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N-Acylethanolamine Metabolism Interacts with Abscisic Acid Signaling in *Arabidopsis thaliana* Seedlings [W] [OA]

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***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 abscisic acid (ABA) levels were depleted during seed germination, and both metabolites inhibited the growth of *Arabidopsis thaliana* seedlings within a similar developmental window. Combined application of low levels of ABA and NAE produced a more dramatic reduction in germination and growth than either compound alone. Transcript profiling and gene expression studies in NAE-treated seedlings revealed elevated transcripts for a number of ABA-responsive genes and genes typically enriched in desiccated seeds. The levels of ABI3 transcripts were inversely associated with NAE-modulated growth. Overexpression of the *Arabidopsis* NAE degrading enzyme fatty acid amide hydrolase resulted in seedlings that were hypersensitive to ABA, whereas the ABA-insensitive mutants, *abi1-1*, *abi2-1*, and *abi3-1*, exhibited reduced sensitivity to NAE. Collectively, our data indicate that an intact ABA signaling pathway is required for NAE action and that NAE may intersect the ABA pathway downstream from ABA. We 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 (NAEs) are bioactive acylamides. In mammalian systems, the metabolism of NAEs is part of the endocannabinoid signaling system wherein NAEs bind to and activate G protein–coupled cannabinoid receptors, which in turn regulate a wide array of physiological and behavioral processes (Howlett et al., 2004). Recently, additional cellular targets of endocannabinoid lipids have been discovered, including ion channels (Movahed et al., 2005; Oz et al., 2005) and transcription factors (LoVerme et al., 2006).

The steady state levels of NAEs are likely important to their signaling function and are determined by the tight control of their formation and degradation. Recent advances in our understanding of the physiological and signaling functions of NAEs in vertebrates have been facilitated by the discovery of key enzymes involved in their synthesis and catabolism. For example, biochemical studies have shown that NAEs are derived from the minor membrane phospholipid *N*-acylphosphatidylethanolamine (NAPE). A novel NAPE, phospholipase D (PLD), was cloned from mammals that exhibited specificity toward NAPE substrates but not to other common membrane phospholipids, indicating that this is one of the enzymes responsible for converting NAPE to NAE in vivo (Okamoto et al., 2004). In plants, PLD-β and -γ isoforms catalyze the formation of NAE from NAPE in vitro (Pappan et al., 1998). Additionally, recent reports indicated that NAE may be generated from NAPE via phospholipase A (Simon and Cravatt, 2006) or phospholipase C (Liu et al., 2006) mediated pathways depending on the source tissue. On the other hand, the signaling activity of NAEs can be terminated by inactivation to ethanolamine and free fatty acids. This task is accomplished by a fatty acid amide hydrolase (FAAH) (Chapman, 2004; McKinney and Cravatt, 2005) or the recently discovered NAE acid amidase (Tsuboi et al., 2005).

The occurrence and metabolism of NAEs is conserved among eukaryotic organisms (Schmid et al., 1996; Chapman, 2004); however, the physiological functions of these lipids have been investigated mostly in vertebrates. In higher plants, NAEs have been identified and quantified in different tissues by mass spectrometry, and they are found at their highest levels in desiccated seeds (Chapman, 2004). NAE types in plants include ethanolamides with acyl chain lengths from 12 to 18C and with zero to three double bonds. The typical numerical designation for *N*-acyl moieties is NAEX:Y, where X is the number of carbon atoms in the acyl chain and Y is the number of double bonds (Venables et al., 2005; Blancaflor and Chapman, 2006). The elevated levels of NAEs in seeds point to the possibility that these lipids may function in processes relevant to seed or seedling development. Evidence in support of this notion comes from our

