibition, the uptake of water is triphasic with a rapid initial uptake (phase 1), followed by a plateau phase (phase 2). A further uptake of water occurs only after germination is complete (phase 3), germination being complete when the radicle penetrates the seed coat, often called visible germination (Bewley, 1997). Upon imbibition, the quiescent dry seed rapidly resumes metabolic activity. In phase 1 DNA and mitochondria are repaired and proteins are synthesized using extant mRNAs. In phase 2 mitochondria are synthesized and proteins are synthesized using new mRNAs. Phase 3 is postgerminative, and at this stage stored reserves are mobilized, radicle cells elongate and cells divide. Some preformed mRNAs that are present within the dry embryo are residual messages associated with previous developmental processes and may be used transiently during germination, such as late embryogenesis abundant (LEA) mRNAs (Han et al., 1997), but in general, germination triggers the end of embryogenic programs and the degradation of embryo-specific mRNAs; this embryo-to-seedling transition process can be halted by an ABA-mediated process (Lopez-Molina et al., 2002).

The role of ABI3 in late embryogenesis has been established in several previous reports (Nambara et al., 1995; McCourt, 1999). A plant growth checkpoint has been identified that takes place within 60 h post stratification and triggered by ABA (Lopez-Molina et al., 2001). Chua and coworkers (Lopez-Molina et al., 2002) showed that

during germination ABA can recruit *de novo* late embryogenesis programs to confer osmotic tolerance in arrested, germinated *A. thaliana* embryos. Western blot analysis of ABI3 protein levels and Northern blot analysis of representative late embryogenesis genes, including RAB18, were conducted, and the results demonstrated that during 3 days of stratification, levels of ABI3 and RAB18 dropped from the highest in desiccated seeds to eventually minimal or undetectable levels, regardless of the presence or absence of ABA. When ABA was added post stratification, ABI3 and RAB18 levels began to increase after 1 day, reaching a plateau in arrested, germinated embryos at 5 days. The data further indicated that these levels were below that of desiccated seeds, but were capable of reactivating late embryogensis pathways after embryo germination in order to acquire osmotolerance.

During germination the extension of the radicle is a turgor driven process that requires the yielding of the walls of the embryonic axis that lie between the root cap and embryonic axis (Cosgrove, 1997). Cell wall loosening may result from cleavage and rejoining of xyloglucan molecules that tether adjacent cellulose microfibrils, which permit expansion by microfibril separation (Bewley, 1997). The activity of xyloglucan endotransglycosylase (XET), an enzyme capable of reversibly cleaving xyloglucan molecules, increases in the apical region of maize seedling roots during their elongation (Wu et al., 1994), but this increase occurs after germination is complete. Another candidate for cell wall loosening proteins are the expansins, these have the ability to disrupt hydrogen bonds between cell wall polymers. Expansins have been strongly implicated in the expansion of cucumber hypocotyls (McQueen-Mason and Cosgrove, 1995; Cosgrove, 1997) and have become generally accepted as part of the mechanism

regulating plant cell growth (Cosgrove, 1997). Cell wall loosening proteins are believed to play key roles in controlling cell wall extension. XET activity is enhanced in the apical 5 mm of water stressed root tips of maize, and this response was shown to be dependent on ABA accumulation (Wu et al., 1994), while expansin gene expression in maize primary roots at low water potential was not ABA dependent (Wu et al., 2001). The transition from dormancy to germination is regulated by external factors such as light quality, moisture, transient exposure to cold, as well as several internal growth regulators (Koornneef et al., 2002). It is a change in the balance between the two main regulators, abscisic acid and gibberellin, that controls the seeds fate. A shift towards ABA maintains the dormancy of seeds, whereas a shift towards GA has the opposite effect breaking dormancy and inducing seed germination (Steber et al., 1998).

It was shown recently that micromolar concentrations of exogenous NAE 12:0 greatly reduced post-germinative growth of *A. thaliana* embryos (Blancaflor et al., 2003), similar to the effects of added ABA. Endogenous NAE levels declined in parallel with ABA levels during germination and post-germinative growth. It is my hypothesis that NAE metabolism and ABA signaling pathways interact to modulate post-germinative seedling growth, and this dissertation research project is directed toward uncovering the molecular mechanisms involved in this interaction.

CHAPTER 2

N-ACYLETHANOLAMINE (NAE) ALTERS *Arabidopsis thaliana* SEEDLING GROWTH AND ABA RESPONSIVE GENE EXPRESSION

Abstract

N-Acylethanolamines (NAEs) are bioactive acylamides that are present in a wide range of organisms. In plants, NAEs are generally elevated in desiccated seeds suggesting that they may play a role in seed physiology. NAE and ABA levels were depleted during seed germination and elevated levels of either class of chemicals inhibited the growth of *Arabidopsis* seedlings. This growth arrest had many of the characteristics of a so-called “secondary dormancy”, and this arrest of seedling growth should be reflected in altered gene expression programs. To investigate this growth arrest at the molecular level NAE treated seedlings were subjected to transcript profiling by whole genome microarray analysis. NAE-treated seedlings exhibited elevated transcripts for a number of ABA-responsive genes and genes typically enriched in desiccated seeds, compared with untreated seedlings. Several genes were selected for detailed investigation by quantitative RT-PCR over the course of post germinative growth, including genes known to be regulated by ABA and associated with dormancy. Transcript levels of the majority, but not all of the selected genes were elevated correspondingly by NAE or ABA, including *ABI3* (At3g24650), which is known to be associated with the embryo-seedling transition. Since many of the genes selected were ABA responsive, endogenous levels of ABA were quantified in NAE treated seedlings. NAE treatments did not cause endogenous levels of ABA to rise, nor did ABA treatments elevate endogenous levels of NAE. Collectively our data suggest that NAE

metabolism interacts with ABA in the negative regulation of seedling development and that normal seedling establishment depends on the reduction of the endogenous levels of both metabolites. I propose that NAE metabolism interacts with ABA in the negative regulation of seedling development and that normal seedling establishment depends on the reduction of the endogenous levels of both metabolites.

Introduction

N-Acylethanolamines (NAE's) are lipid mediators that are produced from the phospholipase D-mediated hydrolysis of *N*-acylphosphatidylethanolamines, a minor membrane lipid constituent of cellular membranes (Schmid et al., 1996). In animal systems NAEs have been implicated in immunomodulation (Buckley et al., 2000) synchronization of embryo development (Paria and Dey, 2000), apoptosis (Krishna Pada et al., 2000) and neurotransmission (Wilson and Nicoll, 2002). These endogenous bioactive molecules lose their signaling activity upon hydrolysis by fatty acid amide hydrolase (FAAH). An increasingly detailed understanding of the degradation of these bioactive NAEs by FAAH has pointed to this metabolic step as a key regulator of NAE levels and hence function *in vivo* (Ueda et al., 2000; Cravatt and Lichtman, 2002; Ueda, 2002).

Several NAE types have been identified in desiccated seeds of a variety of plant species (Chapman et al., 1999). These NAE types identified in seeds contain acyl chains of 12-18C in length and contain up to three double bonds, reflecting the typical acyl moieties prevalent in higher plants. The total NAE content ranges from about 0.5 to 2.0 μg/g fresh weight in desiccated seeds, with species to species variation in total

NAE content similar to subspecies (cultivar) differences in total NAE content (Chapman et al., 1999). The predominant species in desiccated seed are usually 16-18C NAE types, although short chain NAE12:0 and NAE14:0 also are detectable. Some of these minor NAE lipid types found in plant tissues have potent biological activities, which is similar in vertebrates where the relatively minor NAE20:4 (about 1% of the total NAE pool) (Schmid and Berdyshev, 2002) is believed to be responsible for many of cannaboid (CB) receptor-mediated physiological effects of NAE (Di Marzo et al., 2002). One emerging theme is that in both plant and animal systems the largest proportion of NAEs are the common 16:0, 18:1 and 18:2 acylethanolamides, however in terms of bioactivity, plants are particularly responsive to low concentrations of medium chain NAEs. However, animal physiology is largely regulated by low concentrations of long chain polyunsaturated NAEs (e.g. NAE20:4), both of which constitute a relatively minor fraction of the NAE pool in their respective system (Chapman, 2004).

The levels of most NAEs identified in desiccated seeds declined with imbibition and germination, and this was observed for several species (Chapman et al., 1999). For example, total NAE content declined from approximately 1600 to 760 ng/g fresh weight in cotton seeds 4 h after imbibition, and this decline was observed for the major individual NAE types as well (NAE18:2 and NAE16:0) (Chapman, 2004). In cotton, NAE levels continue to drop during seed germination and early post-germinative growth to barely detectable levels by 24 h after imbibition. Under these conditions, the 18-24 h period is after germination (marked by radical emergence), but before the majority of lipid mobilization activity (marked by glyoxylate cycle enzyme acytivities) (Chapman and Sprinkle, 1996). The rapid decline in measurable NAE levels during seed imbibition and

germination suggests that these lipids may play a physiological role in this process (Chapman, 2004).

In desiccated *Arabidopsis* seeds, total NAE content was ≈2,000 ng/g and these levels declined significantly 24-192 after sowing (Wang et al., 2006). It has been determined by quantitative RT-PCR that *AtFAAH* transcript levels are found at different levels in different organs, the lowest being in inflorescence stems. *AtFAAH* transcript levels were 2.5 fold higher in desiccated seed than those in stems, and *AtFAAH* transcript levels were 4- and 19-fold higher in imbibed seeds and 4-d-old seedlings, respectively (Wang et al., 2006). The higher transcript levels in imbibed seeds and seedlings were consistent with the notion that NAE catabolism is activated during seed germination and seedling development. The increase of *AtFAAH*::GUS expression in seeds and seedlings also mirrored the depletion of NAEs during seed germination and seedling growth (Wang et al., 2006).

The rapid depletion of NAEs in imbibing seeds suggests a role for this metabolic pathway in seed germination and seedling growth (Chapman et al., 1999). To further examine this hypothesis, *A. thaliana* (wild type Columbia) seeds were germinated and grown in liquid culture with sustained NAE12:0 levels, (35μM). Several growth abnormalities were evident in seedlings grown in continuous exposure to elevated levels of NAE 12:0, including inhibition of root elongation, increased radial swelling of root tips and reduced root hair numbers (Blancaflor et al., 2003) These morphological effects were selective for NAE type and were concentration dependent. Moreover, these effects were reversible when seedlings were removed from exposure to NAE (Blancaflor et al., 2003), indicating that NAE itself was not toxic to plant cells.

A. thaliana seed germination was not affected by NAE treatment, but there appeared to be a dramatic reduction in growth that was easily quantified by measuring gain in fresh weight (1.1A,D). Growth was arrested in NAE12:0 treated seedlings by approximately 96 h, or shortly after germination. This growth arrest had many of the characteristics of an induced secondary dormancy. Mechanisms responsible for the the arrest of seedling growth, a phenotypic response to exogenous elevated levels of NAE12:0, are unknown. As a first step to understand this process I postulated that altered growth would be a manifestation of altered gene expression patterns. Hence, I took a molecular approach to examine the gene expression programs likely to be involved in NAE-induced growth arrest, first by whole genome microarray analysis and then by more selected transcript analysis. The goal was to identify changes in selective transcript abundance with changes in growth.

Results

ABA and NAE Inhibit Seedling Growth and Their Depletion during Seed Germination Follows Similar Kinetics

Previous studies have suggested that the depletion of endogenous NAE and ABA levels during germination might be a requisite for normal seedling development (Chapman et al., 1999; Jacobsen et al., 2002; Blancaflor et al., 2003; Nakabayashi et al., 2005; Wang et al., 2006). Indeed, seedling growth, measured as gain in fresh weight, was arrested by NAE12:0 in a manner similar to ABA (Figure 2.1A). To determine whether endogenous levels of NAEs and ABA follow a similar time course of depletion during *Arabidopsis* seed germination, I quantified NAE and ABA levels in desiccated seeds and seedlings 1-7 days after imbibition using isotope dilution mass

spectrometry. ABA and NAE levels were highest in desiccated seeds, and the content of both dropped simultaneously after imbibition and then remained low for up to 7 days. Although the endogenous levels of both metabolites dropped in a similar fashion during seed germination, total endogenous NAE was about 10-fold higher than endogenous ABA when expressed in ng/g fresh weight basis (Figure 2.1B). Although maintaining higher levels of exogenous NAE12:0 in the medium inhibited seedling growth (Figure 2.1A), its removal from the medium allowed for seedling growth to resume (Figure 2.1C; (Blancaflor et al., 2003)) indicating that the growth arrest by NAE was reversible.

Endogenous Levels of ABA are not Significantly Altered by Exogenous NAE Treatment

One possible explanation for the NAE-induced growth inhibition was an NAE dependent increase of endogenous ABA. However, endogenous ABA levels in NAE12:0-treated seedlings showed only modestly higher ABA levels at 4 d, and no difference at 8 d (Figure 2.2A), despite the clear impact on seedling growth at these ages (Figure 2.1A). Conversely, the endogenous total NAE content was moderately higher at 4 d ($p < 0.03$), but not substantially affected at 8 d treatment with ABA (Figure 2.2B). These results suggested that total NAE and ABA content are not influenced substantially by one another. Hence, NAE-mediated growth arrest did not appear to be mediated by increased ABA nor did ABA-induced growth inhibition stem from changes in total amounts of endogenous NAE (especially in older seedlings).

Transcript Profiling Links NAE-Induced Growth Inhibition in Arabidopsis *Seedlings to ABA Responses*

To further probe the molecular mechanisms underlying NAE12:0 effects on

seedling growth, I conducted global gene expression profiling using Affymetrix ATH1 whole genome microarrays. Seeds were germinated and seedlings maintained for 4 d in liquid MS media supplemented with 35 μM NAE12:0 prior to RNA isolation. Under these conditions, the growth of seedlings maintained in NAE12:0 was significantly inhibited when compared to seedlings grown without NAE12:0 (Figure 2.1A,C). The hydridization and initial analysis were conducted at Texas A&M University (College Station, TX) microarray service center supervised by Dr. Thomas McKnight. The data from the genome-wide analysis were evaluated using the criteria of increased or decreased expression by two-fold or greater and a quality assessment of each data point. The data in figure 2.3A (replicate 1) shows the genes expressed in both germinated seedling samples plotted in a scatter graph; this represents the genes of the ATH1 Genome gene chip that were expressed in these samples. The data in figure 2.3B is a volcano plot of the 216 genes that showed a 2-fold change or greater in transcript levels and gives an indication of the level of change and quality assessment of each gene (a 95% confidence interval calculated from statistics on all 11 paired probes).

Replicate microarray tests were conducted and the data revealed that expression was elevated for 45 genes in both data sets, and lowered for 19 genes in both data sets (Figure 2.4). These differences between experiments appeared to be due largely to developmental timing since many genes from both experimental sets were also found modulated in subsequent array analysis and RT-PCR experiments. However, to be more rigorous in selecting genes, I focused on those that showed transcript changes in both experiments. The list of genes that were common to both microarray experiments

(Table 2.1) were classified using the data mining web site FATIGO (http://fatigo.bioinfo.cino.es). This tool uses the gene ontogeny (GO) annotation assigned by The *Arabidopsis* Information Resource (TAIR) to classify genes by function and cellular distribution (Figure 2.5). Of the 45 genes with increased expression levels greater than 2 fold, almost half are predicted or known to be associated with the endomembrane. The larger groups classified by function include metabolism, seed storage, embryogenesis and defense genes (11% each of total). Others (34%) included transcription factors, ABA responsive, cell signaling and several more. About 18% of the up-regulated genes were annotated as "unknown", whereas 9% were heat shock proteins (HSP). Of the 19 genes with lower transcript levels by 2 fold or more, 60% were associated with the chloroplast genome and were not be considered further. The next largest group, 25%, was associated with the endomembrane. By function, the genes with lower transcript levels were defense biosynthesis, lipid transport, protease, embryonic development and cell wall associated.

Developmental Changes in Transcript Levels by Quantitative Real Time RT-PCR

Several genes that were differentially regulated by NAE12:0 were selected for a more detailed analysis of expression levels by quantitative, real-time reverse transcriptase-polymerase chain reaction (RT-PCR) during seed germination and seedling growth (Figure 2.6). I was especially interested in the expression of *ABI3* over the time course, since this gene product is known to participate in the regulation of several of the genes in the microarray datasets and it is considered to be a key factor in the regulation of the embryo-seedling transition. Quantification of transcript levels

relative to 18S rRNA generally confirmed the microarray results, and provided a comprehensive view of the temporal changes of these transcripts over a 7-d time course of seed germination and seedling growth. Several of the profiled genes, known to be responsive to ABA based on comparison with published microarray data (Teaster et al., 2007), were higher in NAE-treated seedlings. Many of these ABA-responsive transcripts occurred at higher levels especially in NAE-treated older seedlings. Several other ABA-responsive genes that were examined by real-time RT-PCR showed similar patterns of gene expression and NAE modulation (e.g., *LEAM17, EXGT-4, RAB18*, data not shown). Some genes in the microarray list (Table 2.1) were not known to be ABA-responsive, such as *EXPR3* (an expansin-related gene), and the temporal profile of *EXPR3* transcripts was altered in NAE12:0-treated seedlings, albeit differently than the ABA-responsive transcripts (Figure 2.6). Transcript levels of *AtFAAH*, encoding an NAE-hydrolase, increased during the normal course of seedling establishment (Wang et al., 2006), but were not affected by NAE12:0 (Figure 2.6). A comparison in the fold-difference of four ABA-responsive genes induced by either ABA or NAE 12:0 after 4 d and 7 d showed that transcript levels, quantified by real-time RT-PCR, generally were influenced to a similar extent by both compounds (Table 2.2), except that the transcripts appeared to be up-regulated earlier in development in the ABA-treated seedlings. *EXPR3* was not modulated by ABA, but was induced by NAE, and so might be considered an NAE-specific responsive gene. *AtFAAH* transcripts were not modulated by either ABA or NAE (Figure 2.6, Table 2.2).Values for transcript were averages from two independent measurements at each time point, and results from three independent experiments showed similar profiles.

Discussion

A. thaliana seeds germinated and grown in constant exposure to micro molar concentrations of NAE 12:0 displayed several chronic abnormalities including swollen root tips, reduction in primary root length and defects in root hair formation (Blancaflor et al., 2003; Motes et al., 2005). The severity of the effects were dose dependent and specific for NAE12:0, but not lauric acid nor *N*-palmitoylethanolamine (NAE16:0). Imaging of fixed and living cells in the root tips of seedlings treated with NAE12:0 showed extensive swelling and disorganized cell files, TEM revealed cells with incomplete walls and vesicles accumulated near the plasma membrane (Blancaflor et al., 2003). Yet, the role of NAEs in these effects remains unclear. The information gathered in this study suggests that NAE interacts with the ABA signaling pathway during seedling growth arrest. These insights provide specific molecular targets to monitor and assist in elucidating the role of NAE metabolism in *Arabidopsis* seedlings.

ABA induces a secondary dormancy (growth arrest) in germinating *Arabidopsis*, and this growth arrest occurs only within a developmental sensitive time period (Lopez-Molina et al., 2001; Lopez-Molina et al., 2002). This secondary dormancy is a stress defense mechanism and responds to unfavorable growth conditions, such as water stress. During the normal transition from imbibing seed to germinating seed and seedling growth, ABA and NAE levels drop concurrently (Figure 2.1B). I propose that one mechanism of NAE action in plants is to interact with ABA signaling in the negative regulation of plant growth and development (Figure 2.1A,D). This NAE/ABA induced growth arrest can be monitored molecularly by the induction of ABA responsive genes, particularly *ABI3* (Figure 2.6, Table 2.1, 2.2).

The data gathered in this study suggests that NAE alters seedling growth at least partially through the ABA signaling pathway. First, whole genome microarray studies show that the transcript levels of a number of up-regulated genes in *Arabidopsis* seedlings treated with NAE12:0 are ABA responsive (Table 2.1, (Teaster et al., 2007)). Also, several genes that have elevated transcript levels in desiccated seeds which normally decrease during imbibition and germination remain elevated in NAE or ABA treated seedlings (Teaster et al., 2007). Many of the genes with elevated transcript by both NAE12:0 or ABA treatment encoded for proteins involved in desiccation tolerance (e.g. dehydrins), seed storage reserves and late embryogenesis abundant proteins (Table 2.1 (Teaster et al., 2007)). An additional and more comprehensive microarray analysis was conducted at The Samuel Roberts Noble Foundation in Ardmore Oklahoma using total RNA I provided from samples grown under identical conditions as in the previous microarray study. The results were evaluated from three replicate microarrays for both NAE-treated- and solvent (0.05% DMSO)- treated seedlings, the three replicates enabled a detailed statistical analysis conducted by Dr. Yuhong Tang (Teaster et al., 2007). Their findings reported 8124 genes were shown to be differentially expressed by NAE treatment. Among these 8124 genes, 548 genes in seedlings were differentially regulated by NAE12:0, 280 were up regulated and 268 were down regulated.

The NAE microarray data sets were compared with other published whole-genome microarray studies in ABA-treated seedlings or *Arabidopsis* seeds. Interestingly, 22% of the genes up regulated in NAE-treated seedlings also were up regulated in ABA-treated seedlings (Li et al., 2006). Moreover, compared to a well-

characterized data set developed by (Seki M, 2002), many ABA-induced genes were found in the NAE up-regulated gene list (Teaster et al., 2007). A number of genes in the NAE up-regulated gene list (and ABA-seedling arrays) were annotated as "embryo associated" (e.g. late embryogenesis abundant genes, dehydrins, globulins, oleosins, vicilins). This is reasonable since one well known role for ABA is the activation of embryo-associated genetic programs in maturing seeds (Finkelstein et al., 2002; Nambara and Marion-Poll, 2003; Nambara and Marion-Poll, 2005; Finch-Savage and Leubner-Metzger, 2006). Indeed, 19% of the genes that were significantly elevated in NAE12:0-treated seedlings also were highly expressed in *Arabidopsis* seeds (Nakabayashi et al., 2005). Both NAE microarray studies complement each other in their findings that several of the genes up-regulated by NAE were also ABA responsive. These results are consistent with the notion that NAE and ABA might act similarly to promote embryo-associated gene expression, and retard the seed to seedling developmental transition.

Another interesting finding of the Noble Foundation microarray study was the result of an *in silico* examination of potential *cis* regulatory elements in transcripts that were up regulated by both NAE12:0 and ABA (Teaster et al., 2007). The 1 kb regions upstream of the NAE12:0 up-regulated and down-regulated genes were queried with a statistical motif analysis tool. This search yielded multiple sequences in the upstream region of NAE12:0 up-regulated genes containing the ACGTG core, which is a feature of ABA-response elements (ABREs). As shown by (Nakabayashi et al., 2005), a number of genes expressed in mature *Arabidopsis* seeds contained the CACGTG-related sequence, a typical ABRE, and the CGTGTC-related sequence, which shares a

common motif with coupling element (CE3) to form a functional ABA response complex (Shen and Ho, 1995; Busk and Pagès, 1998). It was found that the 1 kb upstream region in 31.5% of the NAE12:0 up-regulated genes contained these ABRE motifs (Teaster et al., 2007). The results from whole-genome microarray analysis clearly indicate that ABA-responsive gene expression is a major target in NAE-induced, growth-arrested seedlings. The involvement of the ABA signaling pathway in the NAE response is further demonstrated by the observation that a significant number of genes highly expressed in NAE12:0-treated seedlings contained upstream promoter motifs for a functional ABA response complex (Teaster et al., 2007).

ABI3 is considered to be a key transcriptional regulator of embryo maturation and its transcript levels decline rapidly following normal imbibition and seed germination (Finkelstein et al., 2002; Gazzarrini and McCourt, 2003; Nambara and Marion-Poll, 2003; Suzuki et al., 2003; Nambara and Marion-Poll, 2005; Bassel et al., 2006). NAE12:0 treated seedlings had 50 fold greater transcript levels of *ABI3* than untreated seedlings at 7-d (Figure 2.6, Table 2.2). This time point of elevated *ABI3* transcript also corresponds to a period of reduced growth in NAE 12:0 treated seedlings (Figure 2.1A). This relationship between elevated *ABI3* transcript levels and reduced seedling growth occurred in the same time frame for ABA- modulated growth (Figure 2.1A, Table 2.2).