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recent reports showing that NAE12:0 and NAE18:2 have potent growth inhibiting properties when supplied exogenously to *Arabidopsis thaliana* seedlings (Blancaflor et al., 2003; Motes et al., 2005; Wang et al., 2006). Also, in an effort to manipulate endogenous NAE levels in plants, we generated transgenic *Arabidopsis* lines overexpressing At *FAAH* since FAAH has been shown to hydrolyze NAEs in vitro (Shrestha et al., 2002, 2003). As expected, seeds and seedlings of FAAH overexpressors had significantly lower levels of endogenous NAEs than nontransgenic seeds, and this was associated with enhanced seedling growth and larger seedling cell and organ size (Wang et al., 2006). Moreover, depletion of endogenous NAEs is detectable within hours of seed imbibition (Chapman et al., 1999), concomitant with increased NAE hydrolytic activity and FAAH expression (Shrestha et al., 2002; Wang et al., 2006). The above results suggest that the metabolism of NAEs might be necessary for cell expansion that typically accompanies seed germination and early seedling development. However, despite the strong association between NAE metabolism and cell expansion, nothing is known about how NAEs interact with other environmental and endogenous factors that regulate plant development.

In view of the diverse signaling functions for NAEs in vertebrates (Rodriguez de Fonseca et al., 2005), and the tight control of their endogenous levels in seeds and early seedling development (Chapman et al., 1999; Wang et al., 2006), it is possible that these fatty acid amides together with other effectors influence seed and seedling physiology. One potential regulator that could interact with NAE is abscisic acid (ABA), the plant hormone with well-established roles in the signaling cascades that influence seed physiology (Finkelstein et al., 2002; Nambara and Marion-Poll, 2003, 2005; Finch-Savage and Leubner-Metzger, 2006). Much of the recent progress made toward understanding the role

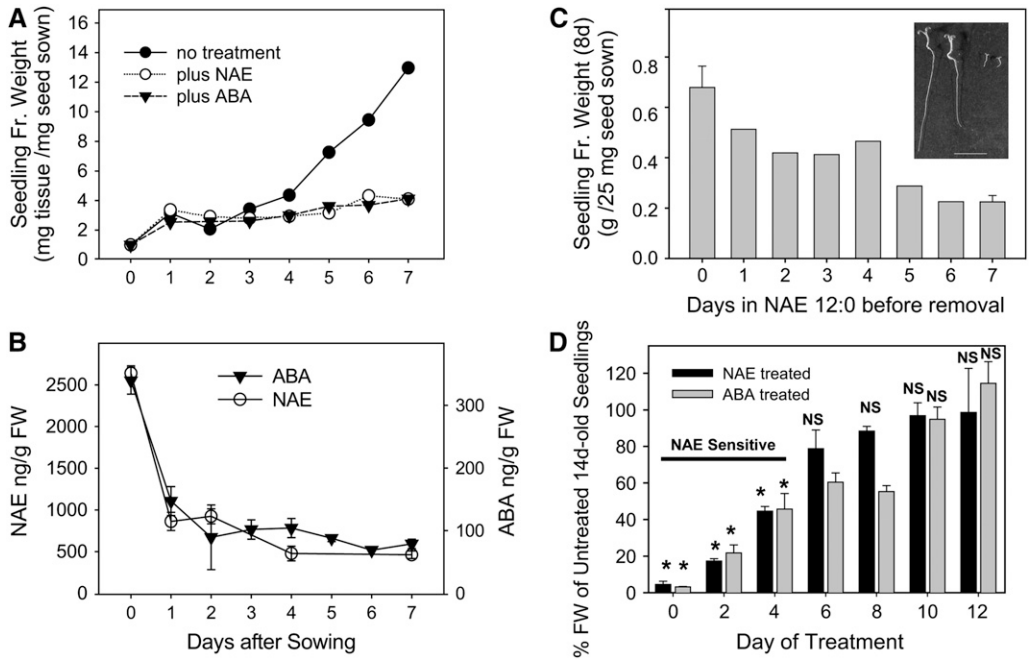

Figure 1. ABA or NAE12:0 Negatively Regulates Seedling Growth, and This Inhibition Is Reversible and Occurs within a Narrow Developmental Window.