NAE12:0 treated seedlings undergo growth arrest and had elevated ABA responsive gene transcript levels, including *ABI3*, *ATEM1, AtHVA22B* and *CRA1,*(Figure 2.6) although, endogenous ABA levels were not elevated. NAE treated seedlings at 7-days of growth displayed growth arrest (Figure 2.1A), ABA levels in 8-d-old NAE treated seedlings were essentially equal to untreated seedlings (Figure 2.2A).

Also, the results for ABA treated seedlings were similar, the seedling growth was arrested (Figure 2.1A) and ABA responsive gene transcript levels were elevated (Table 2.2), yet endogenous levels of NAE at 8-days of growth were the same in treated compared to untreated (Figure 2.2B). These results indicate that even though NAE growth arrest at least partially functions through the ABA signaling pathway it is not mediated by the coincident elevation of ABA levels and vice versa. These results suggest that NAE and ABA levels were not influenced by one another, but, complex changes in NAE composition have been reported in ABA induced growth arrested *Arabidopsis* seedlings (Teaster et al., 2007). The NAE profile of 8-d ABA treated seedlings resembled the NAE profile of 4-d untreated seedlings, suggesting that this change of NAE composition could be the result of ABA, or ABA induced growth arrest. This also emphasizes the need to study the total NAE levels, but also the changes of individual species during developmental changes.

ABRE elements were identified in the promoter region of several genes that are responsive to NAE12:0 (Teaster et al., 2007). Even though many of the genes that NAE12:0 up-regulates contain these ABRE elements, it remains unclear whether NAE acts through these ABRE regulatory elements. Yet the number of genes with ABREs that are induced by both NAE and ABA suggest that both compounds have overlapping targets. One of these targets is ABI3 where NAE likely influences the steady-state levels of *ABI3* transcripts, which in turn supports downstream activation of ABA-responsive genes and a resumption of an embryo-oriented (no growth) program.

The evidence gathered in this study confirms that during normal seed imbibition and germination NAE and ABA levels decline simultaneously (Figure 2.1B). However, if

either metabolite remains elevated, growth arrest occurs, and the commensurate rise in transcript levels of genes associated with growth arrest (Figure 2.1A, Table 2.1). The whole genome microarray study shows that many of the genes up regulated are involved in desiccation tolerance, seed storage reserves and late embryogenesis. Also, a large portion of these genes were found to be ABA responsive (Table 2.1), and several function in the activation of a secondary dormancy program (Lopez-Molina et al., 2001; Lopez-Molina et al., 2002). Similar to ABA, NAE12:0 induces growth arrest in a narrow window of postgerminative growth, yet, neither exogenous NAE nor ABA induces elevated endogenous levels of the other metabolite (Figure 2.2). The evidence of NAE-responsive but non-ABA responsive genes (Figure 2.6, Table 2.2) indicates that growth arrest may be partially mediated in a separate non-ABA responsive pathway. Future experiments into the role of NAE12:0 in growth arrest should include a more in-depth transcript analysis of pivotal genes involved (i.e. *ABI3*), and the effect of other species of NAE during the germination of *A. thaliana*.

Methods

Plant Materials and Growth Assays

Plants were propagated in soil for seed production. For germination and growth experiments, seeds were first surface-sterilized with 95% ethanol followed by 33% commercial bleach for three minutes each and rinsed several times with sterile, deionized water. Seeds were stratified for three days at 4 ^{o}C in the dark and grown in liquid nutrient media (0.5X Murashige and Skoog (MS) salts containing 1% sucrose) as described previously (Wang et al., 2006). Germination and growth proceeded in a controlled environment room with 16h/8 h light/dark cycle (60 $\mu mol \cdot m^{-2} \cdot sec^{-1}$) at 20-22

^{O}C. Liquid cultured seedlings were incubated with shaking (75 rpm) and growth was quantified as fresh weight accumulation after collection and rinsing in a Buchner funnel. ABA or NAEs were added from DMSO stocks to the appropriate final concentrations, and untreated controls contained equivalent final University, College Station, Texas (supervised by Dr. Thomas McKnight). Each gene was assigned absent, present or marginal status based on hybridization to each of the 11 oligonucleotide representing a perfect match compared with hybridization to 11 mismatched oligonucleotides that differed from the perfect oligonucleotide by one base pair in the middle of the sequence. Only genes designated as "present" were considered in this study. A p-value for confidence in the quality of each gene signal was concentrations of solvent alone, 0.01 to 0.05%. In separate experiments, DMSO alone had little observed impact on growth. Concentrations of exogenous ABA were calculated and reported based on the active *cis*-isomer, Calbiochem-Novabiochem Corp. catalogue # 100111. NAE12:0 was synthesized by Dr. Kent Chapman according to (Devane et al., 1992).

Gene Chip Microarray Experiments and Data Analysis

Total RNA was isolated from seed and seedling samples according to (Dunn et al., 1988; Vicient and Delseny, 1999). RNA was quantified and evaluated for purity by UV spectroscopy and agarose gel electrophoresis (Krieg, 1996). Total RNA was analyzed by microarray analysis using the Affymetrix GeneChip® *Arabidopsis* ATH1 Genome Arrays. The tests were conducted at The Microarray Service Facility at Texas A&M assigned by Affymetrix software and only those trancripts with a value below 0.05 were considered for further analysis. Genes that showed a 2-fold or greater change in

transcript level in both replicate microarray analyses were identified. Each gene was classified using FatiGO tool (http://fatigo.bioinfo.cino.es) and Gene Ontology assignment given by The *Arabidopsis* Information Resource (TAIR: www.*Arabidopsis*.org).

Quantitative RT-PCR

Verification of microarray results and quantification of transcripts by quantitative RT-PCR was performed with a Smart Cycler II (Cepheid) instrument using real time one step assay system (Takara Bio Inc) with SYBR^{TM} Green I. The following gene specific primer pairs were used, *ABI3* (At3g24650) (F) 5'GAGCTGGCTCAGCTTCTGCTATG-3' (R) 5'AGGCCAAAACCTGTAGCGCATGTTC-3', *ATEM1* (At3g51810) (F) 5'-CTGAAGGAAGAAGCAAGGGAG-3', (R) 5-' TCCATCGTACTGAGTCCTCCTTTAC-3' *EXPR3* (At2g18660) (F) 5'-CCTACACTAGGTCTGCGTG-3' (R) 5'-GATAACCCGAAAAGCGT-3', *EXGT*-A4 (At5g13870) (F) 5'-CTCTGCCTCACGTTTCTGATTTTG G-3' (R) 5'-GAAAGCCAGTGCCAG TGTACTTGTC-3', *CRA1*(At5g44120)(F) 5'-CACCATTGCGTTTTGACGGAAGATC-3' (R) 5'-GATGACAACCGTGGAAACATTGTCC-3', *AtHVA22B* (At5g62490) (F) 5'-CATCGCTGGACCTGCATTA C-3' (R) 5'-GGATATAATGGGATCCATTCGAGG-'3, *AtFAAH* (At5g64440) (F) 5'-CCATCTCAAGAACCGGAGCATG-3' (R) 5'-GGTGTTGGAGGCTTGTCATAGC-3'. All primers were designed to span one intron to distinguish cDNA amplification from genomic DNA contamination. Relative transcript levels in all samples were normalized using 18S rRNA as a constitutively expressed internal control, with primers (F)5'-TCCTAGTAAGCGCGAGTCATCA-3' and (R) 5'-

CGAACACTTCACCGGATCAT-3' (Dean Rider et al., 2003). Quantitative RT-PCR reactions were performed in duplicate with 0.2 µg total RNA and 0.5 µL of 10 µM gene specific primers in each 25 µL reaction. The reaction mix was subjected to the following RT-PCR conditions, 42 ^{O}C for 15 min, one cycle; 95 ^{O}C for 2 min, one cycle; 94 ^{O}C for 10 sec, 58 ^{O}C for 25 sec (read cycle), 72 ^{O}C for 20 sec, the number of cycles and annealing temperature were experimentally determined for each set of gene-specific for primers. RT-PCR products were examined by gel electrophoresis and by melting curve analysis (60 ^{O}C to 95 ^{O}C at 0.2 ^{O}C /sec) to rule out anomalous amplification products. The $2^{-\Delta\Delta CT}$ cycle threshold (C_T) method was used to calculate relative changes in transcript levels determined from quantitative, real time RT-PCR (Livak and Schmittgen, 2001). The data were analyzed using the equation where $\Delta\Delta C_T = (C_{T,\,Target} - C_{T,\,18s})_{\,Treated} - (C_{T,Target} - C_{T,\,18s})_{\,Not\ Treated}$. "Treated" refers to samples treated with ABA or NAE, and "Not Treated" refers to samples treated with solvent alone. For ease of presentation, in the cases when transcript levels in the "treated" samples were lower than in "not treated" samples, the fractional values obtained by the above formula were converted to fold difference by taking the negative reciprocal.

Metabolite Quantification

Metabolites were quantified by isotope-dilution mass spectrometry. NAEs were extracted from approximately 50-250 mg plant tissue in ground glass homogenizers into 2-propanol/chloroform/water (2/1/0.45, v/v/v). Deuterated NAE standards (NAE12:0, NAE18:0, and NAE20:4) were added as quantitative standards. Total lipid extracts

were fractionated by normal-phase HPLC and individual NAE types were quantified as TMS-ethers (dervatized in BSTFA, Sigma) by GC-MS (Venables et al., 2005).

For ABA analysis, approximately 100 mg of plant tissue was ground in a chilled mortar with 30 mM imidazole buffer in 70% 2-propanol. Deuterated ABA (200 ng) was added as a quantitative standard (kindly provided by Dr. Karl Hasenstein). Three additional 2-propanol extracts of the same tissue were combined and reduced under N_2 in a dry bath at 70^{O}C to about 1-2 ml, removing the 2-propanol. Samples were stored at -20^{o}C until fractionation by HPLC and quantification by GC-MS as described previously (Wang et al., 2001).

ABA levels were quantified in seed and seedling extracts with the assistance of Dr. Karl Hasenstein, University of Louisiana at Lafayette. NAE levels were quantified by Will Wiant with the assistance of Dr. Barney Venables, University of North Texas.

Table 2.1. Genes in Replicate Microarray Analysis of 4 d-old *A. thaliana* seedlings with Transcripts that were Changed 2 Fold or More with NAE Treatment

Diamond indicates known ABA responsive gene.

Locus ID	Fold	Cellular Component	Gene Description	ABA Responsive
At2g18660	42.2	endomembrane	Expansin family protein (EXPR3)	
At3g51810	19.7	unknown	Embryonic abundant protein AtEm1	◆
At2g43590	18.4	endomembrane	Putative endochitinase	◆
At1g78340	8.6	unknown	Glutathione transferase	
At5g44120	7.0	endomembrane	12S seed storage protein (CRA1)	◆
At3g54940	5.7	endomembrane	Cysteine proteinase	◆
At1g75830	5.3	endomembrane	Plant defensin-fusion protein, putative (PDF1.1)	
At5g59720	4.9	unknown	Heat shock protein 18	
At3g01570	4.3	membrane	Putative oleosin	◆
At2g41260	4.0	endomembrane	Late embryogenesis abundant M17	◆
At3g03640	4.0	endomembrane	Beta-glucosidase	
At2g28420	3.7	unknown	Lactoylglutathione lyase family protein / glyoxalase I family protein	
At4g28520	3.5	intracellular organelle	12S cruciferin seed storage protein	◆
At1g05340	3.5	intracellular organelle	Unknown protein	◆
At5g20230	3.2	membrane	Blue copper binding protein	◆
At5g03350	3.0	endomembrane	Legume lectin family protein	◆
At2g32210	3.0	intracellular organelle	Unknown protein	
At4g27670	2.8	unknown	25.3 kDa small heat shock protein	
At3g22640	2.8	endomembrane	Cupin family protein,	◆
At2g43510	2.8	endomembrane	Encodes a defensin-like (DEFL) family protein, putative trypsin inhibitor	◆
At2g05380	2.8	endomembrane	Glycine-rich protein (GRP3S),	
At1g52690	2.8	unknown	Late embryogenesis-abundant protein	◆
At2g23620	2.8	unknown	Putative acetone-cyanohydrin lyase	
At5g06860	2.8	cell wall	Polygalacturonase inhibiting protein 1; PGIP1	
At5g62490	2.6	endomembrane	ABA-responsive protein (HVA22b), identical to AtHVA22b (*Arabidopsis thaliana*)	◆
At2g32190	2.6	intracellular organelle	Unknown protein	
At4g34135	2.6	endomembrane	UDP-glucoronosyl/UDP-glucosyl transferase family protein	
At2g25510	2.6	intracellular organelle	Unknown protein	
At2g21820	2.6	unknown	Unknown protein	◆
At4g25200	2.5	intracellular organelle	*Arabidopsis* mitochondrion-localized small heat shock protein (AtHSP23.6-mito)	

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TAt1g54870	2.3	intracellular organelle	Dormancy related protein, similar to short-chain dehydrogenase/reductase (SDR) family protein	◆
At2g26400	2.3	unknown	Acireductone dioxygenase (ARD/ARD') family protein	
At3g22060	2.3	endomembrane	Domain of unknown function that is usually associated with protein kinase domain	◆
At3g24650	2.1	unknown	Abscisic acid-insensitive protein 3 (ABI3)	◆
At2g44130	2.1	unknown	Kelch repeat-containing F-box family protein	
At5g54740	2.1	endomembrane	Protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	◆
At1g55920	2.1	intracellular organelle	Serine acetyltransferase	
At1g32560	2.0	unknown	Late embryogenesis abundant (LEA) group 1 protein	◆
At5g13870	2.0	endomembrane	Identical to endoxyloglucan transferase EXGT-A4 GI:5533315 from (*Arabidopsis thaliana*)	◆
At3g53230	2.0	unknown	Cell division cycle protein 48, putative / CDC48	
At5g12030	2.0	cytoplasm	17.7 kDa class II heat shock protein 17.6A	◆
At5g51760	2.0	intracellular organelle	Protein phosphatase-2C; PP2C-like protein	◆
At2g43620	2.0	endomembrane	Putative endochitinase	◆
At1g04560	2.0	endomembrane	AWPM-19-like family; similar to late embryogenesis abundant protein (Lea)	◆
Atcg01020	-24.3	chloroplast	Encodes a chloroplast ribosomal protein L32	
Atcg00630	-13.0	chloroplast	PSI J protein	
Atcg00720	-13.0	chloroplast	Encodes the cytochrome b(6) subunit of the cytochrome b6f complex.	
Atcg00470	-9.8	chloroplast	ATPase epsilon subunit	
At3g59930	-9.2	endomembrane	Encodes a defensin-like (DEFL) family protein	
Atcg00340	-7.5	chloroplast	Encodes the D1 subunit of photosystem I and II reaction centers.	
Atcg01040	-6.5	endomembrane	Hypothetical protein	
Atcg00080	-4.0	chloroplast	PSII I protein	
At5g46900	-3.0	endomembrane	Protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	◆
Atcg00540	-2.8	chloroplast	Encodes cytochrome f apoprotein; involved in photosynthetic electron transport chain	
At5g59090	-2.8	intracellular organelle	Subtilisin-like serine protease	
Atcg00300	-2.5	chloroplast	Encodes PsbZ, which is a subunit of photosystem II	
At5g49080	-2.5	endomenbrane	Proline-rich extensin-like family protein	
At5g23020	-2.3	chloroplast	2-isopropylmalate synthase 2 (IMS2)	
Atcg00650	-2.3	chloroplast	Chloroplast-encoded ribosomal protein S18	
Atcg00800	-2.1	chloroplast	Encodes a chloroplast ribosomal protein S3	
At1g71830	-2.1	membrane	Plasma membrane LRR receptor-like serine threonine kinase (SERK1)	
Atcg00070	-2.1	chloroplast	PSII K protein	
At2g39705	-2.0	chloroplast	Expressed protein ; supported by full-length cDNA: Ceres: 27620.	

Microarray analysis was conducted by TAMU service facility supervised by Dr. Thomas McKnight.

Transcripts were judged as absent, present or marginal status based on hybridization to each of the 11 oligonucleotide representing a perfect match compared with hybridization to 11 mismatched oligonucleotides. Only genes designated as "present" and a p-value for confidence below 0.05 were considered for further analysis. Genes that showed a 2-fold or greater change in transcript level in both replicate microarray analyses were identified and classified using FatiGO tool (http://fatigo.bioinfo.cino.es) and Gene Ontology by The *Arabidopsis* Information Resource (TAIR: www.*Arabidopsis*.org).

Table 2.2. Comparison of Fold-Changes in Transcript Abundance in NAE12:0- and ABA-Treated Seedlings at 4-d and 7-d After Sowing Relative to Untreated Seedlings

	4 d		7 d	
Gene product	NAE	ABA	NAE	ABA
ABI3	2	17	48	13
CRA1	98	843	685	465
EXGT-A4	6	4	46	41
AtHVA22B	6	76	46	122
EXPR3	23	2	2	1
FAAH	-3	2	1	-2

Transcript levels were quantified by real-time RT-PCR from seedlings grown in NAE12:0 (35 μM) or ABA (0.5 μM). Fold difference was calculated by the $2^{-\Delta\Delta CT}$ cycle threshold (C_T) method (Livak and Schmittgen, 2001) normalize to 18s rRNA.

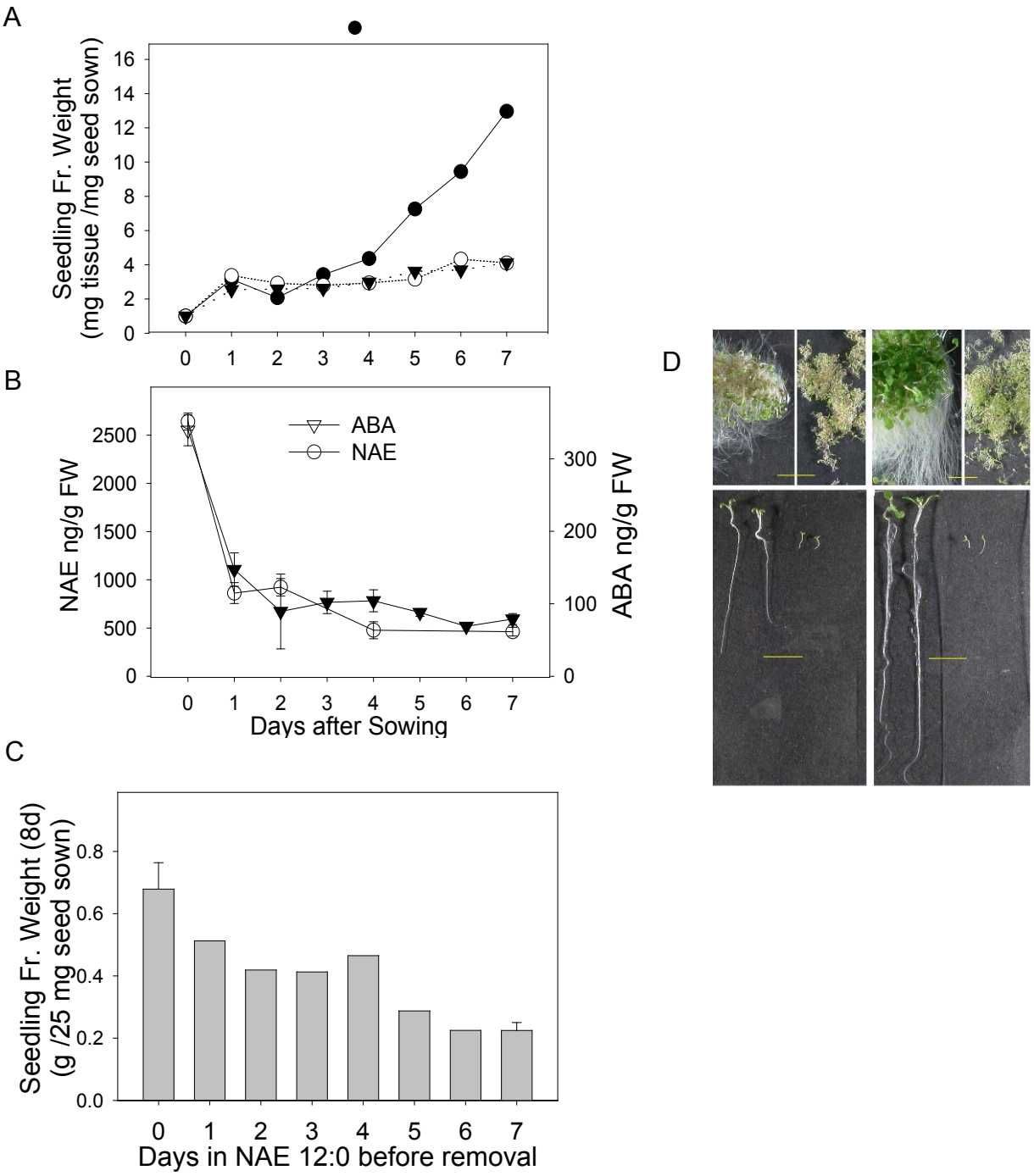

Figure 2.1. ABA or NAE12:0 negatively regulates seedling growth and this inhibition is reversible and occurs within a narrow developmental window.

(A) Treatment of seedlings with 0.5 μM ABA or μ35 M NAE arrests seedling growth. Data shown are representative of a single time course experiment. The trends of

growth suppression by ABA and NAE compared to controls were similar in replicate experiments (six for NAE and five for ABA), although the absolute fresh weight gain at each time point varied somewhat from experiment to experiment.

(B) Endogenous ABA and NAE levels drop precipitously with seedling emergence. Values for NAE are means and standard deviations of 6 independent extractions and were quantified by Will Wiant in Dr. Barney Venables laboratory. Values for ABA are means and standard deviation of three independent extractions, quantified with the assistance of Dr. Karl Hasenstein, University of Louisiana at Lafayette. Under these conditions, radicle emergence occurs at about d 3.

(C) The inhibitory effects of NAE (35 μM) on *Arabidopsis* seedling growth are reversible. Bars show the standard deviation of three different liquid culture experiments to show the general range of variability.

(D) Image compares 7-d old seedlings grown in liquid cultures without (two on left) or with (two on right) NAE12:0. Scale bar represents 10 mm.

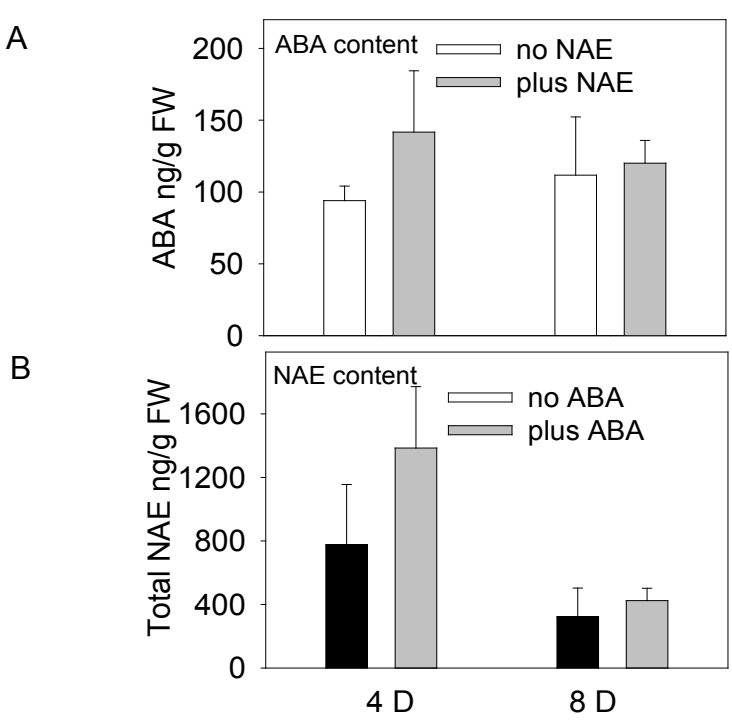

Figure 2.2. ABA and NAE content in seedlings at 4 and 8 d after sowing quantified by isotope-dilution mass-spectrometry.

(A) ABA content in seedlings treated without or with 35 µM NAE12:0. Deuterated ABA standard was added at the time of extraction. Values are means and standard deviations of three independent measurements. Seedling extracts were sent to the lab of Dr. Karl Hasenstein, University of Louisiana at Lafayette for ABA quantification.

(B) NAE content in seedlings treated without or with 0.5 µM ABA. Deuterated NAE (NAE12:0, NAE18:0 and NAE20:4) standards were added at the time of extraction. Values are averages and standard deviations of six independent measurements. NAE levels were quantified by Will Wiant with the assistance of Dr. Barney Venables, University of North Texas.

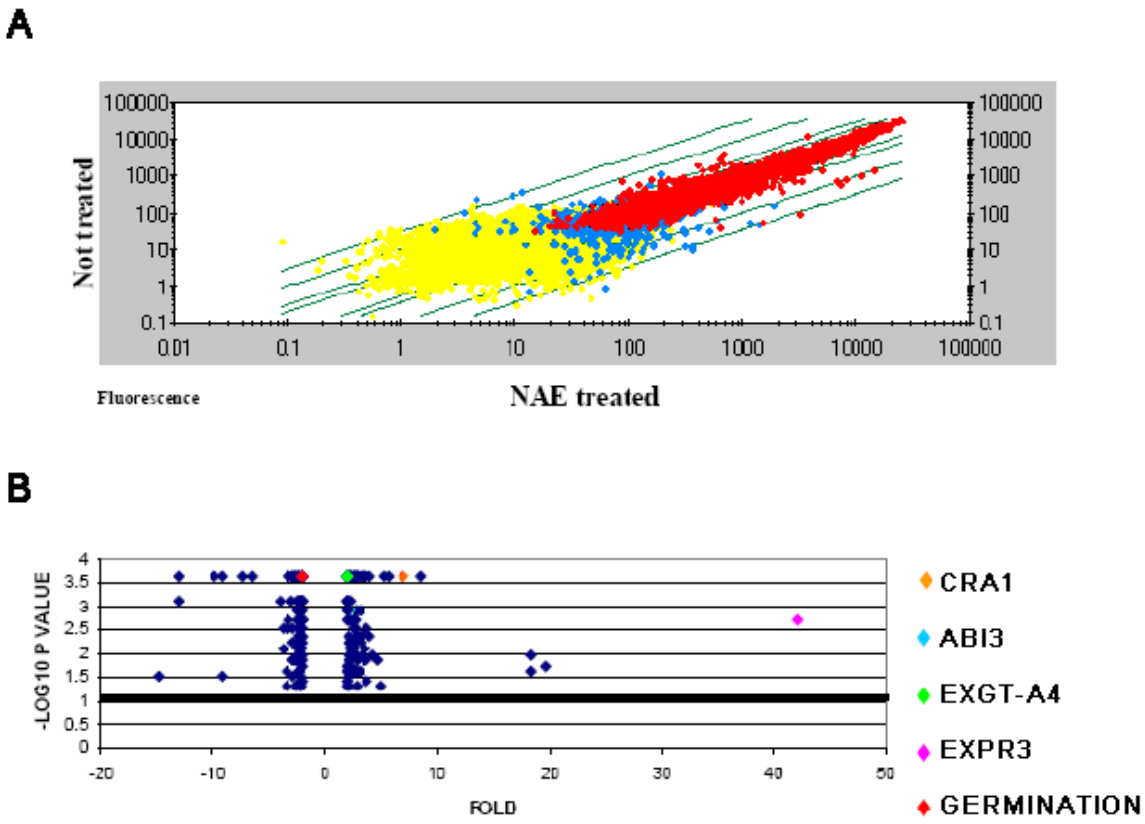

Figure 2.3. Global gene expression analysis of total RNA from 96 h -old, NAE treated and untreated seedlings. Microarray analysis was conducted using the Affymetrix GeneChip® *Arabidopsis* ATH1 Genome Array and the service facility at Texas A&M University, College Station, Texas, supervised by Dr. Thomas McKnight. Experiments, treatments and RNA isolation were conducted at The University of North Texas. (A) Representative scatter graph generated by statistical analysis of the ratio of normalized fluorescent signals for untreated versus NAE 12:0 treated seedlings. Each data point is proportional to the transcript abundance of an individual gene (represented by 11 oligonucleotides in each array). Points above and below the green diagonal line indicate changes in transcript level for that gene. The first set of diagonal lines from the

center represents a 2 fold change in expression, up or down and only those genes were analyzed further.

(B) Volcano plot shows 216 genes with 2 fold or greater change that surpassed the quality assessment of the fluorescence signal (above the bold line). Data points higher on Y-axis and farther on X-axis represents genes with high quality assessment and a greater fold change in expression, respectively. Color coded data points are the genes selected for further investigation.

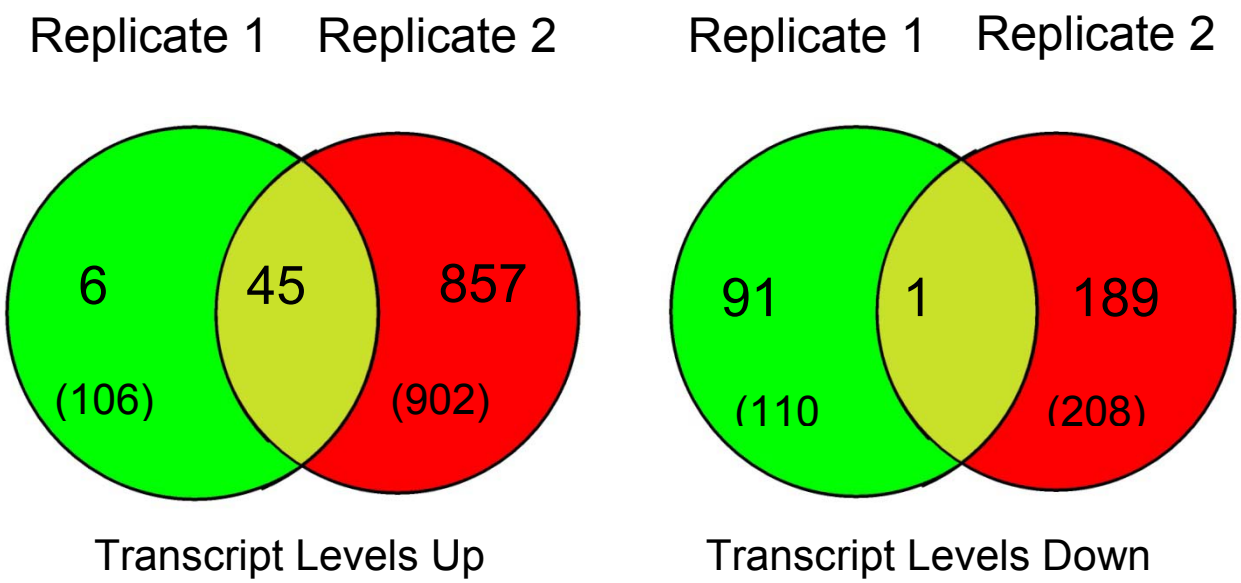

Figure 2.4. Diagram of common genes in duplicate sets of microarrays. The Venn diagram displays the number of genes that are common to replicate microarrays. Genes with an increase in transcript levels have 45 genes in common between replicate microarrays. Nineteen genes with a decrease in transcript levels were common in both microarrays. The numbers in parenthesis are the total number of genes (up or down) with transcript changes greater than 2 fold.

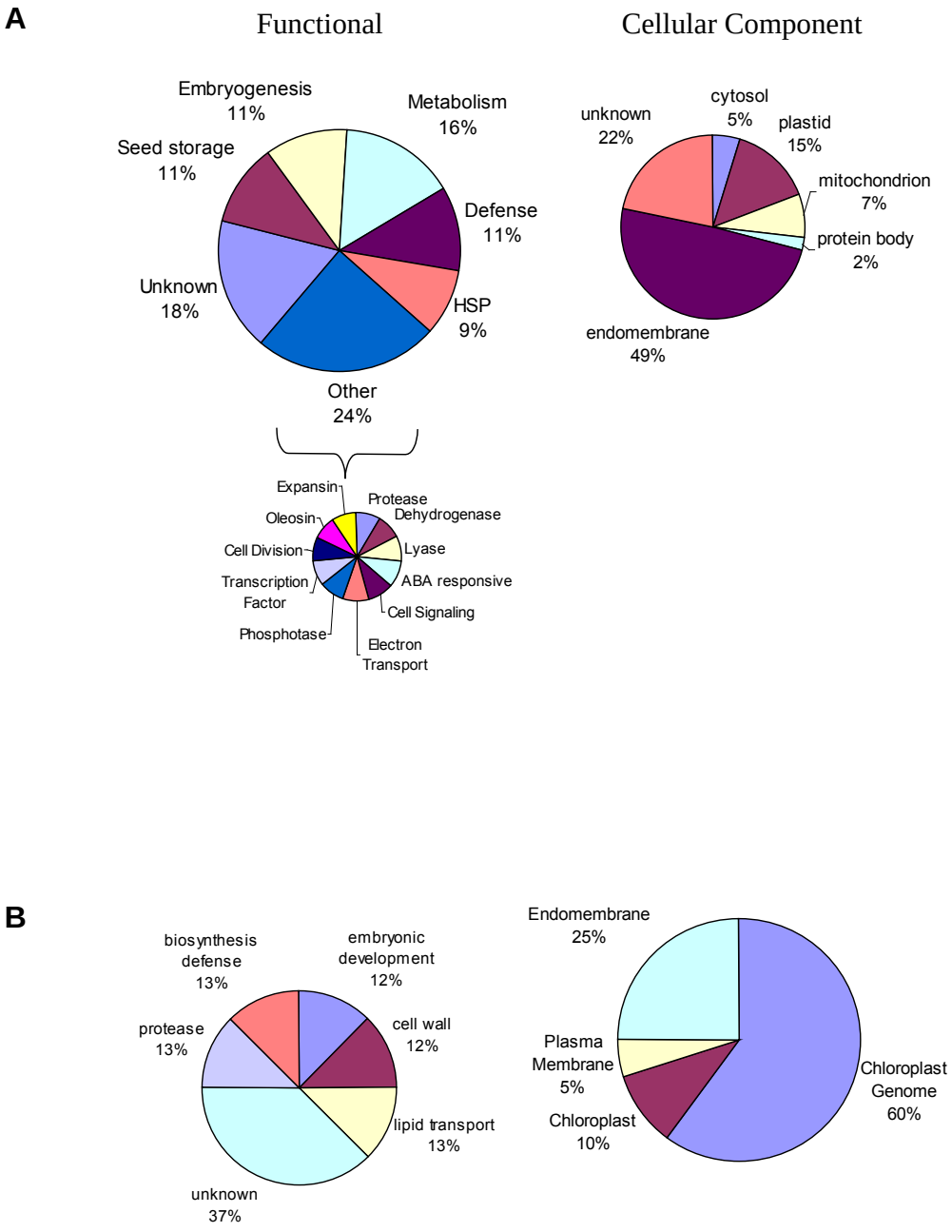

Figure 2.5. Pie chart of gene function and cellular component from microarray analysis.

(A) The 45 genes with transcript levels higher in treated seedlings.

(B) The 19 genes with transcript levels lower in treated seedlings.

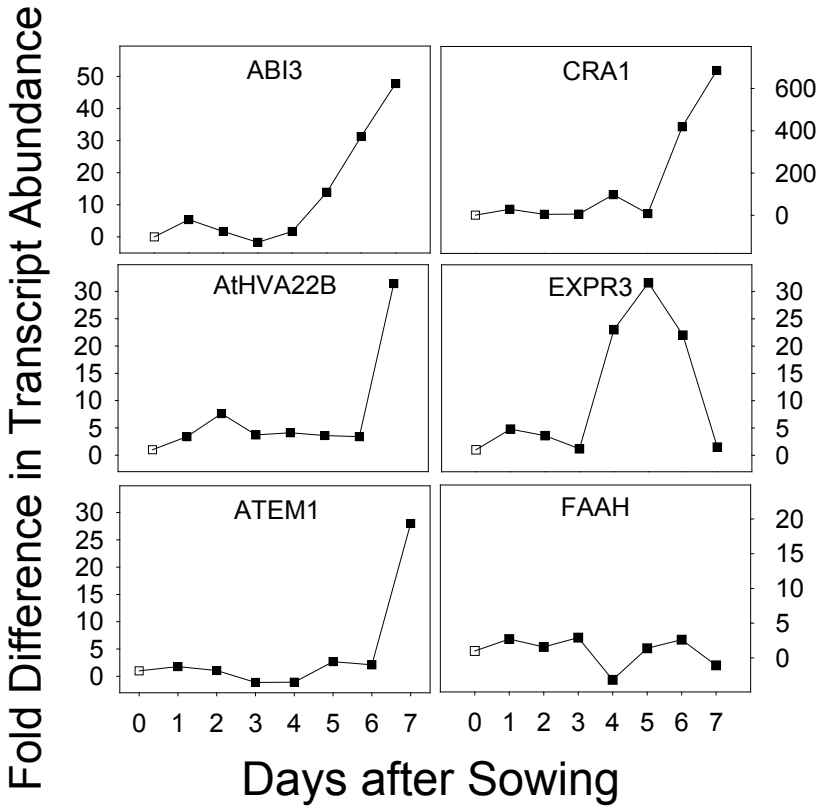

Figure 2.6. Time course of NAE-modulated changes in transcript abundance for several ABA-responsive (*ABI3, HVA22B, ATEM1, and CRA1*) and ABA-non-responsive (*EXPR3* and *FAAH*) genes.

Transcript levels were quantified in total RNA extracts by real-time RT-PCR against 18S rRNA and plotted as -fold difference (NAE treated vs. untreated) using the $\Delta\Delta$CT method (Livak and Schmittgen, 2001). Transcripts were quantified in total RNA extracts from seeds or seedlings at designated days after sowing in liquid media (same stages as in figure 2.1). RNAs were targeted for amplification with gene specific primers (see methods). Values shown are averages of duplicate samples and results from three independent experiments showed similar profiles. Oldest seedlings showed the greatest increase in ABA-responsive transcripts in NAE12:0-treated (35 μM) seedlings.

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CHAPTER 3

N-ACYLETHANOLAMINE (NAE) INDUCES GROWTH ARREST IN *Arabidopsis thaliana* SEEDLINGS VIA ABA DEPENDENT AND ABA-INDEPENDENT SIGNALING PATHWAYS

Abstract

Exogenous treatments of NAE 12:0 and ABA induce growth arrest in *Arabidopsis thaliana* seedlings within a similar developmental window. The transcription factor ABI3 is considered to be a key regulator of the seed-to-seedling transition. Here, I conducted additional gene expression studies with *ABI3* during NAE 12:0 and ABA induced growth inhibition. Overexpression of the NAE degrading enzyme fatty acid amide hydrolase (FAAH) resulted in seedlings that were tolerant to NAE, with increased sensitivity to ABA, while the FAAH knockout (*faah*) was hypersensitive to NAE. The tolerance to NAE of the FAAH OE was determined to be its ability to deplete the NAE from the growth media. Transcript profiling confirmed that the levels of *ABI3* were inversely associated with ABA/NAE modulated growth of WT and FAAH altered seedlings, but only within an early developmental window. NAE12:0 has been shown to induce dose dependent inhibition of growth, yet, transcript levels of associated genes were not elevated until higher doses of NAE12:0 (35 μM). The ABA insensitive mutant (*abi3-1*) does not undergo growth inhibition when treated with ABA, yet NAE will inhibit growth. Also, transcript levels of genes associated with inhibited growth are lower, or not detectable in the NAE treated seedlings. Genetic and molecular evidence suggest that NAE induced growth arrest operates in a parallel pathway, yet, operates in concert with the ABA signaling pathway. Collectively our data suggest that NAE metabolism interacts in an

ABA dependent and independent negative regulation of seedling development and that normal seedling establishment depends on the reduction of the endogenous levels of both metabolites.

Introduction

N-Acylethanolamines (NAEs) are fatty acid amides that are derived from an *N*-acylated phosphatidylethanolamine precursor, a minor membrane lipid constituent of plant and animal cells. NAEs were first reported as constituents of soy lecithin and peanut meal in the 1950s (Kuehl et al., 1957). The occurrence and metabolism of NAEs is conserved among the eukaryotic organisms (Schmid et al., 1996), (Chapman, 2004); however, the physiological functions of these lipids have been investigated mostly in vertebrates. In animal systems NAEs have been determined to have roles as regulators of important physiological processes such as embryo development, cell proliferation, immune responses and apoptosis (De Petrocellis et al., 2004).

There is accumulating evidence that plants also use NAEs to regulate important physiological processes. This is supported by the identification of NAEs in a variety of plant tissues (Chapman, 2000, 2004), and the fact that levels vary due to environmental and growth conditions. NAEs in plants, like animals, have potent biological activities at low concentrations (Chapman, 2004; Kilaru et al., 2007), such as defense gene activation by NAE14:0. Sub-micromolar to low micromolar concentrations added to cell suspensions and leaves of tobacco plants increased phenylalanine-lyase (*PAL2*) transcript abundance (Tripathy et al., 1999). In other work, *Arabidopsis* seedlings germinated in, and maintained on, micromolar concentrations of NAE12:0 show marked

developmental abnormalities in young roots, and these effects were specific for short/medium chain for acylethanolamides (Blancaflor et al., 2003). Endogenous levels of NAEs drop dramatically from the micromolar typically found in seeds to nanomolar levels during imbibition and germination in a variety of plant species, and remain low during subsequent seedling growth (Chapman et al., 1999). This rapid depletion of NAEs suggests that for normal seedling development to occur a system for NAE metabolism is required.

Key to understanding the functional roles of these lipids in all organisms is to identify the mechanisms that regulate their accumulation. NAEs in animal systems appear to be modulated by their degradation to free fatty acids and ethanolamine by fatty acid amide hydrolase (FAAH), a member of the amidase superfamily (McKinney and Cravatt, 2005). Thus, FAAH modulation of NAE levels has become an important focus for understanding the mechanistic action of the “endocannabinoid” signaling system in vertebrates (Cravatt et al., 2001).

Bioinformatics approaches and heterologous protein expression led to the molecular identification of an amidohydrolase from *Arabidopsis thaliana* that hydrolyzed a wide range of acylethanolamides, including those in desiccated seeds (Shrestha et al., 2003). Biochemical data supported the notion that the *Arabidopsis* gene At5g64440 encoded a homologue of the mammalian FAAH (Shrestha et al., 2003). Although there was limited primary amino acid sequence identity over the length of the *Arabidopsis* protein compared with the rat protein (18%), there was substantially higher similarity within the amidase catalytic domain both at the primary (37-60% depending on the lengths compared) and secondary structural levels (Shrestha et al., 2003). Research in

the last decade has made it apparent that NAE metabolism occurs in plants by pathways analogous to those in vertebrates and invertebrates (Chapman, 2000; Shrestha et al., 2002), pointing to the possibility that these lipids may be part of an evolutionarily conserved mechanism for the regulation of physiology in multicellular organisms. With the evidence of conserved enzymatic machinery in plants for the formation and degradation of NAEs, and the potent biological effects caused by altered exogenous NAE levels, it is now important to begin to address the question of how altered FAAH expression would affect NAE signaling in plants.

With the recent discovery that a functional homologue of FAAH in *Arabidopsis* converted a wide range of NAEs to their corresponding free fatty acids and ethanolamine (Shrestha et al., 2003), and homologues of the *Arabidopsis* FAAH (*AtFAAH*) were also identified in *Oryza sativa* and *Medicago truncatula*, a common mechanism for the regulation of NAE hydrolysis in diverse plant species has emerged (Shrestha et al., 2006). To further examine the role of this FAAH in NAE metabolism *in situ* there have been several lines of transgenic *AtFAAH* over expressing (OE) lines generated by the E. Blancaflor lab (Samuel Roberts Noble Foundation) with the full-length *AtFAAH* cDNA cloned down stream from the CaMV 35S promoter. *AtFAAH* transcript levels and enzyme activity in these OE lines were substantially higher than WT (Wang et al., 2006). Also available is an AtFAAH knockout (KO; Salk_095108) obtained from the *Arabidopsis* Biological Resource Center (ABRC), homozygous for T-DNA insertion in the 16th exon, had *AtFAAH* mRNA levels below detection, and almost no detectable FAAH activity in seedlings (Wang et al., 2006). The manipulation of AtFAAH activity altered the physiological responses of *Arabidopsis* seedlings to

exogenously applied NAEs (Wang et al., 2006). The enhanced seedling growth of *AtFAAH* OEs, the hypersensitivity of *AtFAAH* knockouts to exogenous NAE, and the predictable alterations in expression and enzymatic activity of AtFAAH, which coincided with NAE depletion during seed germination, all were consistent with the notion that these fatty acid amides may be negative regulators of seedling growth.

Interestingly, ABA is a known negative regulator of seedling growth (Lopez-Molina et al., 2002), and many NAE responsive genes were also ABA responsive. For example, microarray studies of ABA treated *Arabidopsis* seedlings (Li et al., 2006) show that the transcript levels of a number of upregulated genes are also upregulated in NAE treated seedlings (Teaster et al., 2007). Many of the genes upregulated by both NAE and ABA treatments encoded for proteins involved in desiccation tolerance, seed storage proteins and late embryogenesis abundant proteins. The involvement of ABA and NAE response is further demonstrated by the significant number of genes highly expressed in NAE treated seedlings that contain upstream promoter motifs for a functional ABA response complex (Teaster et al., 2007). Also, the hypersensitivity to ABA found in the FAAH OEs (Teaster et al., 2007), which also contain lower endogenous levels of NAE (Wang et al., 2006), suggests interaction between the two pathways. The similarity of molecular targets and signaling intermediates of ABA and NAE provide us with a strong basis to hypothesize that these two classes of compounds might somehow interact in regulating plant development. Perhaps more compelling is the fact that both metabolites are negative regulators of seedling growth (Lopez-Molina et al., 2002; Blancaflor et al., 2003; Motes et al., 2005) and that endogenous ABA and NAE, which are typically elevated in desiccated seeds, are depleted during germination

and early seedling growth in a comparable time course (Chapman et al., 1999; Jacobsen et al., 2002; Nakabayashi et al., 2005; Wang et al., 2006; Teaster et al., 2007). Comparison of stress-induced gene expression in ABA biosynthesis and response mutants has demonstrated that there are both ABA-dependent and – independent signaling pathways (Rock, 2000). Isolation of mutants with defects in stress- and ABA-induced signaling has provided evidence for cross-talk among even the “independent” signaling pathways (Ishitani et al., 1997).

To test the hypothesis that NAE and ABA interact during plant development, and to explore in more detail the potential mechanism(s) of NAE action in seedling growth arrest, I focused my attention on seed germination and early seedling establishment. Collectively, our results provide molecular and genetic evidence that NAE regulation of seedling growth works in an ABA dependent and independent signaling pathways. Seedling growth arrest can be induced by elevating NAE or ABA content leading to the resumption of embryo-specific gene expression. However, induction of this growth arrest/embryo program can be initiated only within a narrow window of early seedling establishment. These data suggest that NAE metabolism acts in concert with ABA to negatively regulate seedling development in *Arabidopsis*, and that normal seedling growth proceeds after sufficient depletion of both NAE and ABA and a developmental reduction in the sensitivity of seedling tissues to these regulators. These results provide evidence that NAE metabolism, which until now has been implicated mostly in the regulation of animal physiology, impacts a major hormone signaling pathway in plants.

Results

Arabidopsis thaliana *Seedlings with FAAH-Altered Metabolism Affects Sensitivity to NAE12:0*

The substantial amounts of NAEs in desiccated seeds and their depletion upon imbibition (Chapman et al., 1999) likely requires an active enzyme to metabolize them. An enzyme in *A. thaliana* responsible for hydrolyzing a range of NAEs to free fatty acids is encoded by the gene At5g64440, fatty acid amide hydrolase (FAAH) (Shrestha et al., 2003). Growth studies were conducted with exogenously applied NAE 12:0 and the results demonstrated NAE tolerance of the FAAH OE, and a higher degree of sensitivity in the FAAH knock out (*faah*) compared to wild type (WT) (Figure 3.1). The NAE12:0 treated FAAH OE had a 3-fold increase in growth (quantified by fresh weight) at 14-d compared to treated WT, while the *faah* showed no gain in fresh weight (arrested growth) during the 14-d time course. Without NAE treatment, the growth of the three types of seedlings without treatment was similar among all genotypes.