(A) Treatment of seedlings with 0.5 μM ABA or 35 μM NAE arrests seedling growth. Data shown are representative of a single time course experiment. The trends of growth suppression by ABA and NAE compared with 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 SD of six independent extractions. Values for ABA are means and SD of three independent extractions. Under these conditions, radicle emergence occurs at about day 3. FW, fresh weight.

(C) The inhibitory effects of NAE (35 μM) on *Arabidopsis* seedling growth are reversible. Bars show the SD of three different liquid culture experiments to show the general range of variability. Inset compares 8-d-old seedlings grown in liquid cultures without (two on left) or with (two on right) NAE12:0. Bar = 10 mm.

(D) Seedling growth responses to NAE12:0 fall within a narrow window of developmental sensitivity (<6 d), similar to the window of growth arrest previously characterized for ABA (Lopez-Molina et al., 2001, 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 ~2500 individuals (50 mg seed) each. Asterisks indicate a significant difference compared with 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.

of ABA in seed physiology comes from genetic studies in *Arabidopsis* where ABA biosynthesis and ABA-insensitive (ABI) mutants display seed germination and seedling phenotypes (Finkelstein et al., 2002).

Interestingly, some components of the ABA signaling pathways have been shown to be targets of NAE signaling. For instance, as noted earlier, NAEs in animal systems bind to G protein–coupled receptors to trigger various neurological and physiological responses (Fowler, 2003). Evidence is now accumulating that ABA mediates some of its physiological effects on seed germination via heterotrimeric G proteins (Ullah et al., 2002; Pandey and Assmann, 2004; Pandey et al., 2006; Liu et al., 2007). Downstream products of PLD signaling, such as phosphatidic acid (PA), also have been implicated in modulating ABA responses in guard cells (Jacob et al., 1999; Zhang et al., 2004; Mishra et al., 2006) and seed germination (Katagiri et al., 2005). In this regard, it is worth noting that NAEs were found to be potent, noncompetitive inhibitors of PLD-α enzyme activity in vitro, with short-chain saturated species being most potent (effective concentrations in the nanomolar range). In fact, NAE12:0 was used by several groups to inhibit PLD-α–mediated processes in vivo (Austin-Brown and Chapman, 2002; Dhonukshe et al., 2003; Motes et al., 2005; Komis et al., 2006). In addition, NAE in mammals and ABA in plants also have been shown to exert their effects on various physiological processes through calcium and potassium channels, reactive oxygen species, nitric oxide release, Glu receptors, and sphingolipids (Coursol et al., 2003; Kang et al., 2004; Bright et al., 2006; Chai et al., 2006).

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) (Figure 1). 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, we focus our attention here on seed germination and early seedling establishment. Collectively, our results provide biochemical, molecular, and genetic evidence that NAE regulation of seedling growth requires an intact, functional ABA signaling pathway. Seedling growth arrest can be induced by elevating NAE and/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. Our 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. Our 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

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

Previous studies have shown 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