NAE 12:0 induced a dose dependent reduction in growth in WT *Arabidopsis* seedlings (Blancaflor et al., 2003). To determine if the NAE tolerant OE and the NAE sensitive *faah* would have a similar dose dependent reduction in growth, seedlings were germinated and grown under constant exposure at increasing concentrations of NAE12:0 for 7-d (Figure 3.2A,B). At a relatively low NAE12:0 concentration of 10 μM the *faah* seedlings showed a reduction of growth by approximately 25%, compared to essentially 0% in WT (quantified by fresh weight), indicating an increased sensitivity of *faah* to exogenous NAE. While the FAAH OE displayed an increased tolerance toward NAE12:0 at 20 μM, a 10% reduction of growth in the FAAH OE compared to approximately 70% in WT. Growth was reduced in all genotypes as NAE12:0

concentration increased, with the FAAH OE displaying tolerance to NAE12:0 in concentrations as high as 50 μM (Figure 3.2B). Taken together these results confirmed that FAAH functions in planta to metabolize NAE, and that the plants with altered FAAH expression have the predicted growth phenotypes associated with FAAH expression.

The transcription factor ABI3 is considered to be a key regulator of the seed to seedling transition, and it has also been shown to be NAE responsive (Chapter 1), hence it was selected for the investigation of NAE dose-dependent gene expression by quantitative real-time RT-PCR. In addition to *ABI3*, *AtHVA22B* and *RD29B* transcript levels were elevated by NAE12:0 in a previous microarray study (Teaster et al., 2007), and these two genes have been shown to be regulated in part by ABI3. Quantification of transcript levels relative to 18S rRNA provided the transcript abundance for the NAE responsive genes in 7-d old seedlings of WT, FAAH OE and *faah* seedlings grown at increasing concentrations of NAE12:0 (Table 3.1). The results show that transcript levels for *ABI3* in WT increased in a dose dependent manner, with a dramatic rise at 35 μM in all three genotypes (to varying degrees associated with NAE sensitivity). Somewhat puzzling were the moderately higher levels of *ABI3* transcripts in the FAAH OE, relative to WT at lower concentrations of NAE. At 35 μM NAE12:0 growth is substantially reduced in all genotypes, with *faah* the most severely reduced, FAAH OE the least affected, and WT in between (Figure 3.2A). Generally, at the higher NAE concentrations, 35 μM and above, where growth differences are clearest among genotypes, gene transcript levels associated with growth arrest reflected this same trend, *faah* having the most elevated levels, FAAH OE the lowest and WT in between. It appears that transcript levels became elevated at some critical concentration (above 20

μM) rather abruptly instead of in a dose dependent fashion for *AtHVA22B* and *RD29B,* perhaps reflecting some threshold level of regulation.

Growth Arrest and Associated Gene Expression are Induced Only within a Strict Time Period after Germination

The transcription factor ABI3 is considered to be a key regulator of the seed-to-seedling transition (Parcy et al., 1994; Nambara et al., 1995; Parcy et al., 1997); therefore, I conducted more detailed time course studies with ABI3 during NAE12:0-induced growth inhibition (Figure 3.4). Interestingly, *ABI3* expression was associated inversely with NAE12:0-modulated growth. This relationship was demonstrated in a dramatic manner with AtFAAH OEs, which are tolerant to NAE12:0-induced growth arrest and AtFAAH T-DNA knockouts (*faah*, Salk 095108), which are hypersensitive to NAE12:0 (Wang et al., 2006). Differences in *ABI3* transcript levels were associated with growth differences in these different genotypes (Figure 3.5). Highest levels of *ABI3* transcripts were associated with the least growth (*faah* grown in NAE12:0), and in contrast, the lowest *ABI3* transcript levels were associated with the most robust growth (AtFAAH OEs).

Previous work by Chua and coworkers (Lopez-Molina et al., 2001; Lopez-Molina et al., 2002) helped define the developmentally sensitive window for seedling growth arrest by ABA. Therefore, I tested whether seedlings showed a similar developmental sensitivity to exogenous NAE treatment. Wild type seedlings after 14-d growth that were treated with NAE12:0 earlier than 8 d after germination were inhibited in growth substantially compared to untreated seedlings (Figure 3.3). There was no significant difference in growth of seedlings treated with NAE after eight days of growth. The

sensitivity of seedlings to NAE was significant ($p<0.001$) up to day 6, but not significant (NS) thereafter. ABA sensitivity overlapped NAE sensitivity up to day 6, but persisted several days longer, until it too was abolished by day 10. Thus NAE and ABA appeared to target a similar developmental growth program in young seedlings.

Further, I predicted a developmental sensitivity in NAE- and ABA-induced *ABI3* expression, since there was a clear developmental sensitivity to NAE and ABA in terms of seedling growth arrest (e.g. Figure 3.4A, B). Indeed, increased *ABI3* expression was evident only in NAE12:0- and ABA- treated seedlings that had been treated at early stages (e.g., 2 d) where growth inhibition was most pronounced, but not at later stages (e.g., 10 d) of development, where both compounds had minimal growth inhibitory effects (Figure 3.4C). Taken together these results suggest that similar to ABA (Lopez-Molina et al., 2002), arrest of seedling growth by NAE12:0 is associated with *ABI3* gene expression (Figure 3.5).

To determine if an altered NAE metabolism would change this window of sensitivity to NAE/ABA I investigated growth and gene expression in the early and late stages of seedling development of the FAAH altered mutants. Growth of wild-type and FAAH-altered 14-d-old seedlings in response to exogenous NAE 12:0 and ABA was quantified by fresh weight gain (Figure 3.6). Growth was reduced by both treatments in the early stage of seedling development (2-d), the FAAH OE showed a tolerance to the NAE treatment, while the *faah* displayed an increased sensitivity (Figure 3.6A). The ABA treatment in the early stage reduced tissue accumulation equally in the WT and *faah* mutant, but revealed a heightened sensitivity in the FAAH OE (Figure 3.6 B). Growth was not substantially reduced by treatment with NAE or ABA in the later stage

of seedling development (10-d) (Figure 3.6A, B) in any genotype. These results indicate that the window of sensitivity was not appreciably affected by altering NAE metabolism.

To expand on these results with *ABI3* expression and growth, and I examined the gene expression levels of two other NAE/ABA responsive genes, *AtHVA22B* and *RD29B* (Teaster et al., 2007). The elevated levels of *ABI3* transcripts were, again, inversely associated with growth arrest (Figure 3.7), *faah* seedlings treated at 2-d with NAE display the least growth and highest levels of gene expression for all three genes, consistent with their hypersensitivity to NAE. The FAAH OE treated with NAE at 2-d had much more growth and no transcripts were detected for any of the three ABA-responsive genes, consistent with their tolerance to NAE. NAE-treated wild type seedlings (treated at 2-d) showed some growth, with low levels of *ABI3* transcripts. All three genotypes treated with ABA at the early stages of seedling development had varying degrees of reduced growth, and all had elevated transcript levels of the three genes, suggesting that although NAE could induce a differential growth/gene expression program in FAAH mutants, the responses to ABA were largely intact.

Little to no effect can be observed in the images of seedlings treated with ABA or NAE at 10-d of seedling development in any of the three genotypes (Figure 3.7A). The only gene displaying elevated transcript levels in older seedlings was *RD29B* (Figure 3.7B), which is ABA responsive outside the sensitivity window (Uno et al., 2000) (and is why it was selected for this study). Interestingly, NAE treatment at 10-d does not induce *RD29B* transcript, which would indicate that NAE12:0 may not entirely overlap with ABA signaling pathways outside the sensitivity window.

NAE Induced Growth Arrest can Occur Independent of an Intact ABA Signaling Pathway

The *abi3-1* mutant is ABA insensitive and recently ABA insensitive mutants were shown to be somewhat tolerant to NAE in early development (Teaster et al., 2007). Growth data confirmed the ABA insensitivity of the *abi3-1* mutants (Figure 3.8), with growth quantified as gain in fresh weight. The Landsberg *erecta* ecotype showed growth arrest when treated at the early stage of development, similar to that previously observed for Columbia WT. However Landsberg showed greater inhibition of growth when treated at the late stage of seedling development with ABA than the Columbia ecotype (Figure 3.6B). Nevertheless, treatment with NAE at the early stage of development resulted in growth arrest of both wild type (Ler) and *abi3-1*, while treatment at the late stage showed no inhibition of growth in either wild type or *abi3-1,* quantified as gain in fresh weight (Figure 3.8, 3.9A). Figure 3.9 shows the growth arrest of wild type seedlings treated with ABA or NAE and a corresponding increase in transcript levels of the three surveyed genes (*ABI3*, *AtHVA22B* and *RD29B*), which are associated with growth arrest. The ABA insensitivity of the *abi3-1* mutant (seedlings treated at the early stage of development with ABA), is in stark contrast to its sensitivity when treated with NAE. The pattern of gene expression is definitely altered in the *abi3-1*. As expected there were no detectable *ABI3* transcripts, and since the ABI3 transcription factor drives the expression of AtHVA22B, there were no transcripts for it either (Figure 3.9B). However, the *abi3-1* mutant undergoes growth arrest when treated with NAE at the early stage of seedling growth, indicating that NAE induced growth arrest, at least partially, works outside the ABA signaling pathway.

FAAH Overexpressor Tolerance is Associated with NAE Depletion in the Growth Media

FAAH OEs were tolerant to NAE perhaps due to the increased capacity by which NAE12:0 was metabolized to free fatty acids. The FAAH OE lines showed visible signs of recovery from NAE12:0 treatment at about 7-d of growth, WT showed similar recovery a few days later, and *faah* did not show any signs of recovery even after 14-d (Figure 3.1). To determine if NAE12:0 being metabolized from the growth media and that enabled the earlier recovery of FAAH OE seedlings, the concentration of NAE12:0 in the growth media at two time points, 6 and 12-d of growth was quantified. The initial concentration in the growth media was 55 μM, similar to the "no seed" control indicating no spontaneous degradation of NAE during the 12-d time course. After 6-d, NAE concentration was reduced in the media containing WT seedlings by 18%, and the media containing the FAAH OE seedlings was lowered by 45%, while the media containing *faah* seedlings lost only 13% (Figure 3.10). By 12-d the NAE12:0 content of the media containing WT or FAAH OE seedlings was below 1% of the initial concentration, while media containing the *faah* seedling was 53% of the initial concentration. This depletion of NAE12:0 from the growth media correlates well with the recovery of growth for NAE treated seedlings seen in figure 3.1.

Discussion

A. thaliana seed germination was not affected by NAE treatment, but there appeared to be a dramatic reduction in post germinative growth that was easily quantified by measuring gain in fresh weight (Figure 3.1). Severe morphological and cellular defects accompanied treatment of *Arabidopsis* seedlings with micromolar

concentrations of NAE12:0 (Blancaflor et al., 2003; Motes et al., 2005), but the primary mechanism(s) underlying these actions was/were unclear. Exogenous ABA treatment of seedlings, or the application of desiccation stress, induced seedling growth arrest comparable to NAE12:0, and this growth arrest may be mediated by ABA (Lopez-Molina et al., 2001; Lopez-Molina et al., 2002). NAE12:0 growth inhibition was dose dependent (Blancaflor et al., 2003), as observed in liquid and solid media (Figure 3.2). Transcript levels that are associated with growth arrest (*ABI3*) were also induced in a dose dependent manner in WT at lower levels of NAE12:0, and increased exponentially at higher levels of 35 μM NAE12:0 treatment (Table 3.1). *AtHVA22b* and *RD29B* transcript levels did not increase until application of NAE12:0 above 20 μM.

ABI3 is considered to be a key transcriptional regulator of embryo maturation and its transcript levels decline rapidly following normal imbibition and seed germination (Finkelstein et al., 2002; Gazzarrini and McCourt, 2003; Nambara and Marion-Poll, 2003; Suzuki et al., 2003; Nambara and Marion-Poll, 2005; Bassel et al., 2006). The treatment of seedlings with ABA/NAE12:0 in the early stage of seedling development resulted in higher steady-state levels of *ABI3* transcripts, which were particularly evident in older seedlings (e.g. 14-d old, Figure 3.4). The data gathered suggests an inverse relationship between *ABI3* transcript levels and NAE12:0 modulated growth (Figure 3.5). The highest levels of ABI3 transcript were found in the *faah* seedlings, which exhibited the most severe growth arrest, and the lowest level of *ABI3* transcript in the NAE tolerant FAAH OE lines, with the highest level of growth (Figure 3.5). I found that the FAAH OE's tolerance to NAE12:0 was, in part, due to its ability to metabolize NAE12:0 from the growth media (Figure 3.10). The FAAH knockout (*faah*), with its

capability to metabolize NAE greatly reduced, was not able to resume growth (Figure 3.1). I propose that one mechanism of NAE action in plants is to interact with ABA signaling in the negative regulation of plant growth and development. Here this concept is manifested in the arrest of early seedling growth when maintaining seedlings on elevated levels of either of these compounds.

It has been documented in literature (Lopez-Molina et al., 2001; Lopez-Molina et al., 2002) that ABA-induced growth arrest and desiccation tolerance occur only within a narrow window of early post-germinative growth. This arrest of seedling growth is proposed to function as a stress defense mechanism for seedlings in the activation of a secondary dormancy program. Under the normal course of seed germination and seedling growth, ABA and NAE levels decrease concomitantly in seedlings to allow the seed-to-seedling transition (Teaster et al., 2007). The sensitivity of the tissues to either ABA or NAE changed with progression of seedling development (Figure 3.3). This change can be marked at the molecular level by the occurrence and elevation of *ABI3* transcript levels (Figure. 3.4). The higher levels of *ABI3* transcripts in the seedlings treated at the early stage of development likely led to increased expression of other the ABA-responsive genes, *RD29B* and *AtHVA22B* (Figure 3.7). The data gathered shows that the sensitivity window is intact in the FAAH altered mutants, but they do have differing sensitivities to NAE and ABA (Figure 3.6). NAE12:0 induces gene expression higher in *faah* than the FAAH OE, relative to WT (Table 3.1), supplying molecular evidence of an increased sensitivity in *faah* and tolerance in the FAAH OE. Interestingly, while the FAAH OE displays a tolerance to NAE it has heightened sensitivity to ABA (Figure 3.6B, 3.7). Seedlings (Columbia genotype) treated at the late stage of seedling

development do not suffer from growth inhibition or elevated transcript levels of *ABI3*. I believe that when seedlings are exposed to elevated levels of NAE or ABA, within the window of sensitivity, a delay of the normal development of seedlings is induced by maintaining the expression, or resumption of expression, of genes that are normally associated with the ungerminated, desiccation-tolerant state.

As stated previously, ABI3 is pivotal in the transition from embryo to seedling growth, and I have demonstrated the inverse relationship between *ABI3* transcription levels and growth. Seeds of severe *abi3* mutants in *Arabidopsis* accumulate less storage protein, are desiccation-intolerant and germinate precociously (Nambara et al., 2000). There also are problems of seed viability after extended storage with *abi3* mutants. Also, it has been established in the literature that ABA insensitive mutants do not have a window of sensitivity for ABA (Lopez-Molina et al., 2001), and display a partial tolerance to NAE12:0 (Teaster et al., 2007). Interestingly, I found a window of sensitivity to NAE12:0 in the *abi3-1* ABA insensitive mutant when treated at the early stage of seedling development (2-d) (Figure 3.8, 3.9). The *abi3-1* mutant underwent growth arrest without any detectable transcript levels of *ABI3* or *AtHVA22B*. This suggests that NAE12:0 induced growth arrest is not entirely mediated by the same pathway as ABA growth arrest, yet both share many of the same characteristics, including overlapping gene expression (Teaster et al., 2007). This NAE induced growth arrest in an ABA signaling mutant supports my hypothesis that NAE functions in ABA-dependent and ABA-independent signaling pathways. The new information presented here on NAE function in *Arabidopsis* seedlings supports an intersection between NAE

metabolism and ABA signaling pathways, and provides a molecular context in which to probe the precise, primary targets of this interaction.

The evidence suggests that both NAE and ABA can act to arrest early seedling development. I postulate two parallel pathways of NAE-induced seedling growth arrest, one that is ABA dependent and another that is ABA independent (Figure 3.11). The model provides a diagrammatic summary to describe this interaction. During the normal course of germination and seedling growth, levels of ABA and NAE decline (Teaster et al., 2007); however if the levels of either ABA or NAE are elevated in early seedling development, there is an arrest in seedling growth (Figure 3.3). NAE arrested growth is not mediated by the coincident elevation of ABA levels and vice versa (Teaster et al., 2007). Nonetheless, NAE arrested growth at least partially involves a functional ABA signaling pathway including ABI1, ABI2, ABI3, and ABI5, and is associated with increased transcript levels of well-characterized ABA-responsive genes (Figure 3.11) (Teaster et al., 2007). On the other hand, NAE mediated growth arrest in the *abi3-1* (ABA insensitive) mutant, suggests that seedling growth is likely to be arrested by NAE, at least partially, by mechanisms outside the ABA signaling pathway, in an *ABI3*-independent manner (Figure 3.11). Our data suggest that NAE12:0 functions in concert with ABA as a negative regulator of germinating seed/seedling growth. These results provide evidence that NAE metabolism impacts a major phytohormone signaling pathway in plants, placing this lipid pathway in an important regulatory role. Future work should endeavor to identify specific targets of NAE in seedlings. This would aid in defining the role(s) of NAEs and NAE metabolism in regulating germinating seed and seedling growth.

Methods

Plant Materials and Growth Assays

Plants were propagated in soil for seed production. For growth experiments, seeds were first surface-sterilized with 95% ethanol followed by 33% commercial bleach for three minutes each and rinsed several times with sterile, deionized water. Seeds were stratified for three days at 4 ^{o}C in the dark and grown in liquid or solid nutrient media (0.5X Murashige and Skoog (MS) salts containing 1% sucrose) as described previously (Wang et al., 2006). Germination and growth proceeded in a controlled environment room with 16h/8 h light/dark cycle (60 $\mu mol \cdot m^{-2} \cdot sec^{-1}$) at 20-22 ^{O}C. Liquid cultured seedlings were incubated with shaking (75 rpm) and growth was quantified as fresh weight accumulation. ABA or NAEs were added from DMSO stocks to the appropriate final concentrations, and untreated controls contained equivalent concentrations of solvent alone. Concentrations of exogenous ABA were calculated based on the active *cis*-isomer, Calbiochem-Novabiochem Corp. catalogue # 100111.

Quantitative RT-PCR

Quantification of transcript levels by quantitative RT-PCR was performed with a Smart Cycler II (Cepheid) instrument using real time one step assay system (Takara Bio Inc) with SYBR^{TM} Green I. The following gene specific primer pairs were used, *ABI3* (At3g24650) (F) 5‘GAGCTGGCTCAGCTTCTGCTATG-3’ (R) 5‘AGGCCAAAACCTGTAGCGCATGTTC-3’, *AtHVA22B* (At5g62490) (F) 5’-CATCGCTGGACCTGCATTA C-3’ (R) 5’-GGATATAATGGGATCCATTCGAGG-‘3,

RD29B (At5g52300) (F) 5'-CATAAAGGTGGAGAAGCTGGAGTA-3' (R) 5'-CCTCCAAATCTTGCCGGAGAATTC-3'. All primers were designed to span one intron to distinguish cDNA amplification from genomic DNA contamination. Relative transcript levels in all samples were normalized using 18S rRNA as a constitutively expressed internal control, with primers (F)5'-TCCTAGTAAGCGCGAGTCATCA-3' and (R) 5'-CGAACACTTCACCGGATCAT-3' (Dean Rider et al., 2003). Quantitative RT-PCR reactions were performed in duplicate with 0.2 µg total RNA and 0.5 µL of 10 µM gene specific primers in each 25 µL reaction. The reaction mix was subjected to the following RT-PCR conditions, 42 ^{O}C for 15 min, one cycle; 95 ^{O}C for 2 min, one cycle; 94 ^{O}C for 10 sec, 58 ^{O}C for 25 sec (read cycle), 72 ^{O}C for 20 sec, the number of cycles and annealing temperature were experimentally determined for each set of gene-specific for primers. RT-PCR products were examined by gel electrophoresis and by melting curve analysis (60 ^{O}C to 95 ^{O}C at 0.2 ^{O}C /sec) to rule out anomalous amplification products. The $2^{-\Delta\Delta CT}$ cycle threshold (C_T) method was used to calculate relative changes in transcript levels determined from quantitative, real time RT-PCR (Livak and Schmittgen, 2001). The data were analyzed using the equation where $\Delta\Delta C_T = (C_{T,\,Target} - C_{T,\,18s})_{\,Treated} - (C_{T,Target} - C_{T,\,18s})_{\,Not\ Treated}$. "Treated" refers to samples treated with ABA or NAE, and "Not Treated" refers to samples treated with solvent alone. For ease of presentation, in the cases when transcript levels in the "treated" samples were lower than in "not treated" samples, the fractional values obtained by the above formula were converted to fold difference by taking the negative reciprocal.

Metabolite Quantification

Metabolites were quantified by isotope-dilution mass spectrometry. NAEs were

extracted from approximately 100 µL MS growth media into 2-propanol/chloroform/water (2/1/0.45, v/v/v) (Chapman et al., 1999). Deuterated NAE standards (NAE12:0, NAE18:0, and NAE20:4) were added as quantitative standards. Extracted lipids were partitioned into chloroform and NAEs were fractionated by normal-phase HPLC and were quantified as TMS-ethers (derivatized in BSTFA, Sigma) by GC-MS (Venables et al., 2005).

Table 3.1. Gene Transcript Abundance in Seedlings Treated with Increasing Concentrations of NAE12:0 at 7-d after Sowing

	WT					*faah*					OE11				
μM NAE	0	10	20	35	50	0	10	20	35	50	0	10	20	35	50
ABI3	1	4	13	541	524	70	18	69	2410	1687	17	32	17	96	312
RD29B	1	1	1	53	51	5	2	4	180	99	1	1	1	12	20
AtHVA22B	1	1	1	57	52	6	5	6	187	124	1	1	1	10	30

Transcript levels were quantified in WT and FAAH-altered seedlings by quantitative real time RT-PCR. Values are shown as fold change relative to WT at 0 μM NAE12:0. Values are averages of duplicate samples, and independent experiments show similar trends.

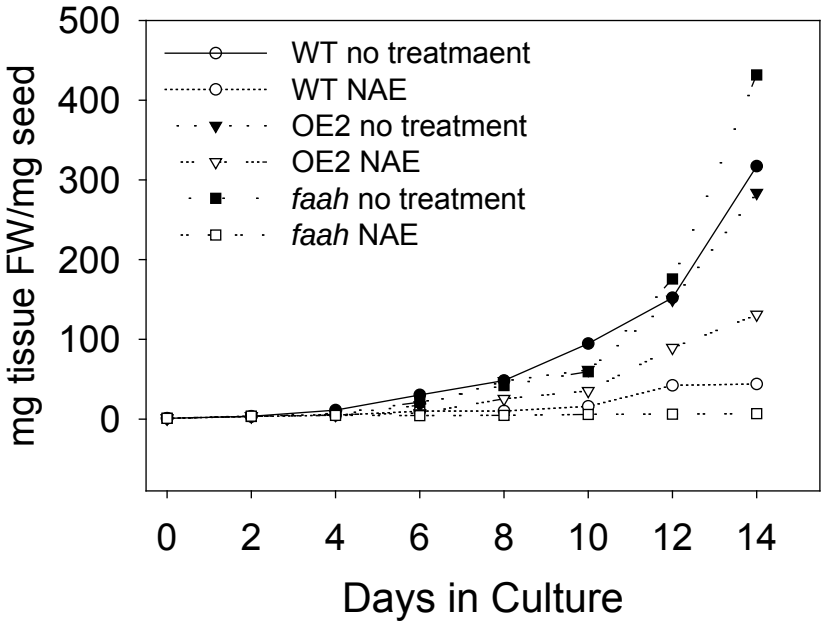
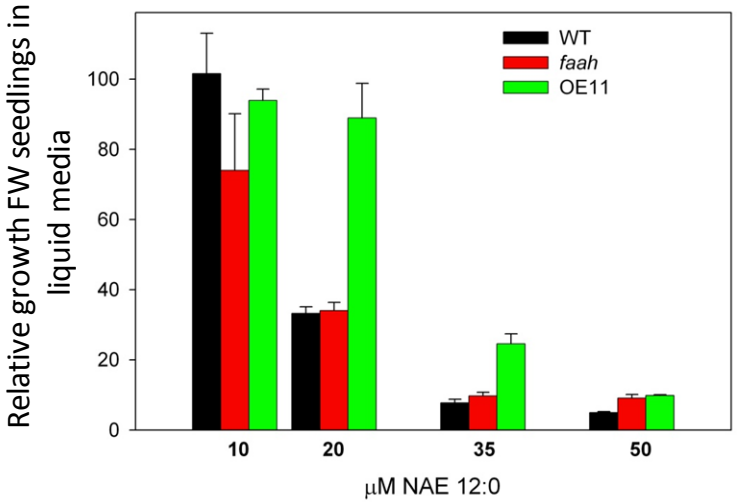


Figure 3.1. NAE12:0 negatively regulates seedling growth.

Effect of 35 μM NAE 12:0 treatment on the growth of *A. thaliana* (ecotype Columbia) seedlings during 14 days in liquid culture. Growth was severely reduced in wild type (WT) and *faah* NAE treated seedlings but not in the FAAH OE. Growth of the three types of seedlings without treatment was similar.

A



B

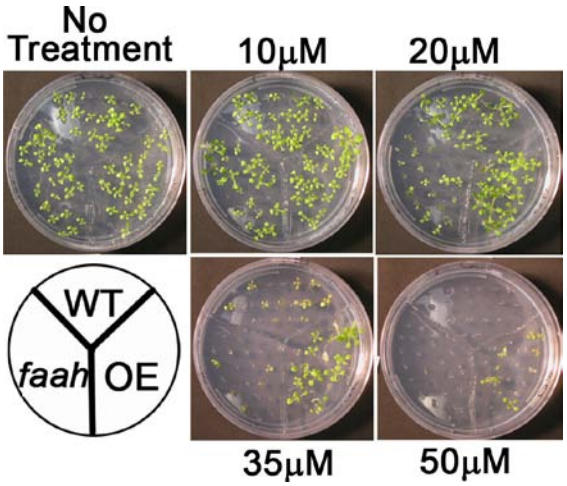


Figure 3.2. NAE 12:0 induces a dose dependent inhibition of seedling growth.

(A) Growth of wild-type and FAAH-altered seedlings in increasing concentrations of NAE 12:0 (measured after 7-d continuous treatment). Values for gain in fresh weight are relative to untreated seedlings and are the mean and SE of triplicate experiments.

(B) Representative images of 10-d-old seedlings at increasing concentrations of NAE12:0 exhibit the tolerance of the FAAH OE and the sensitivity of the *faah*.

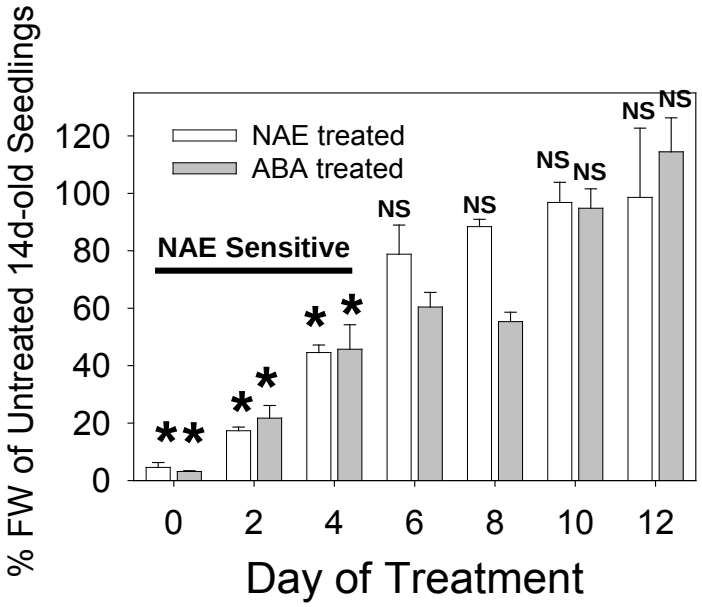
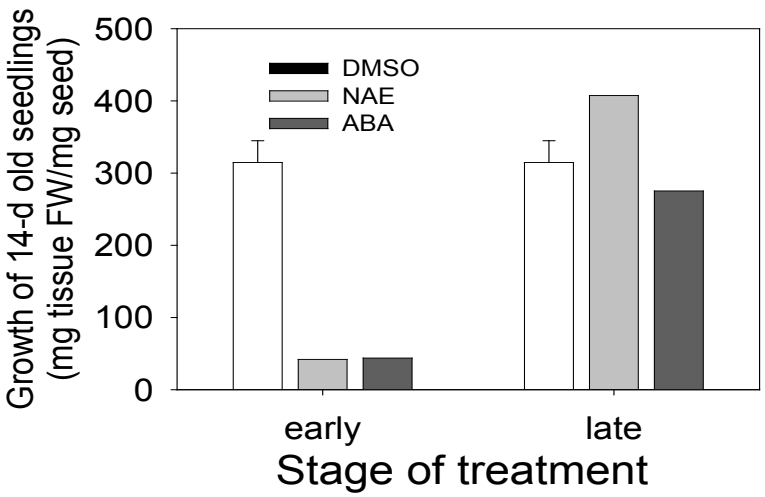


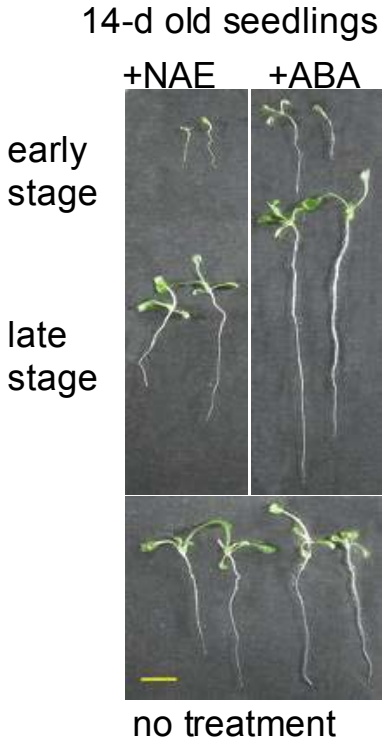
Figure 3.3. Seedling growth responses to NAE12:0 fall within a narrow window of developmental sensitivity.

This window of sensitivity (less than 6 d) is similar to the window of growth arrest previously characterized for ABA (Lopez-Molina et al., 2001; Lopez-Molina et al., 2002). For example, treatment of 2-d old seedlings with ABA (0.5 μM) or NAE (35 μM) severely stunts growth (measured after 14 d), whereas treatment of 10-d old seedlings does not impact growth much at all (measured after 14 d). Seedling growth was normalized to original seed weight and plotted as a percentage of fresh weight of untreated seedlings after 14 d. Results for each time point are averages from three replicate experiments containing approximately 2500 individuals (50 mg seed) each. Asterisks indicate a significant difference compared to untreated seedlings which was determined by t-test. The sensitivity of seedlings to NAE was statistically significant ($p<0.001$) up to day 6, but not significant (NS; $p > 0.1$) thereafter. ABA sensitivity overlapped NAE sensitivity up to day 6, and it, too, was abolished by day 10.

A


Growth of 14-d old seedlings
(mg tissue FW/mg seed)
500
400
300
200
100
0
DMSO
NAE
ABA
early
late
Stage of treatment

B


14-d old seedlings
+NAE
+ABA
early
stage
late
stage
no treatment

C

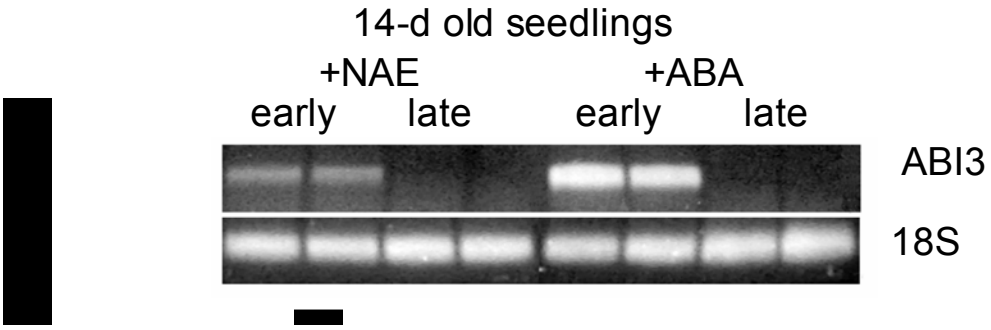
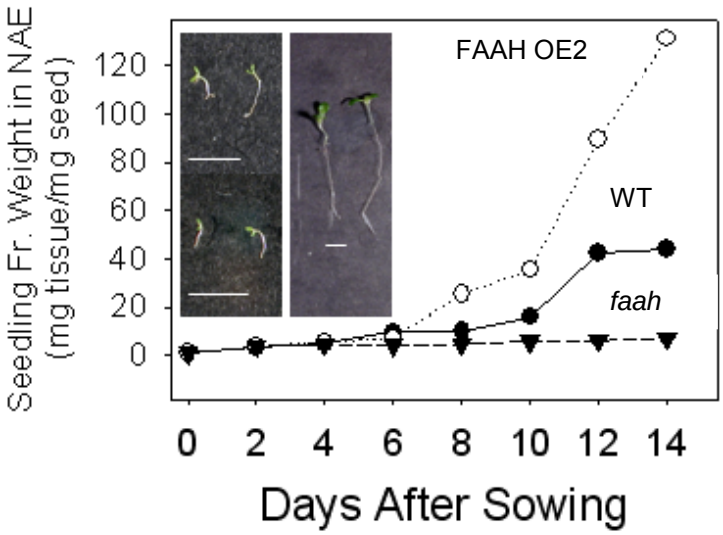

14-d old seedlings
+NAE
+ABA
early
late
early
late
ABI3
18S

Figure 3.4. ABI3 expression is associated with growth arrest and is responsive to NAE 12:0 (35 μM) or ABA (0.5 μM) treatment only within the NAE/ABA-sensitive interval (up to 6 d).

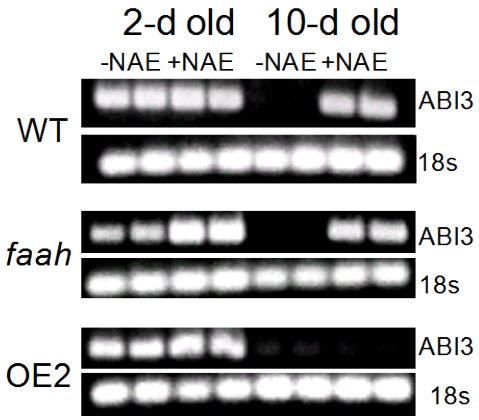
(A-B) Treatment of WT (Col.) early-stage seedlings (e.g., 2-d old) arrests growth (measured after 14 d); whereas treatment of late-stage seedlings (e.g., 10-d old) does not inhibit seedling growth (measured after 14 d). ABA treatment of late stage seedlings shows characteristic promotion of root growth, but this is not evident in NAE-treated, late-stage seedlings.

(C) Expression of *ABI3* is apparent only in NAE- or ABA-arrested seedlings (treated at early stage of seedling development) after 14 day of growth. A representative agarose gel of duplicate samples following semi-quantitative, RT-PCR with either *ABI3*- or 18S rRNA-gene specific primers.

A


Seedling Fr. Weight in NAE
(mg tissue/mg seed)
120
100
80
60
40
20
0
FAAH OE2
WT
faah
0 2 4 6 8 10 12 14
Days After Sowing

B


2-d old
10-d old
-NAE +NAE
-NAE +NAE
WT
ABI3
18s
faah
ABI3
18s
OE2
ABI3
18s

C

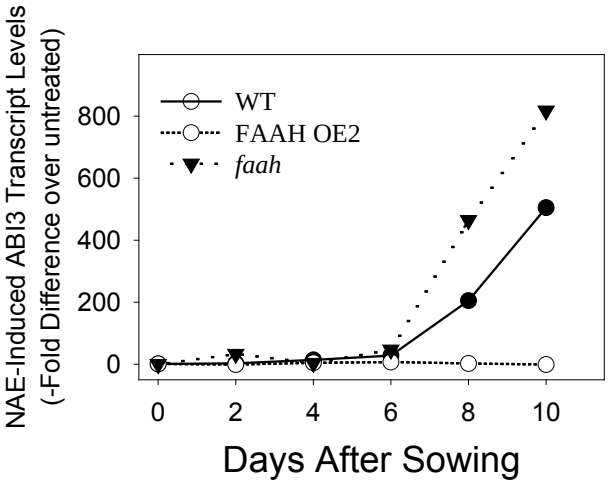

NAE-Induced ABI3 Transcript Levels
(-Fold Difference over untreated)
800
600
400
200
0
WT
FAAH OE2
faah
0 2 4 6 8 10
Days After Sowing

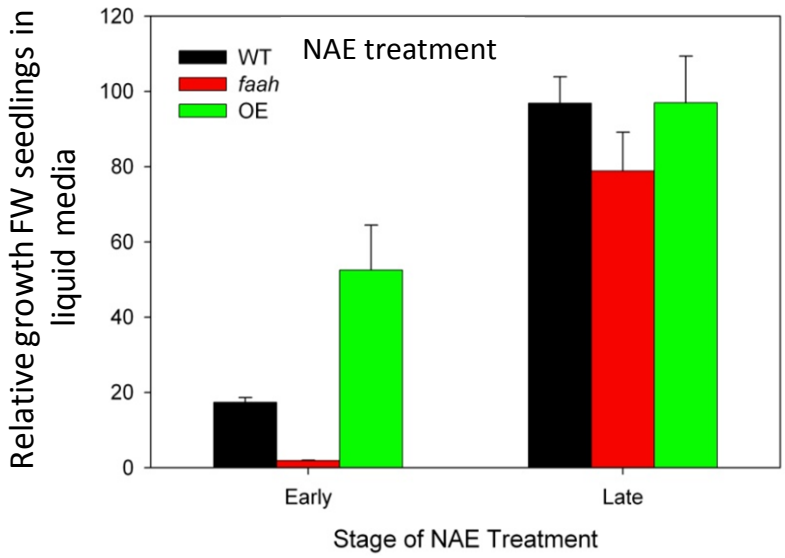
Figure 3.5. NAE-induced *ABI3* expression is inversely associated with NAE-regulated seedling growth.

(A) Growth of wild-type and FAAH-altered seedlings in response to exogenous NAE12:0 (35 μM). Values for gain in fresh weight are from a representative experiment, and replicate experiments exhibited similar trends (i.e., FAAH OEs tolerate NAE12:0, FAAH knockouts do not, and WT are intermediate; (Wang et al., 2006). Insets show seedlings of WT (upper left), knockout (lower left), or AtFAAH OE, OE2, grown in 35 μM NAE12:0 for 14 d. Scale bar = 0.5 cm.

(B) Representative agarose gel analysis of *ABI3* amplification products in 2- or 10-d old seedlings grown in 35 μM NAE12:0 (analyzed by semi-quantitative RT-PCR).

(C) Quantification of transcripts by real-time RT-PCR. Values for transcripts were averaged from two independent measurements at each time point (normalized to 18S rRNA) and are plotted as -fold difference between NAE-treated (35 μM) and untreated seedlings.

A


Relative growth FW seedlings in liquid media
120
100
80
60
40
20
0
WT
faah
OE
NAE treatment
Early
Late
Stage of NAE Treatment

B

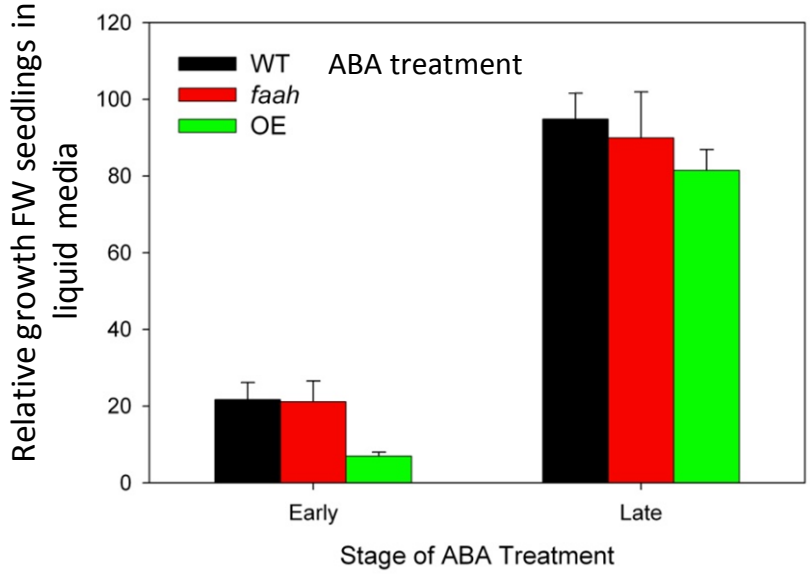
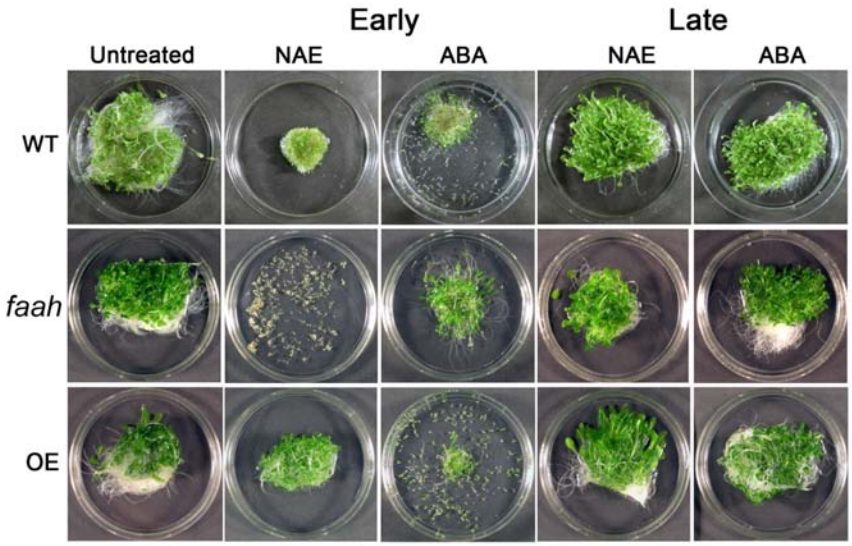

Relative growth FW seedlings in liquid media
120
100
80
60
40
20
0
WT
faah
OE
ABA treatment
Early
Late
Stage of ABA Treatment

Figure 3.6. ABA and NAE 12:0 inhibit seedling growth only within the NAE/ABA window of sensitivity (up to 6-d).

(A), (B) Growth of wild-type and FAAH-altered 14-d-old seedlings in response to exogenous NAE 12:0 (35 μM) and ABA (0.25 μM). ABA treatment of early stage seedlings (e.g., 2-d-old) inhibits growth, whereas treatment of late-stage seedlings (e.g., 10-d-old) do not. NAE Treatment of early stage seedlings inhibits growth in wild-type and FAAH knockout seedlings, while the FAAH OEs tolerate NAE 12:0. NAE treatment of late-stage seedlings does not inhibit growth. The FAAH knockout shows an increased sensitivity to NAE 12:0 during the early-stage of growth, while the FAAH OE displays sensitivity to ABA during the early-stage of growth, compared to wild-type. Values for gain in fresh weight are the mean and SE of triplicate experiments.

A



B

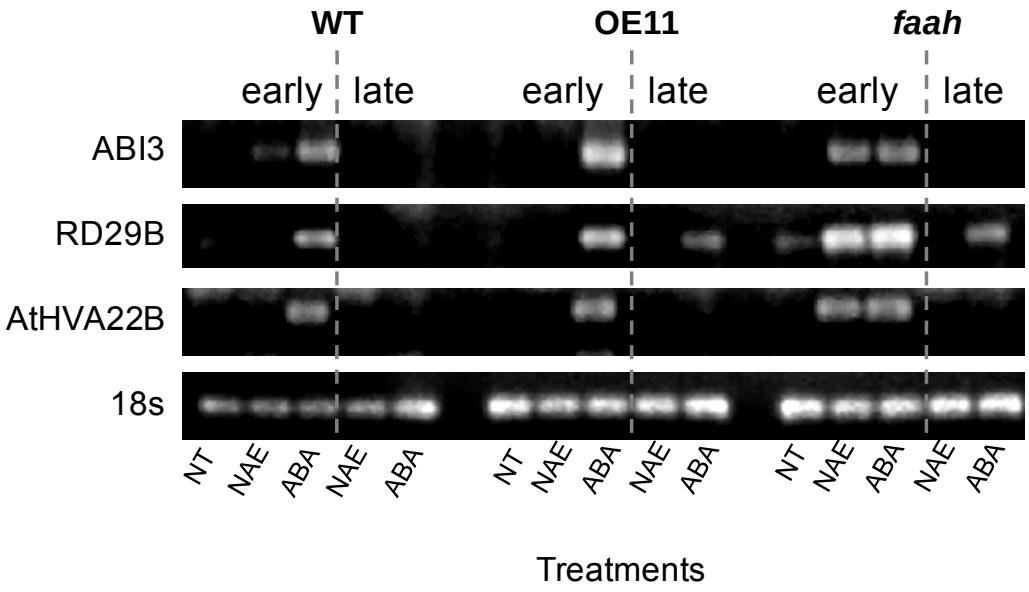


Figure 3.7. Gene expression is associated with growth arrest in FAAH-altered *Arabidopsis* seedlings only within the ABA/NAE-sensitve interval (up to 6d).

(A) Images of 14-d-old wild-type and FAAH-altered seedlings in response to exogenous NAE 12:0 (35 μM), ABA (0.25 μM) and NT (not treated). ABA treatment of early stage seedlings (e.g., 2-d-old) inhibits growth, whereas treatment of late-stage seedlings (e.g.,

10-d-old) did not. The heightened sensitivity to NAE12:0 of FAAH knockout seedlings and the tolerance of the FAAH OEs are clearly evident in the early stage seedlings, also the increased sensitivity to ABA of the FAAH OE is visible in the early stage. NAE/ABA treatment of late-stage seedlings does not inhibit growth.

(B) Agarose gel analysis of semiquantitative RT-PCR using gene specific primers to analyze gene expression in 14-d-old seedlings (treated at early or late stage of seedling development with 35 μM NAE 12:0 or 0.25 μM ABA).

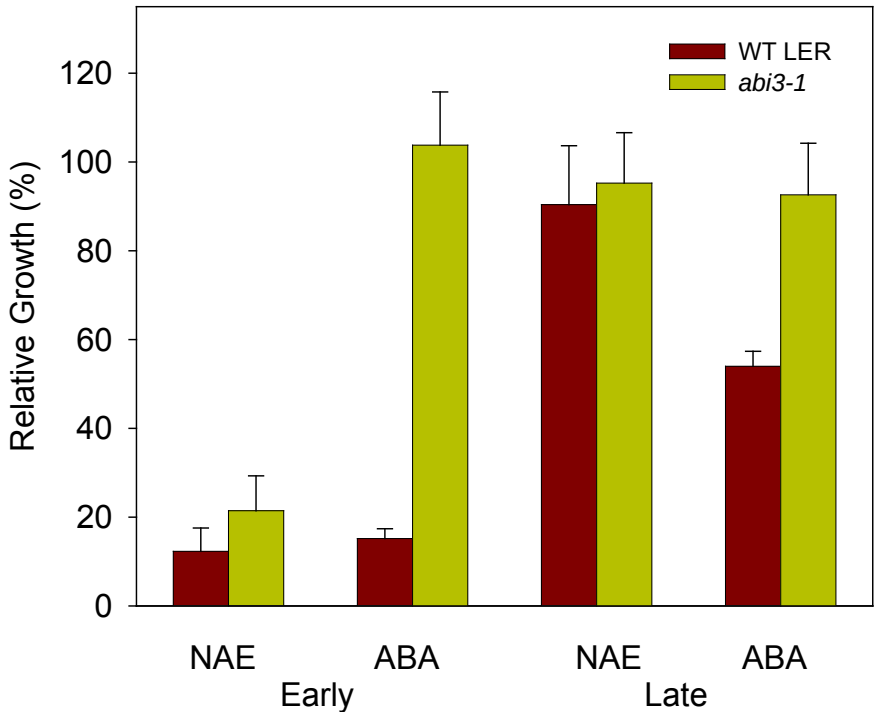
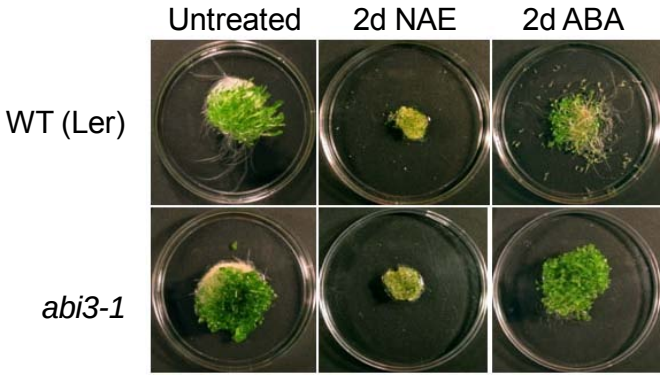


Figure 3.8. NAE 12:0, but not ABA, induces growth inhibition in ABA insensitive mutant (*abi3-1*).

Treatment of wild-type (Landsberg *erecta*) with NAE12:0 (35 µM) or ABA (0.25 µM) at early stage of seedling development reduces growth 14-d-old seedlings, quantified by fresh weight gain. While growth in the ABI mutant is not inhibited by ABA treatment during the early stage of seedling development, growth is inhibited by NAE12:0 treatment. Values for gain in fresh weight are the mean and SE of triplicate experiments.

A



B

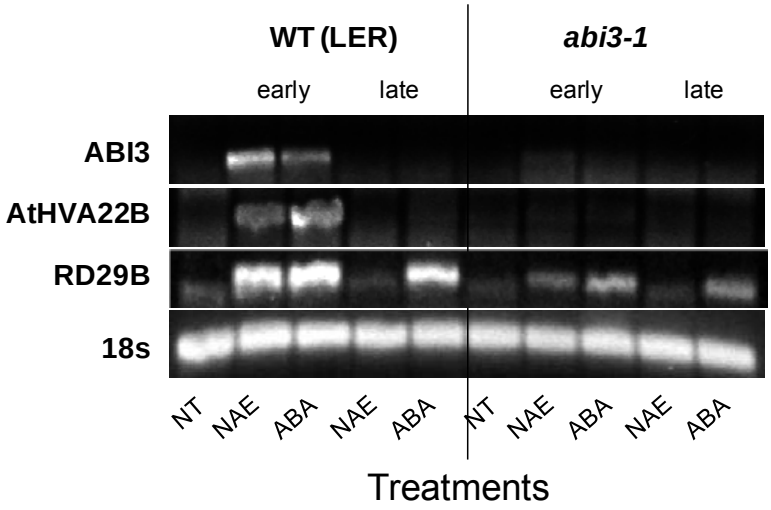


Figure 3.9. NAE 12:0 induces growth inhibition in ABA insensitive mutant (*abi3-1*), but not the associated pattern of gene expression.

(A) Images of 14-d-old wild-type (Landsberg *erecta*) and *abi3-1*seedlings in response to exogenous NAE 12:0 (35 μM) and ABA (0.25 μM). NAE/ABA treatment of early stage wild-type seedlings (e.g., 2-d-old) inhibits growth. The *abi3-1* seedling are insensitive to ABA, yet NAE induces growth arrest in seedlings treated at the early stage of seedling development.

(B) Agarose gel analysis of semiquantitative RT-PCR using gene specific primers to analyze gene expression in 14-d-old seedlings (treated at early or late stage of seedling development with 35 μM NAE 12:0 or 0.25 μM ABA).

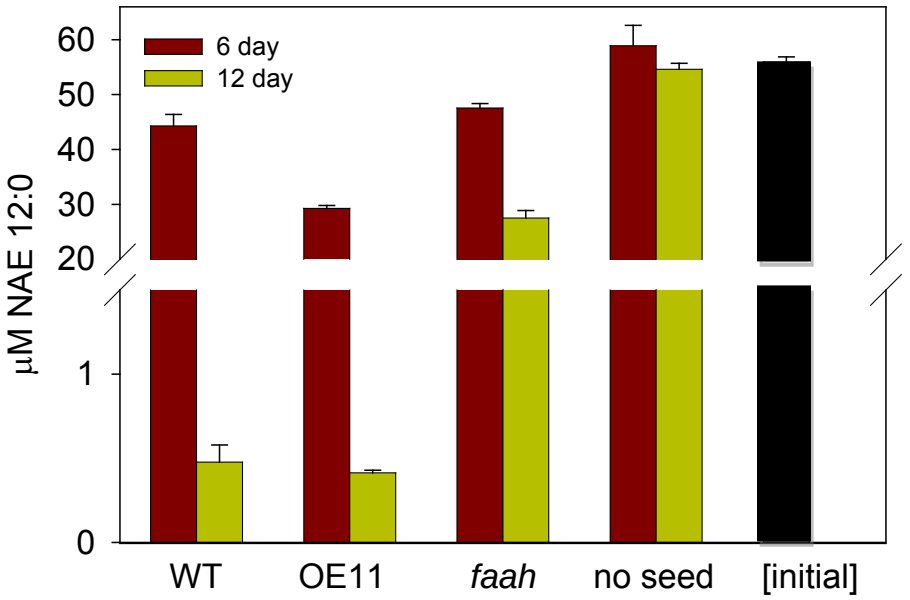


Figure 3.10. NAE12:0 depletion in MS liquid growth media at 6 and 12-d after sowing, quantified by isotope –dilution mass spectrometry.

Quantification of NAE12:0 MS treated liquid growth media showed that the initial concentration [initial] (black bar) and final concentration (12-day "no seed" control) are essentially equal, indicating no spontaneous loss of NAE12:0 during 12-day trial. MS growth media containing WT and FAAH OE seedlings show the greatest depletion of NAE12:0, and growth media with *faah* seedlings the least. Deuterated NAE12:0 standards were added at the time of extraction. Values are averages and standard deviations of three replicate experiments.

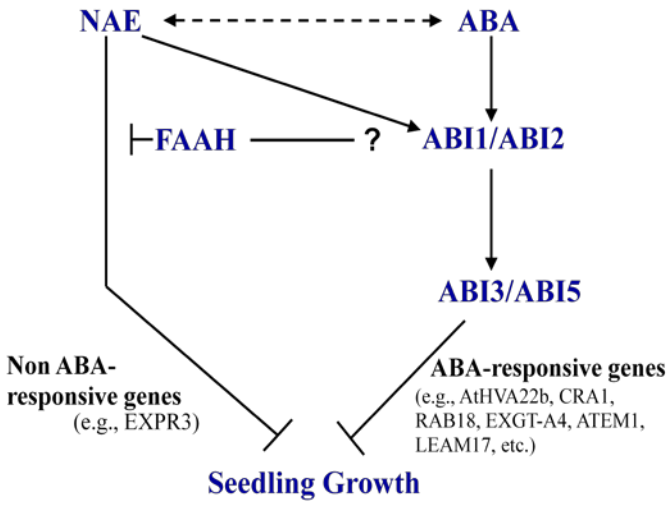


Figure 3.11. Summary diagram describing the hypothetical action of elevated levels of NAE on seedling growth, and its relationship to ABA action.

NAE arrests seedling growth via a pathway dependent upon *ABI1, ABI2, ABI3* and *ABI5*, and this growth arrest is associated with up regulation of ABA-responsive genes such as (*AtHVA22b, CRA1, RAB18, EXGT-A4, ATEM1, LEAM17*) (Teaster et al., 2007). This suppression of seedling growth is reminiscent of the ABA-induced secondary dormancy program discovered by Chua and co-workers (Lopez-Molina et al., 2002) which is operable in young seedlings. I propose that NAE12:0 intersects the ABA signaling pathway downstream from ABA, and that *ABI3* expression is key to the suppression of growth induced by NAE through this pathway. Independent of ABA signaling, NAE12:0 can influence gene expression in seeds and seedlings and suppress seedling growth. Ectopic overexpression of AtFAAH can reverse the growth suppression by NAE, but the interaction with ABA signaling in AtFAAH OEs is complex, since AtFAAH OEs are hypersensitive to ABA. The *abi3-1* mutant provides evidence that NAE only partially works in the ABA signaling pathway, and that it may be possible there are multiple pathways are involved.

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CHAPTER 4

IMPACT OF ALTERED NAE METABOLISM ON SEED GERMINATION IN *Arabidopsis thaliana*

Abstract

Desiccated seeds of a variety of plant species contain NAEs with chain lengths of 12C to 18C in length and zero to three double bonds (Chapman, 2004). Because NAE levels in seeds decline during imbibition (Chapman et al., 1999) similar to ABA, a physiological role was predicted for these metabolites in seed germination and seedling development. There is also a corresponding increase of *AtFAAH* (fatty acid amide hydrolase), transcript levels and activity (Wang et al., 2006), during seed germination which metabolizes NAE to ethanolamine and free fatty acids. Here, *Arabidopsis thaliana* lines overexpressing *AtFAAH* (FAAH OE) displayed a reduced rate (percentage) of germination compared with wild type, which could be accelerated (rescued) by stratification. On the other hand, FAAH knockout (*faah*) seeds displayed accelerated rate of germination relative to wild type, without stratification. This led us to investigate how altered NAE metabolism might affect seed germination in *A. thaliana*. In desiccated seeds of the FAAH altered mutants I quantified lipid, protein and ABA content. Levels of these compounds were found to be similar to wild type (WT). Also, transcript levels of several ABA responsive genes that are associated with mature desiccated seed were examined by quantitative RT-PCR, and these were similar between FAAH altered seeds, and WT. Since stratification rescued the delay in germination in FAAH OE, I examined lipid levels and composition in stratified and non-stratified seed of WT, FAAH OE and *faah,* as well as 48 h after sowing. Lipid levels were lower in stratified seeds of

WT, FAAH OE and *faah* compared with non-stratified seeds; however, levels between genotypes were generally similar for each treatment. The different genotypes exhibited no substantial difference in lipid composition (mostly triacylglycerols, TAG) in stratified or non-stratified seeds, or at 48 h after sowing for both treatments. Transcript levels of the ABA responsive gene *AtHVA22B* were highest in desiccated seeds. I examined transcript levels of *AtHVA22B* at several time points during imbibition and germination, and found no differences between genotypes that correlated with delays in germination suggesting that FAAH OE delay in germination was not attributable to higher ABA responsiveness. Interestingly, the FAAH OE seeds were found to have increased sensitivity to GA, which rescued the reduced germination rate in a manner similar to stratification. By contrast, *faah* displayed no altered response to GA treatment during germination, indicating a surprising insensitivity to GA. These results suggest that NAE metabolism may impact the progression of normal germination by interacting with GA-mediated processes rather than substantially influencing the catabolic processes associated with germination and seedling growth.

Introduction

During normal seed development, embryo arrest and dormancy are reversed upon germination, which occurs when proper environmental conditions are provided and the dry seed imbibes water. Seed dormancy in *Arabidopsis thaliana* can be overcome by, or seed germination is activated by, a set of common germination factors such as ripening, light, cold treatment (also called stratification) and chemicals such as gibberellins (GA) and KNO_3 (Derkx and Karssen, 1993b). None of these exogenous

factors are an absolute requirement for germination because the need for one factor depends on the other factors, as shown for the interaction between light and temperature by (Cone and Spruit, 1983). This requirement for exogenous factors depends very much on genotype. Non-dormant seeds that are exposed for some time to unfavorable germination conditions (imbibed seeds kept at relatively high temperature in darkness for example) may enter a state of dormancy again, which is called secondary dormancy (Cone and Spruit, 1983; Derkx and Karssen, 1993a).

In seed germination mutants, properties of germination and dormancy are affected, which sometimes are accompanied by pleiotropic effects that are specific for maturation such as desiccation intolerance (Goldberg et al., 1994; Koornneef and Karssen, 1994). The *abi3* (abscisic acid-insensitive3), *fus3* (fusca3), and *lec1* (leafy cotyledon1) mutants produce seeds that are intolerant to desiccation (Nambara et al., 1992; Keith et al., 1994). Accumulation of seed storage proteins (SSP; such as CRA1) and late embryogenesis abundant (LEA; such as LEA M17) mRNAs is severely reduced in these mutants (Nambara et al., 1992; Keith et al., 1994; Vicient et al., 2000) and the profile of the global pattern of gene expression is also altered during mid- to late-embryogensis. During these stages, SSP and LEA genes are down regulated and genes normally expressed during or after germination are precociously activated (Parcy et al., 1994; West et al., 1994; Parcy et al., 1997; Nambara et al., 2000). Transgenic *A. thaliana* harboring a CaMV35S::*ABI3* construct expresses some of the SSP genes ectopically in response to exogenous ABA, demonstrating that ABI3 is one of the essential elements for seed-specific gene expression (Parcy et al., 1994).

Carbon storage in the form of triacylglycerol (TAG), is a ubiquitous feature of seed plants, which is stored in organelles known as oil bodies (Penfield et al., 2006). Cells of the embryo and endosperm are full of oil bodies, which comprise up to 45% of *A. thaliana* seed weight. TAG accumulation depends on genes that promote seed dormancy and embryo identity, including *LEAFY COTLYEDON1*, *FUSCA 3* and *ABI3*. The expression of oleosins are also regulated by the transcription factor ABI3 (Crowe et al., 2000) and is restricted to certain tissues at specific developmental stages (Keddie et al., 1994). When OLEO1 is suppressed in *A. thaliana,* the seed oil bodies were larger, and a delay in germination (reversible upon stratification) was revealed. Also, large oil bodies could be found up to 8 d post-germination in OLEO1 suppressed *A. thaliana*. Whereas in WT, oil bodies were scarcely found as tiny particles past 3 d (Siloto et al., 2006). There was also a decrease in the amount of lipids and a compensatory increase in total protein in the OLEO1 suppressed *A. thaliana* (Siloto et al., 2006).

The completion of seed germination is highly regulated by both internal and external cues that determine the dormancy status and the potential for germination (defined as the final percentage of germination) (Holdsworth et al., 2008). In *A. thaliana* dormancy is classified as physiologically non-deep, meaning embryos released from surrounding structures grow normally and dormancy is lost through moist chilling (stratification) or after-ripening (Baskin and Baskin, 2004). Seed dormancy has long been studied in *A. thaliana* and these investigations revealed many components that are required for dormancy induction, maintenance and the completion of germination, including light, temperature and plant hormones. A major conclusion from these studies is that the plant hormone abscisic acid (ABA) is a positive regulator of dormancy, while

gibberellins (GA), release dormancy and promote the completion of germination, counteracting the effects of ABA (Bewley, 1997).

ABA is an important positive regulator of both the induction of dormancy during seed maturation and the maintenance of the dormant state in imbibed seeds. Deficiency of ABA during seed development is associated with the absence of primary dormancy in the mature seed, whereas the over-expression of ABA biosynthesis genes can increase seed ABA content and enhance seed dormancy or delay germination (Finkelstein et al., 2002; Nambara and Marion-Poll, 2003). Abscisic acid produced by the seed itself during seed development can impose a lasting dormancy, whereas maternal ABA, or ABA application during seed development, fails to induce lasting seed dormancy (Groot and Karssen, 1992; Koornneef and Karssen, 1994). During seed development, different programs of gene expression have been identified (Parcy et al., 1994). The maturation class includes major seed storage protein (*SSP*) genes (including 2S albumins and 12S globulins), and the late embryogenesis abundant (*LEA*) class, which includes primarily genes involved in the acquisition of desiccation tolerance (Wobus and Weber, 1999; Hoekstra et al., 2001).

During dry storage of seeds (after ripening), dormancy is reduced until seeds are able to germinate. Several factors can affect the rate at which this is accomplished, depending on environmental conditions during maturation, seed storage and germination conditions (Donohue et al., 2005). Dry mature seeds contain a large number of stored mRNA species whose function remains unknown. It is believed that these are not just remnants from embryogenesis and seed maturation, but provides important RNA species for protein synthesis during early germination.

The *A. thaliana* family of DELLA proteins was originally defined following the cloning of GAI (GA insensitive) (Peng et al., 1999). This family now consists of five proteins, GAI, RGA, RGL1, RGL2, and RGL3, and all contain a DELLA amino acid motif (Sun and Gubler, 2004). Due to their location in the nucleus and their action to repress GA responses during development it is believed that they are transcription associated factors (Sun and Gubler, 2004). RGL2 has a major role in regulating germination potential, the *rgl2* mutant are insensitive to paclobutrazol (GA biosynthesis inhibitor). The repression of germination (Lee et al., 2002), and removal of RGL2 in the *ga1* mutant background restoring germination (Lee et al., 2002) demonstrates that RGL2 is a repressor of germination in the absence of GA. The four remaining DELLAs may respond to environmental cues; seeds lacking all four show light-independent and cold independent germination (Cao et al., 2006).

Seed dormancy or germination is dependent on the balance between ABA, GA, and other environmental factors. These pathways are interconnected at several different levels and also interact with other plant hormones such as ethylene and brassinosteroids. These pathways interact to influence the ABA-GA balance that affects germination. The cross talk between these pathways contributes flexibility of seeds to respond to developmental and environmental cues (Brady and McCourt, 2003; Chiwocha et al., 2005; Kucera et al., 2005; Weiss and Ori, 2007). Our data suggest that by altering FAAH expression I have shifted the ABA-GA balance (or perception) and that this has affected the timing of germination, especially in unstratified seeds. In chapters 1 and 2, I demonstrated that altering FAAH expression caused a dysfunction in normal ABA-mediated processes in seedling development. Here I look specifically at

seed germination and examine the role of NAE metabolism (FAAH) in ABA mediated processes including visible germination, oil mobilization and GA interaction.

Results

AtFAAH OEs Have Delayed Germination That Can be Rescued by Stratification

Germination assays were conducted to determine if the altering of the FAAH expression levels in *A. thaliana* would affect germination, or if stratification (3 d cold treatment) would impact seed germination differentially in FAAH mutants. Figure 4.1 shows the percent germination for WT (Col-0), *faah* and three lines of AtFAAH OEs, with and without stratification. Stratification of the seeds clearly increases the pace at which the seeds germinate in all cases, but there is a gradient of this increase in germination. All stratified seeds had reached (or were very close to) their highest % germination by 60 h, but this also included the non-stratified *faah*. At 36 h the non-stratified *faah* germinated seed was near 0 percent, but by 48 h the percent that germinated was greater than 70 percent, as high as some stratified FAAH OEs. While the non-stratified WT reached its highest % germination between 72-84 h and the FAAH OEs between 72-96 h. The data indicate that the altered NAE metabolism due to altered FAAH activity has reduced the need for a cold treatment in the *faah* and increased the requirement for cold treatment in the AtFAAH OEs relative to WT. Seeds of AtFAAH OEs have lower endogenous levels of NAEs and the *faah* has somewhat higher levels of NAE compared to WT (Wang et al., 2006). Altered levels of endogenous NAEs may have affected seed maturation or germination mechanisms.

Lipid, Protein, ABA and ABA Responsive Gene Transcript Levels Were Similar in Desiccated Seeds

Two properties of seeds that could be affected by an altered seed maturation program is accumulation of lipid and protein seed reserves. WT *A. thaliana* seeds are approximately 40% lipid and 25% protein on a fresh weight basis (Siloto et al., 2006). A quantification of total lipids in desiccated seeds of WT, AtFAAH OE, and *faah* was conducted by ^{1}H nuclear magnetic resonance (NMR). Pulsed-field low resolution NMR spectrometers were originally developed for the margarine industry as a routine method for solid fat content determination. It is a nondestructive method for quantifying seed oil content, and was used to determine total oil content in 185 accessions of *Cuphea* (Phippen et al., 2006). I used seed samples of approximately 1 gram (50,000 *A. thaliana* seeds) to quantify total lipid content ensuring a homogenous sample for each seed batch and line. Plants harvested for seed analyses were grown under controlled growth conditions to lessen environmental impact on seed reserve accumulation. Each sample was analyzed three times by NMR and an average determined. The results for oil content (Figure 4.2A) varied little (all lines were close to 30%) between seed type or batch, indicating that there was little difference in total lipid among WT or FAAH altered mutants.

Total seed protein content of WT, AtFAAH OE, and *faah* was quantified using the Lowry DC protein assay (Bio-Rad) (Lowry et al., 1951) to indicate any appreciable differences in seed storage protein content. Several biological replicates were extracted for total protein and assayed from each line. The results for protein content (Figure 4.2B) varied little (all lines were close to 25%) between seed type or batch, indicating that there was little difference in total protein among WT or FAAH altered mutants.

Taken together it appeared that altering FAAH expression did not influence accumulation of storage reserves in seeds. Hence a difference in reserve content was unlikely to be the cause of the delay in germination exhibited by FAAH OE in the absence of stratification.

NAE and ABA levels have to decline for post germinative growth to proceed normally, and NAE levels were altered in seeds of AtFAAH mutants (Wang et al., 2006). It was possible that ABA levels were different in desiccated seeds of *A. thaliana* WT, *faah* and AtFAAH OE (Figure 4.3). ABA extractions were made in triplicate and ABA quantification was conducted by GC-MS (Wang et al., 2001). ABA levels in all seeds were very similar, suggesting that, altered ABA levels per se in desiccated seeds was not the reason AtFAAH OEs had a slower germination rate in the absence of stratification.

Since ABA levels do not vary much in the desiccated seeds, it may be that the altered NAE metabolism has affected the inherent sensitivity to ABA (Wang et al., 2006) and that this altered sensitivity to ABA is responsible for the germination delay. This increased sensitivity to ABA may be reflected in altered gene expression affecting the seed maturation program in *A. thaliana*. To determine if AtFAAH altered mutants have a different seed transcript profile *AtFAAH* and several ABA responsive genes that are associated with desiccated seeds were surveyed (Figure 4.4). Total RNA was extracted from mature desiccated seeds of *A. thaliana* WT, AtFAAH OE and *faah* and quantitative real time RT-PCR analysis was conducted using gene specific primers to examine extant mRNA levels. All transcript levels examined in *faah* and AtFAAH OE were relatively similar to WT, with the exception of *AtFAAH*, which was overexpressed in the

AtFAAH OE and not present in *faah* (Figure 4.4). These results suggest that altered NAE metabolism has not changed ABA responsive gene transcript levels in mature desiccated seeds, and that altered ABA sensitivity may not be related to the delayed germination of the FAAH OEs.

AtHVA22B *Transcript Depletion and Lipid Mobilization are Similar in All Genotypes*

AtHVA22B transcript levels can be modulated by exogenous ABA (Chapter 1,2) and so using transcript levels as a reporter of endogenous ABA status, *AtHAV22B* transcript levels were profiled over a time course of germination by quantitative real time RT-PCR (Figure 4.5). All three genotypes (AtFAAH OE, *faah* and WT) had higher transcript levels of *AtHVA22B* in desiccated seeds than was found in stratified seeds. Transcript levels in all genotypes were diminished to similar levels (WT slightly higher) over the first 36 h. At 48 h a large difference in percent germination can be observed between stratified and non-stratified genotypes (except *faah*) (Figure 4.1), yet, no large corresponding difference was seen in *AtHVA22B* transcript levels between genotypes, stratified or not (Figure 4.5). Based on the general similarity in the patterns of disappearance of *AtHVA22B* transcripts, it seems unlikely that the delayed germination in the absence of stratification of FAAH OEs is due to enhanced ABA sensitivity.

Seed germination is associated with degradation and mobilization of reserves accumulated during maturation (Bewley, 1997). The efficiency of reserve mobilization during germination, and hence, seed establishment itself, apparently depends on the extent of reserve accumulation during seed maturation (Fait et al., 2006). This general observation suggests that the transition between dormancy and germination is

associated with the activation of initial important metabolic processes needed for seed germination. While oil and protein reserves are long documented to be mobilized following radical protrusion (Bewley, 1997; Eastmond et al., 2000), recent results show that active metabolic processes already initiate during seed imbibition (or stratification) (Fait et al., 2006). I investigated lipid mobilization (and composition) in WT and FAAH altered *A. thaliana* seeds, stratified and non-stratified, and after 48 h of sowing where a large difference in percent germination was found (Figure 4.1). There was a substantial drop in lipid reserves when comparing stratified seeds to non-stratified seeds, but they were similar among genotypes within treatments (Figure 4.6). After 48 h, lipid depletion was fairly similar among genotype. Yet when comparing stratified to non-stratified seeds, the FAAH OE showed a moderate but significant difference, although the magnitude of difference was similar in WT and *faah*. The FAAH OE had relatively more residual lipid if seeds were not first stratified, perhaps a consequence of delayed germination rather than a cause.

Lipid composition was examined and no difference was found between genotype desiccated or stratified, or after 48 h. TAG was the predominant neutral lipid component as expected (Figure 4.7). These data overall suggest that lipid mobilization lags somewhat in the non-stratified FAAH OE, and that germination rate can be rescued by stratification, allowing time for the oil metabolism to “catch up”. The differences, though were trending similar for *faah* and WT (but not significant) so it’s not clear if these differences in oil content between 48 h old seeds that had been stratified versus not stratified are the primary reason for the delay in germination.

Altered FAAH Affects GA Sensitivity in Germinating A. thaliana *Seeds*

The plant hormone abscisic acid (ABA) is needed to induce seed dormancy, whereas the hormone gibberellin (GA) is often required to stimulate seed germination, and mutants with increased ABA sensitivity show increased seed dormancy (Finkelstein et al., 2002; Okamoto et al., 2006). GA does not appear to directly control dormancy, but acts as an antagonist to ABA by stimulating radicle emergence (Bewley, 1997). I asked the question, if ABA sensitivity was altered in the FAAH mutants, could GA sensitivity be altered as well, thereby, affecting germination? To address this question I germinated non-stratified seeds for WT, FAAH OE and *faah* in the presence or absence of GA (Figure 4.8). WT and FAAH OE responded to GA application by improved percent germination at each time point examined, i.e. GA accelerated germination rate. The *faah* did not respond to GA application and was equal (or behind) compared to the "no GA" at all time points, suggesting insensitivity to or a lack of required for GA. Comparing WT percent germination to FAAH OE at increasing concentrations of GA, the FAAH OE displayed an increased sensitivity to GA (Figure 4.9). The FAAH OE without GA has the lowest percent germination through most of the time course, especially the latter part. Yet, the FAAH OE responds to the lowest concentration of GA, 1 μM, with improved germination similar to WT without GA, while WT at this concentration showed little or no response. At higher concentrations of GA, 10 and 25 μM, both WT and FAAH OE germination increased above the 1 μM treatment. These results show that the non-stratified FAAH OE germination were rescued by stratification or GA, while the *faah* germination, which did not require stratification, also was not affected by GA. The responsiveness of FAAH OE to GA suggests that perhaps the delay in germination in

these lines in the absence of stratification is because of a partial disregulation of GA-mediated processes. Given the interaction of NAE metabolism with ABA signaling identified in the first two chapters and the antagonistic relationship between ABA and GA in seed germination, it seems reasonable that altered NAE metabolism may influence the balance of plant responses between these two classical hormones.

Discussion

Altered NAE metabolism affected the germination rate of non-stratified *A. thaliana* seeds. The non-stratified AtFAAH OEs germinated slower than the non-stratified *faah* or WT, and this delayed germination was reversible upon stratification (Figure 4.1). In seed germination mutants, properties of germination and dormancy are affected, which sometimes are accompanied by pleiotropic effects that are specific for maturation such as desiccation intolerance (Goldberg et al., 1994; Koornneef and Karssen, 1994). The *abi3* (abscisic acid-insensitive3), *fus3* (fusca3), and *lec1* (leafy cotyledon1) mutants produce seeds that are intolerant to desiccation (Nambara et al., 1992; Keith et al., 1994). Accumulation of seed storage proteins (SSP; such as CRA1) and late embryogenesis abundant (LEA; such as ATEM1) mRNAs is severely reduced in these mutants (Nambara et al., 1992; Vicient et al., 2000). I examined seed transcript levels of several genes including *CRA1*, *ATEM1* and *ABI3* in the AtFAAH mutants. The results showed transcript levels that were similar to WT, indicating that altered NAE metabolism had not affected desiccated seed mRNA levels.

Seeds of severe *abi3* mutants in *A. thaliana* accumulate less storage protein, are desiccation-intolerant, and germinate precociously (Nambara et al., 2000). When

OLEO1 is suppressed in *A. thaliana,* the seed oil bodies were larger, and a delay in germination (reversible upon stratification) was revealed. There was a decrease in the amount of lipids and a compensatory increase in total protein in the OLEO1 suppressed *A. thaliana* (Siloto et al., 2006). Quantification of lipid content in the FAAH OE and *faah* showed no difference than in WT (Figure 4.2A), also, total protein levels in the three genotypes were similar (Figure 4.2B). Oil body distributions were observed to be similar in the embryos of all three, WT, FAAH OE, and *faah* (Figure 4.2C). The analysis of gene transcript levels, oil and protein content in the desiccated seeds of the AtFAAH mutants had not revealed any altered levels that may have caused a delayed germination in the non-stratified FAAH OE seeds.

ABA establishes and maintains dormancy in seeds and its levels are required to decline for germination to proceed (Nambara and Marion-Poll, 2003). This led us to ask the question of whether ABA levels in desiccated seeds of *A. thaliana* WT, *faah* and AtFAAH OE were similar. ABA quantification was conducted by isotope-dilution mass spectrometry in the three genotypes, and ABA levels in all seeds were very similar (Figure 4.3). Since ABA levels did not vary much in the desiccated seeds, I asked the question if the delayed germination could be attributed to the increased sensitivity of the FAAH OE to ABA(Wang et al., 2006). To examine this I used transcript levels of a known ABA responsive gene, *AtHVA22B,* as a reporter of increased ABA sensitivity. Transcript levels were quantified in stratified and non-stratified seeds of the three genotypes up to 96 h after sowing (Figure 4.5), transcript levels did not reveal any increased ABA sensitivity that could be associated with the delayed germination of the FAAH OE. ABA levels were similar in seeds of all three genotypes and transcript levels

of the reporter gene revealed no increased sensitivity to ABA, these data indicate that the delayed germination is not attributable to altered ABA levels or sensitivity.

Seed germination is associated with degradation and mobilization of reserves accumulated during maturation (Bewley, 1997). Lipid mobilization and composition was examined in stratified and non-stratified WT and FAAH altered seeds, and after 48 h of sowing. Stratification initiates mobilization of lipid reserves, as shown by substantially lower lipid levels in the stratified seeds compared to the non-stratified seeds (Figure 4.6), and levels were similar in all genotypes. At 48 h only the FAAH OE showed a significant, but modest, difference in lipid levels between stratified (lower) and non-stratified seeds. TAG was the predominate neutral lipid found in all genotypes, desiccated, stratified, or after 48 h. The relatively lower levels of lipids in the stratified FAAH OE at 48 h may be a consequence of delayed germination rather than a cause. The FAAH OE delayed germination was reversible upon stratification and recent results show that active metabolic processes already initiate during seed imbibition (or stratification) (Fait et al., 2006). Yet, it is not clear if the differences in lipid levels found here between the stratified and non-stratified FAAH OE are the primary reason for the delayed germination.

The plant hormone abscisic acid (ABA) is needed to induce seed dormancy during embryo maturation, whereas the hormone gibberellin (GA) is needed to stimulate seed germination. GA does not appear to directly control dormancy per se but acts as an antagonist to ABA by stimulating radicle emergence (Bewley, 1997). I questioned if GA application rescued the delayed germination of the FAAH OE. To investigate this I germinated non-stratified seed of WT, *faah* and FAAH OE in the presence and absence

of GA. There was a substantial increase in the rate of germination in both WT and FAAH OE with GA treatment, while the *faah* showed no improvement (or was inhibited) with GA treatment (Figure 4.8). The *faah* mutant had shown some insensitivity to ABA (unpublished observations), now it displayed insensitivity to GA. The WT and FAAH OE germination both improved with GA application, although the FAAH OE was more sensitive to GA at lower concentrations, 1 μM (Figure 4.9). At this concentration the FAAH OE displayed an improved rate of germination while the WT with 1 μM GA showed little or no effect. These results indicate that FAAH may have a role in regulating the effects of ABA and GA during germination. The increased sensitivity to ABA and GA of the FAAH OE, and the insensitivity of *faah* to ABA and GA supports a role as a modulator of the effects of these two antagonistic hormones. This role for FAAH would seem to be totally unrelated to its ability to metabolize NAE, which confers NAE tolerance to the FAAH OE's (Chapter 2).

Methods

Plant Materials and Growth Assays

Plants were propagated in soil for seed production. Seeds were placed on three layers of moistened filter paper in a Petri dish, the stratified were placed in 4^{O} C in the dark for three days, stratified and non-stratified were placed in a growth chamber at 22^{O} C, 16/8 h light/ dark. GA_3 treated seeds were germinated on filter paper moistened with the appropriate concentration of GA_3 solution (Sigma G1025-16 > 50% GA_3).

Quantitative RT-PCR

Verification of microarray results and quantification of transcripts by quantitative RT-PCR was performed with a Smart Cycler II (Cepheid) instrument using real time one step assay system (Takara Bio Inc) with SYBR^{TM} Green I. The following gene specific primer pairs were used, *ABI3* (At3g24650) (F) 5'GAGCTGGCTCAGCTTCTGCTATG-3' (R) 5'AGGCCAAAACCTGTAGCGCATGTTC-3', *ATEM1* (At3g51810) (F) 5'-CTGAAGGAAGAAGCAAGGGAG-3', (R) 5-' TCCATCGTACTGAGTCCTCCTTTAC-3', *CRA1*(At5g44120)(F) 5'-CACCATTGCGTTTTGACGGAAGATC-3' (R) 5'-GATGACAACCGTGGAAACATTGTCC-3', *AtHVA22B* (At5g62490) (F) 5'-CATCGCTGGACCTGCATTA C-3' (R) 5'-GGATATAATGGGATCCATTCGAGG-'3, *RD29B* (At5g52300) (F) 5'-CATAAAGGTGGAGAAGCTGGAGTA-3' (R) 5'-CCTCCAAATCTTGCCGGAGAATTC-3' *LEA M17* (At2g41260) (F) 5'-CGATGCCACACACGATGAAGTG-3' (R) 5'-AGTCTTGCTGGGGCTCTACAAC-3' *AtFAAH* (At5g64440) (F) 5'-CCATCTCAAGAACCGGAGCATG-3' (R) 5'-GGTGTTGGAGGCTTGTCATAGC-3'.

All primers were designed to span one intron to distinguish cDNA amplification from genomic DNA contamination. Relative transcript levels in all samples were normalized using 18S rRNA as a constitutively expressed internal control, with primers (F)5'-TCCTAGTAAGCGCGAGTCATCA-3' and (R) 5'-CGAACACTTCACCGGATCAT-3' (Dean Rider et al., 2003). Quantitative RT-PCR reactions were performed in duplicate with 0.2 µg total RNA and 0.5 µL of 10 µM gene specific primers in each 25 µL reaction. The reaction mix was subjected to the following RT-PCR conditions, 42 ^{O}C for 15 min, one cycle; 95 ^{O}C for 2 min, one cycle; 94 ^{O}C for 10 sec, 58 ^{O}C for 25 sec (read cycle), 72

^{O}C for 20 sec, the number of cycles and annealing temperature were experimentally determined for each set of gene-specific for primers. RT-PCR products were examined by gel electrophoresis and by melting curve analysis (60 ^{O}C to 95 ^{O}C at 0.2 ^{O}C /sec) to rule out anomalous amplification products. The $2^{-\Delta\Delta CT}$ cycle threshold (C_T) method was used to calculate relative changes in transcript levels determined from quantitative, real time RT-PCR (Livak and Schmittgen, 2001). The data were analyzed using the equation where $\Delta\Delta C_T = (C_{T,\,Target} - C_{T,\,18s})_{\,Treated} - (C_{T,Target} - C_{T,\,18s})_{\,Not\ Treated}$. "Treated" refers to samples treated with ABA or NAE, and "Not Treated" refers to samples treated with solvent alone. For ease of presentation, in the cases when transcript levels in the "treated" samples were lower than in "not treated" samples, the fractional values obtained by the above formula were converted to fold difference by taking the negative reciprocal.

Metabolite Quantification

Metabolites were quantified by isotope-dilution mass spectrometry. For ABA analysis, approximately 100 mg of plant tissue was ground in a chilled mortar with 30 mM imidazole buffer in 70% 2-propanol. Deuterated ABA (200 ng) was added as a quantitative standard. Three additional 2-propanol extracts of the same tissue were combined and reduced under N_2 in a dry bath at 70^{o}C to about 1-2 ml, removing the 2-propanol. Samples were stored at -20^{o}C until fractionation by HPLC and quantification by GC-MS as described previously (Wang et al., 2001). ABA levels were quantified in seed extracts with the assistance of Dr. Karl Hasenstein, University of Louisiana at Lafayette.

Quantification of Total Seed Protein

A. thaliana seeds are homogenized in acetone and after centrifugation at 16,000g for 5 min the supernatant is discarded and the pellet air dried. The pellet is resuspend in extraction buffer (50 mM Tris-HCl, pH 8.0, 250 mM NaCl, 1 mM EDTA and 1% (W/V) SDS) and incubated for 2 h at room temperature. The total protein extract was centrifuged at 16,000g for 5 min and the supernatant was used for protein measurement with the Lowry DC protein assay (Bio-Rad) (Lowry et al., 1951), BSA was used for calibration.

Seed Oil Quantification

Approximately 1 g of desiccated *A. thaliana* seed is placed in the Bruker Minispec MQ20 NMR analyzer, and the Bruker minispec Software for Windows will be used to calculate oil content. Cotton seed oil is used for calibration of oil quantification program. The determination of oil in oilseeds by NMR is based upon the fact that that the proton transverse relaxation time T_2 is much longer in oils than in protein, carbohydrate or water, which are the main constituents of oilseeds. The T_2 of oil is about 100ms. This makes it possible to measure the oil in FID signal without any interference from the other parts of the seeds. The signal is converted into the percent of oil in seeds by weighing the seeds and using a linear calibration curve. The calibration curve is valid because the variation of hydrogen in oil from different varieties of the same crop is within 1%, provided cultivation is under similar climatic conditions (Tiwari).

Lipid Extraction and Analysis

Lipids were extracted into chloroform according to Bligh and Dyer (1959) (Bligh and Dyer, 1959)modified by Chapman and Moore, 1993 (Chapman and Moore, 1993). Add 2 ml of hot 2-propanol (70ºC) to the tissue and homogenize then heated at 70ºC for 30 min. One ml of chloroform was added to the mixtures, and lipids were extracted at 4ºC overnight. One ml of chloroform and 2 ml KCl (1M) were added to induce phase separation. The aqueous layer was aspirated off and the organic layer was washed two times with 2 ml KCl (1M) and once with deionized H_2O (MilliQ UF Plus). The organic phase was collected and dried down under nitrogen. Lipid classes were separated by TLC (Hexane:Diethyl ether:Acetic acid; 60:40:5 v/v/v). Identification was done by comigration with known standards.

Microscopy Analysis

A. thaliana mature embryos were isolated from dry seeds and infiltrated with BODIPY to visualize neutral lipids. Images were acquired using a Zeiss Axiovert 200M optical microscope equipped with McBain instruments and CSV10 Yokogawa confocal scanner.

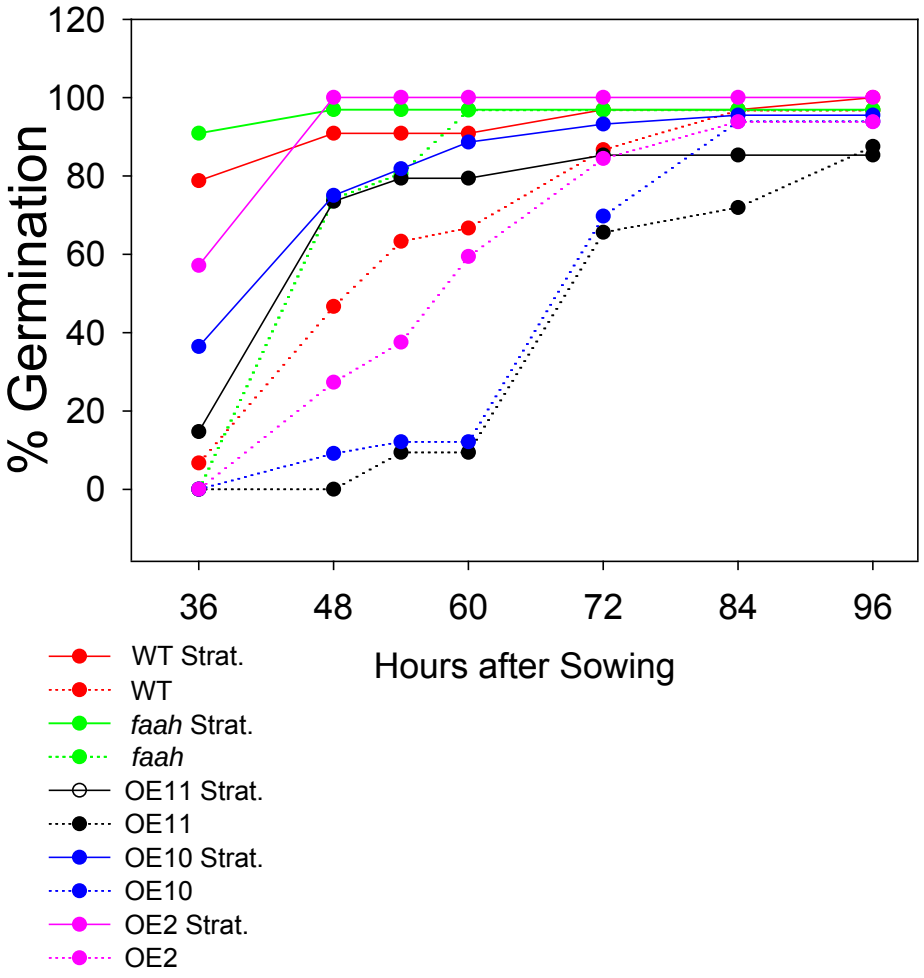

Figure 4.1. *Arabidopsis* altered in FAAH expression have delayed germination rate. Germination (radicle protrusion) counts were taken every six hours for 60 h then every 12 h for stratified and non-stratified WT, three AtFAAH OEs and *faah*. Seeds were placed on three layers of moistened filter paper in a Petri dish, the stratified were placed in 4^{O} C in the dark for three days, stratified and non-stratified were placed in a growth chamber at 22^{O} C, 16/8 h light/ dark.

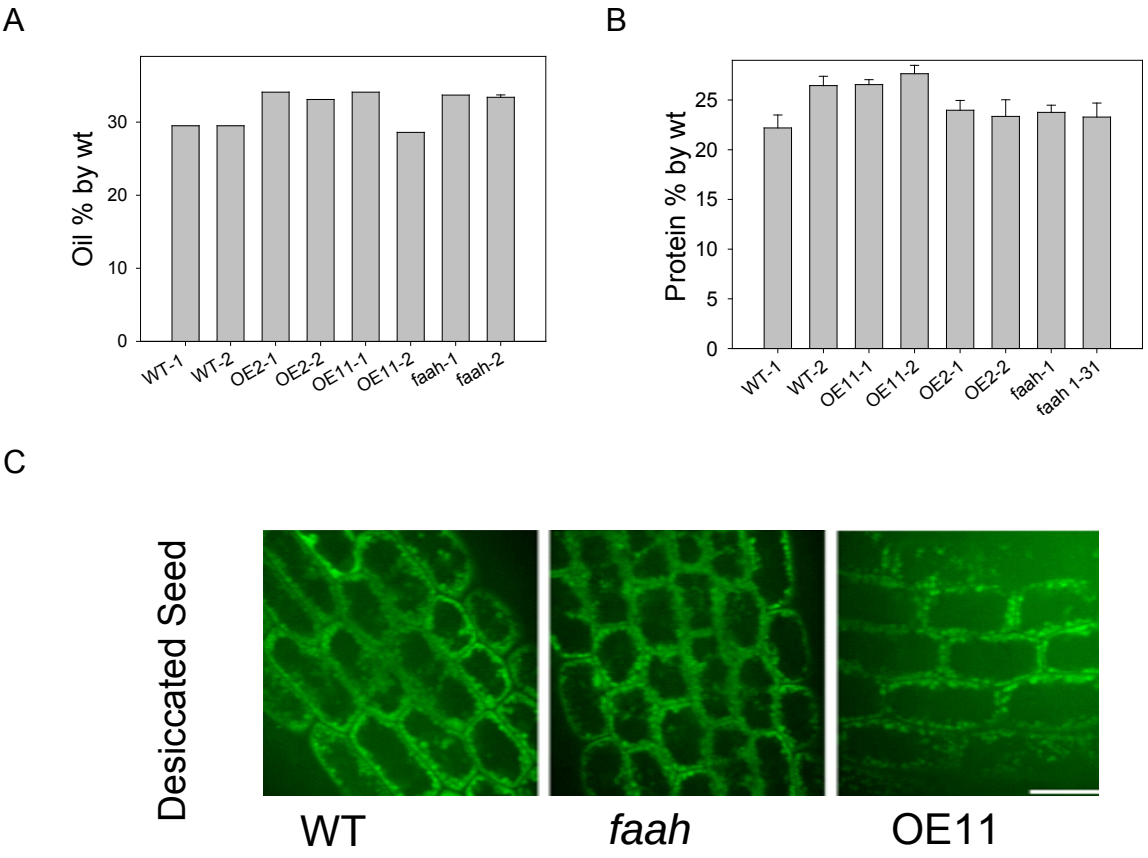

Figure 4.2. Oil and protein content in desiccated seeds of wild type (Col.) and FAAH altered plants.

(A) Percent oil by seed weight was determined using NMR with multiple lines or batches of seed. The values shown are the mean and standard error of triplicate samples.

(B) Total protein by seed weight was determined by employing the Lowry DC protein assay with multiple lines and batches of seed. The values shown are the mean and standard error of six replicate samples.

(C) Oil body distribution was examined by confocal microscopy in living mature *A. thaliana* embryos from desiccated seeds of wild type (Col.) and FAAH Altered Mutants, embryos are stained with BODIPY. Bar = 20μm.

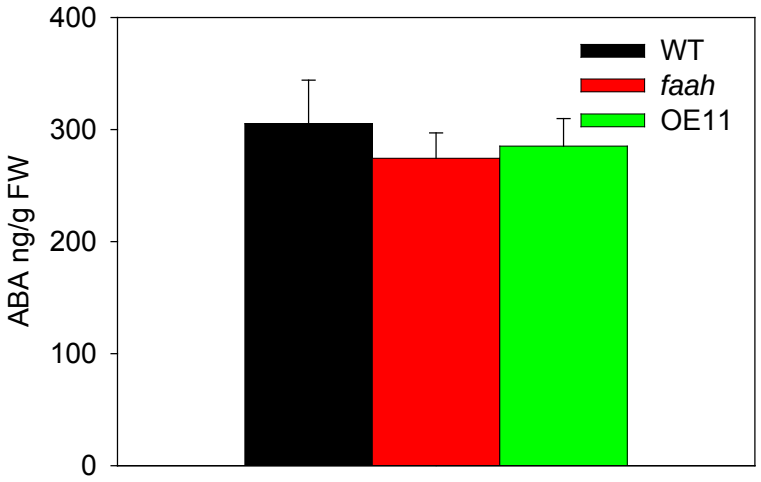

Figure 4.3. ABA content in desiccated *A. thaliana* seed quantified by isotope-dilution mass spectrometry.

ABA levels are similar in all three seed types, WT, *faah* and AtFAAH over expressor. Deuterated ABA standard was added at the time of extraction. Bars represent the standard deviation in triplicate extractions.

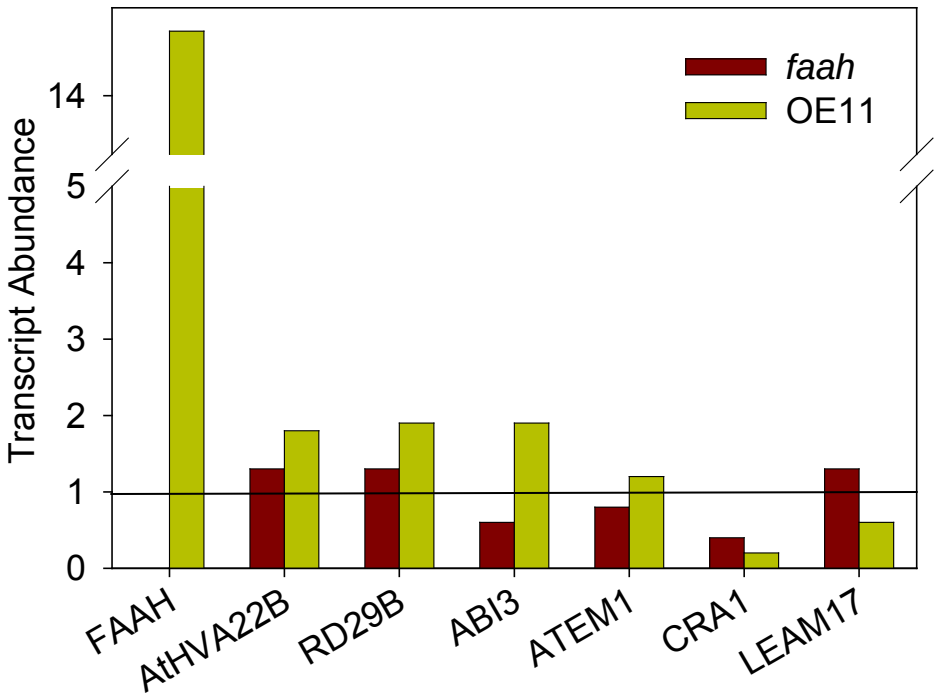

Figure 4.4. Transcript abundance in desiccated seeds of FAAH and several Genes known to be regulated by ABA.

Transcript levels were quantified in total RNA extracts by real-time RT-PCR against 18S rRNA and are plotted relative to wild type (black line) transcript levels using the ΔΔCT method (Livak and Schmittgen, 2001). Transcripts were quantified in total RNA extracts from seeds and RNAs were targeted for amplification with gene specific primers (see methods). Values shown are averages of duplicate samples.

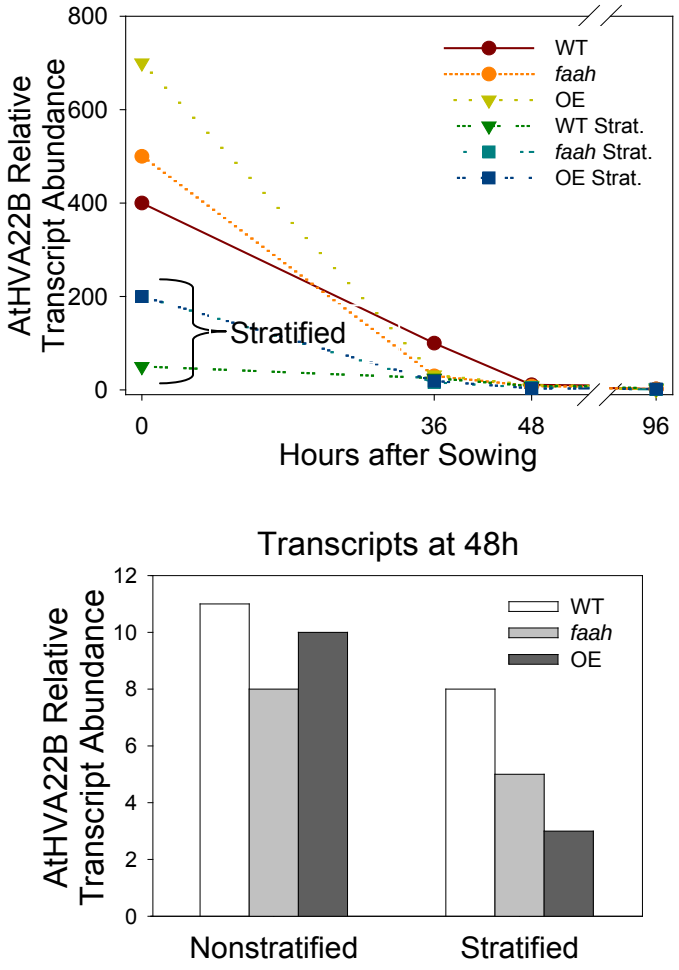

Figure 4.5. Comparison of transcript abundance for AtHVA22B (At5g62490) in wild type and FAAH altered seed and seedlings (Stratified or Non-Stratified).

Transcripts were quantified in total RNA extracts from seeds or seedlings at designated hours after sowing in liquid media. RNAs were targeted for amplification with gene specific primers (see methods). Quantification of transcripts was conducted by real-time RT-PCR and are relative to values measured for WT at 96 h (not stratified). Values for transcripts were averaged from duplicate measurements at each time point (normalized to 18S rRNA). Due to the large drop of transcript levels the histogram displays data gathered at 48 h for greater detail.

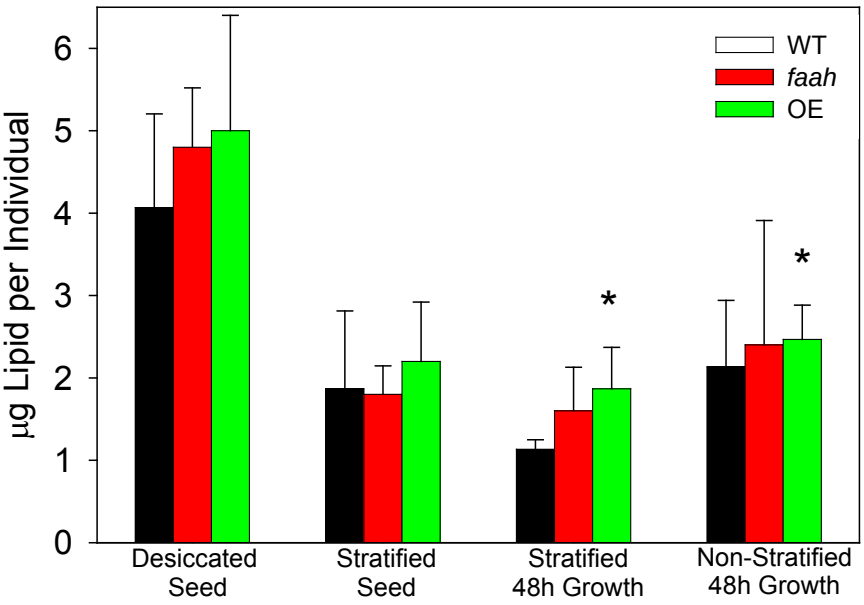

Figure 4.6. Total lipid levels in desiccated seed, stratified seed and at 48h growth of wild type (Col.) and FAAH altered mutants.

Total lipid content was determined using gravimetric quantification. Biological triplicates were analyzed from total individuals starting with 10 mg seed (approximately 500), data is presented as µg lipid per individual. Samples were grown on moistened filter paper in a Petri dish at 22°C 16/8h light/dark for 48h then collected, stratified seeds were given a 3d cold treatment (4°C). T-test comparison of 48h growth stratified to non-stratified found a modest, but significant difference only in the OE's, $p < 0.03$ (*).

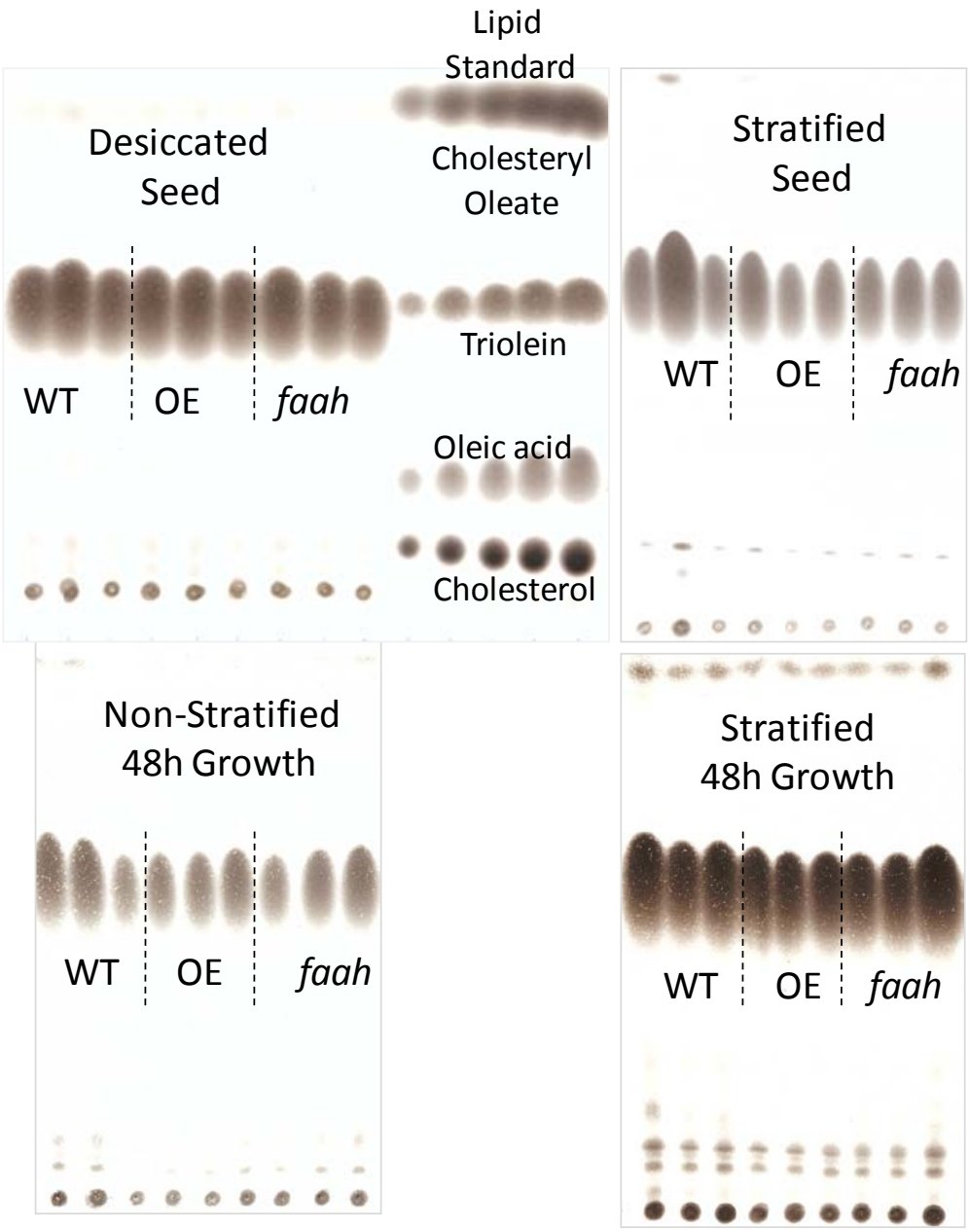

Figure 4.7. TLC separation of total lipid extracts from desiccated seed, stratified seed and at 48h growth of wild type (Col.) and FAAH altered mutants.

Equal concentrations of lipid from biological triplicate extracts were loaded on the TLC plate (200µg), with increasing concentrations of lipid standards containing various species (50-250µg). Lipids were visualized by treating plates with chromic acid solution and charring (heating treated plate to 180°C).

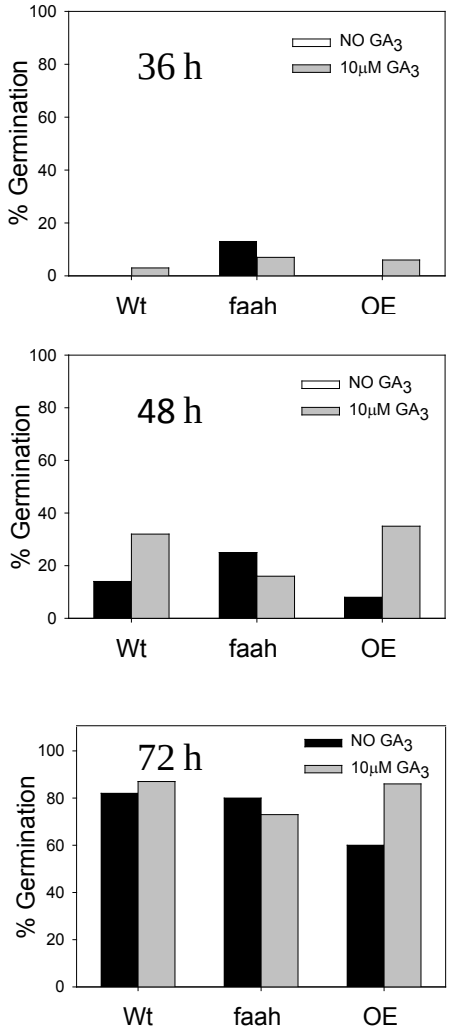

Figure 4.8. GA improves germination in non-stratified *Arabidopsis* seeds of WT and FAAH OE, but not *faah*.

Germination (radicle protrusion) counts were taken at 36, 48 and 72 hours for non-stratified WT, AtFAAH OE and a *faah A. thaliana* seed. Seeds were placed on three layers of moistened filter paper in a Petri dish treated with or without (control) 10μM GA and placed in a growth chamber at 22^{O} C, 16/8 h light/ dark. Non-stratifed FAAH OE's display slower germination (Figure 4.1) than WT or *faah*, the application of GA promotes the FAAH OE germination to similar levels as treated WT, yet the *faah* display no benefit from the application of GA.

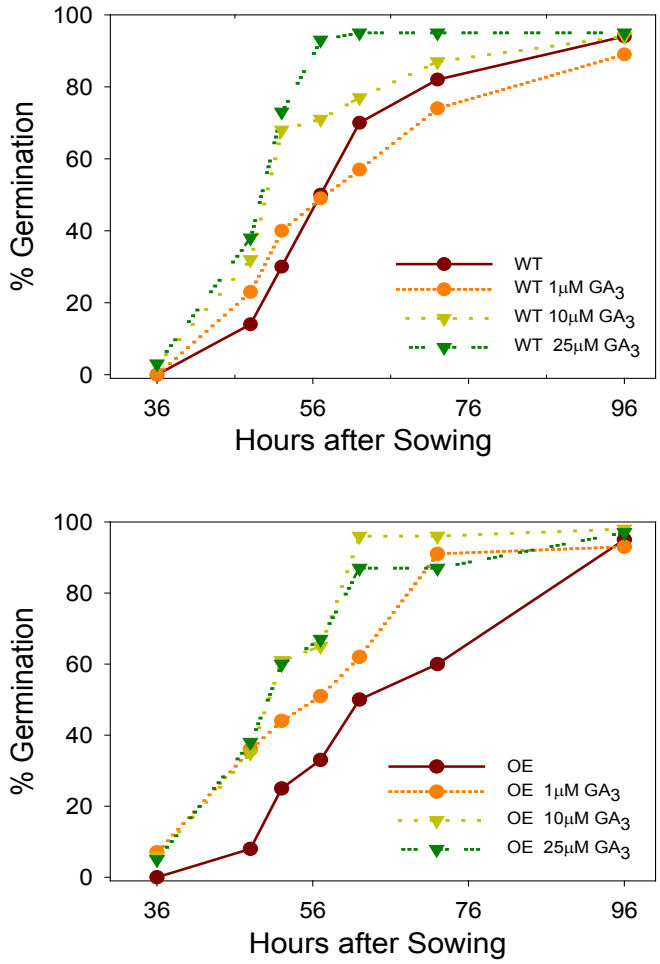

Figure 4.9. FAAH OE Requires Lower GA Concentration (1μM) than WT to Improve Germination in Non-stratified *Arabidopsis* Seeds.

Germination (radicle protrusion) counts for non-stratified WT (top panel) and AtFAAH OE (bottom panel) *A. thaliana* seed on increasing concentrations of GA. Seeds were placed on three layers of moistened filter paper in a Petri dish treated with GA, or without (control), and placed in a growth chamber at 22^{O} C, 16/8 h light/ dark. FAAH OE's heightened germination response to GA at $1\mu M$ indicates an increased sensitivity compared to WT which shows no response to GA at $1\mu M$.

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CHAPTER 5

SUMMARY AND SIGNIFICANCE

NAEs are a minor lipid constituent of plants and animal cells. Their role in animal physiology has been studied for more than three decades, however studies on the function of NAEs in plants has only been initiated more recently. NAEs were first identified in seed derived products in the 1950s but were not identified as endogenous metabolites until the 1990s. The occurrence of NAEs in various plant species (Chapman, 2004), the recent cloning of a plant enzyme involved in the hydrolysis of NAE (Shrestha et al., 2003) and the identification of a high-affinity NAE binding protein in plant tissues (Tripathy et al., 2003), indicate parallels between the role of NAEs in plant and animal systems.

A. thaliana seeds germinated and grown in constant exposure to micromolar concentrations of NAE12:0 exhibited several morphological and cellular defects (Blancaflor et al., 2003; Motes et al., 2005), but the primary mechanisms of these actions are unclear. Exogenous ABA treatment of seedlings, or the application of desiccation stress, induced growth arrest within a narrow window of postgerminative growth (Lopez-Molina et al., 2001; Lopez-Molina et al., 2002). This growth arrest is proposed to function as a stress defense mechanism for seedlings in the activation of a secondary dormancy program. NAE12:0 treatment induced similar growth arrest and only within a narrow window of early seedling development, without elevating endogenous ABA levels. Global transcriptome analyses by whole genome microarrays in *A. thaliana* revealed that many of the genes with elevated transcripts induced by NAE, were also elevated by ABA. Several of these genes encoded for proteins involved

in desiccation tolerance, seed storage reserves and late embryogenesis abundant. Also, many of these genes contain upstream promoter motifs for a functional ABA response complex (Teaster et al., 2007), such as ABRE regulatory elements. The number of genes that contained these regulatory complexes suggests that these metabolites had overlapping targets. These results indicate that elevated levels of NAE maintained expression of ABA responsive genes normally associated with ungerminated, desiccation-tolerant state.

NAE12:0 appeared to affect seedling growth in a similar developmental window as ABA. This sensitivity was monitored at the molecular level by the occurrence and elevation of *ABI3* transcript levels. ABI3, is ABA responsive and the key regulator of the transition from embryo to seedling growth and its transcript levels decline rapidly during normal imbibition and seed germination (Finkelstein et al., 2002; Gazzarrini and McCourt, 2003; Nambara and Marion-Poll, 2003; Nambara and Marion-Poll, 2005; Bassel et al., 2006). Exogenously elevated NAE resulted in sustained *ABI3* transcript levels in seedlings and arrested seedling growth. An inverse relationship between *ABI3* transcript levels and growth was found, while *ABI3* transcript levels remained elevated, growth was inhibited. NAE altered gene expression similar to ABA, yet it did not elevate ABA levels, nor did ABA elevate NAE levels. This suggests that NAE growth arrest at least partially functions through the ABA signaling pathway, yet it is not mediated by the coincident elevation of endogenous levels of ABA and vice versa.

The enzyme in *A. thaliana* responsible for hydrolyzing NAEs to free fatty acids is AtFAAH, a fatty acid amide hydrolase (Shrestha et al., 2003). The over-expression of this gene (FAAH OE) conferred a tolerance to NAE in the seedlings, and an increased

sensitivity to ABA. Whereas, seedlings with AtFAAH knocked out (*faah*) acquired an increased sensitivity to NAE (and somewhat reduced sensitivity to low levels of ABA, unpublished results). As was found in WT, there was an early developmental window in the FAAH altered mutants during which growth arrest could be induced by either NAE or ABA. This window was also delineated by *ABI3* transcript levels, and other growth arrest associated genes *AtHVA22B* and *RD29B*, higher transcript levels were associated with growth arrest and only induced within the sensitivity window. When treated with NAE the FAAH OE had the lowest *ABI3* transcript levels and the highest levels of growth, while the *faah* showed the highest *ABI3* transcript levels with the lowest levels of growth, and WT in between. The tolerance of the FAAH OE was determined to be its increase capacity to metabolize NAE from the growth media and thus removing the NAE effect. WT also had this capacity at a reduced rate, which could be observed by a slower recovery of growth. The *faah* seedlings did not display any recovery during our time course, but NAE levels were slowly being reduced, indicating another mechanism (or pathway) for NAE metabolism.

ABA treatments induced similar gene expression, *ABI3* transcript levels were elevated within the sensitivity window along with other growth arrest associated genes (*AtHVA22B* and *RD29B*). The FAAH OE displayed an increased sensitivity to ABA, and this was reflected in the greater reduction of growth than WT or *faah*. The *abi3-1* ABA insensitive mutant, which was tolerant to ABA and did not undergo growth arrest with ABA treatment, did show growth inhibition with NAE treatment. The ABA tolerance and NAE sensitivity of the *abi3-1* was confirmed by growth studies, also NAE growth arrest occurred within the early stage of seedling development. Gene transcript analysis

showed that in WT (Ler), NAE and ABA induced *ABI3* and growth arrest associated gene transcript levels within the sensitive window, yet in the *abi3-1* mutant only *RD29B* transcripts were induced, and at lower levels than WT. This would suggest that NAE growth arrest works, at least partially, outside the ABA signaling pathway. The evidence gathered in this study suggests that NAE and ABA interacted to arrest early seedling development and if either metabolite remains elevated during early seedling development, growth arrest occurs. NAE-arrested growth involves a functional ABA signaling pathway and is associated with increased transcript levels of well characterized ABA responsive genes. However, evidence suggest an ABI3 independent growth arrest mechanism, which ABA does not seem to function through. Hence, I postulate two parallel pathways of NAE-induced seedling growth arrest: one that is ABA-dependent and another that is ABA-independent.

ABA and NAE levels decline before seed germination, and both metabolites were shown to be negative regulators of seedling growth. The seed of the FAAH OE was found to have lower levels of endogenous NAE (Wang et al., 2006), and germinates slower than WT when not stratified. Several properties of desiccated seeds such as ABA, lipid and protein reserves and selective gene transcript levels associated with desiccated seeds were quantified to determine if altered NAE metabolism had affected their levels. All the properties examined were similar to WT levels in desiccated seed. Lipid mobilization and composition was examined in stratified and non-stratified seeds, also 48 h after sowing; where a large difference in germination was observed. The results showed a drop in lipid levels during stratification but no significant differences between genotypes, at 48 h only the stratified FAAH OE seed showed a modest but

significant difference in lipid levels, lower than the non-stratified seed. In all cases the vast majority of lipid present was TAG. To determine if the higher level of lipid in the FAAH OE at 48 h could be due to its increased sensitivity to ABA I examined the transcript levels of *AtHVA22B*. *AtHVA22B* is a known ABA responsive gene, and was used as a reporter for increased ABA sensitivity in the FAAH OE. Transcript levels were slightly lower in stratified seeds, but by 48 h transcript levels were similar in all genotypes, suggesting that it is not increased sensitivity to ABA responsible for slower germination in the FAAH OE.

Since GA stimulates seed germination, I tested if application of GA can rescue the non-stratified FAAH OE slower germination. Low levels of GA (1 μM) improved germination in FAAH OE but not WT, indicating an increased sensitivity to GA. Higher levels of GA improved germination of the FAAH OE to the levels of some lines of stratified FAAH OEs. Interestingly, the application of GA (at any concentration used) did not improve the rate of the *faah* germination, suggesting insensitivity to GA. Although, the germination rate of non-stratified *faah* was near the rate of some stratified lines. These results suggest that the altered NAE metabolism (FAAH) may have affected the transition between ABA and GA regulation during seed germination.

The evidence gathered in this study suggests that NAE acts in concert with ABA as a negative regulator of early seedling development. During the course of normal seedling development and seedling growth both metabolites drop, if either metabolite remains elevated growth arrest occurs. NAE arrested growth is not mediated by an increase in ABA levels and vice versa. NAE growth arrest is associated with increased transcript levels of several well characterized ABA responsive genes indicating that

NAE does function through the ABA signaling pathway. I conclude that, in part, NAE metabolism regulates seedling growth through the modulation of the ABA signaling pathway by the transcriptional activation of *ABI3* and ABA responsive gene products. On the other hand, the *abi3-1* study demonstrates that NAE can induce growth arrest independent of the ABA signaling pathway. Hence, I postulated two parallel pathways of NAE-induced growth arrest; one ABA dependent and another ABA independent. Also, the FAAH altered mutants have displayed altered sensitivity to NAE, ABA and GA, this suggests the possibility of crosstalk between a lipid metabolic pathway (NAE metabolism) and ABA and GA signaling. Future studies will be aimed at delineating the targets of NAE action and the role of FAAH as a mediator of crosstalk between metabolites. The results from this study should contribute to the overall understanding of the regulation of seed development, germination and seedling growth, and may provide insights into other physiological processes in plants likely to involve NAE such as environmental stress.

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