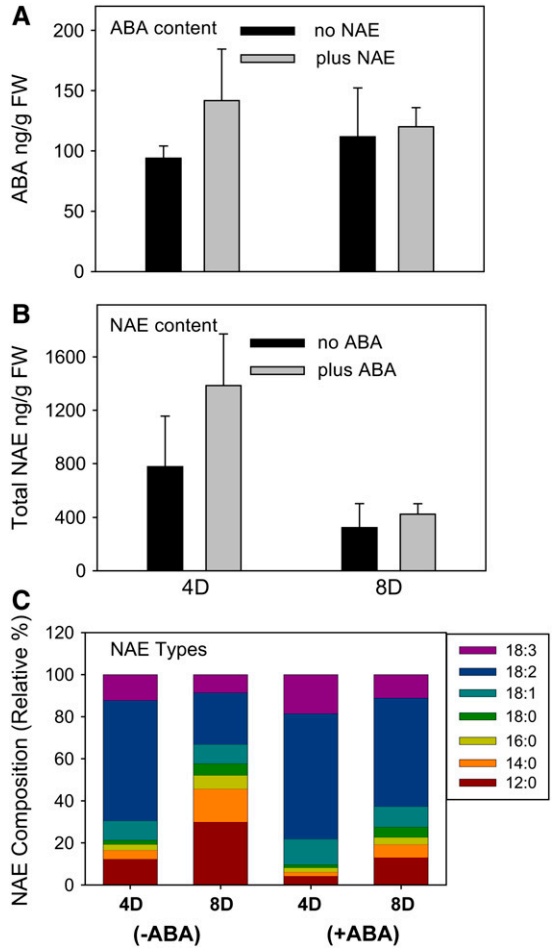

Figure 2. ABA and NAE Content in Seedlings at 4 and 8 d after Sowing Quantified by Isotope-Dilution Mass Spectrometry.

(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 SD of three independent measurements.

(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 SD of six independent measurements.

(C) NAE composition in ABA-treated and untreated seedlings. NAE contents in **(B)** were summed from individual NAE types, and NAE composition was calculated based on the relative percentage of each type. See **(B)** for total NAE content at 4 or 8 d without or with ABA.

fresh weight, was arrested by NAE12:0 in a manner similar to ABA (Figure 1A). To determine whether endogenous levels of NAEs and ABA follow a similar time course of depletion during *Arabidopsis* seed germination, we measured NAE and ABA levels in desiccated seeds and seedlings 1 to 7 d 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 d. Although the endogenous levels of both metabolites dropped in a similar fashion during seed germination, total endogenous NAE was ~10-fold higher than endogenous ABA when expressed in a nanograms/grams of fresh weight basis (Figure 1B). Although maintaining higher levels of exogenous NAE12:0 in the medium inhibited seedling growth (Figure 1A), its removal from the medium allowed for seedling growth to resume (Figure 1C; Blancaflor et al., 2003), indicating that the growth arrest by NAE was reversible.

Previous work by Chua and coworkers (Lopez-Molina et al., 2001, 2002) helped define the developmental window for seedling growth arrest by ABA, and we asked whether seedlings showed a similar developmental sensitivity to exogenous NAE treatment. Seedlings that were treated with NAE12:0 earlier than 8 d after germination were inhibited in growth substantially compared with untreated seedlings (Figure 1D). There was no significant difference in growth of seedlings treated with NAE after 8 d of growth. The sensitivity of seedlings to NAE was significant ($P < 0.001$) up to day 6 but not significant 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 program in young seedlings.

Endogenous Levels of ABA Are Not Significantly Altered by Exogenous NAE Treatment

One possible explanation for the NAE-induced growth inhibition was an NAE-related 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 2A), despite the clear impact on seedling growth at these ages (Figure 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 2B). Total NAE content declined during postgerminative growth, and changes in NAE profiles accompanied this decline. Older seedlings, without ABA, had a lower proportion of polyunsaturated NAE types and a relatively higher proportion of saturated medium/long-chain types (Figure 2C), but this change in NAE composition was not as evident when seedlings were grown in ABA. In fact, the relative

Table 1. NAE12:0-Induced Genes in 4-d-Old *Arabidopsis* Seedlings That Were Also Enriched in Desiccated Seeds (Nakabayashi et al., 2005) and Upregulated by ABA in Seedlings (Li et al., 2006)

Locus ID	Predicted Function	Ratio (NAE:DMSO)
At3g15670	Late embryogenesis abundant protein, putative/LEA protein, putative	10.09
At1g04560	AWPM-19-like membrane family protein	4.87
At5g07330	Expressed protein	4.82
At3g17520	Late embryogenesis abundant domain-containing protein/LEA domain–containing protein	4.57
At4g36600	Late embryogenesis abundant domain-containing protein/LEA domain–containing protein	4.33
At2g36640	Late embryogenesis abundant protein (ECP63)/LEA protein	4.23
At5g62490	ABA-responsive protein (HVA22b)	4.06
At1g52690	Late embryogenesis abundant protein, putative/LEA protein, putative	3.36
At3g51810	Em-like protein GEA1 (EM1)	3.23
At5g66780	Expressed protein	3.21
At2g35300	Late embryogenesis abundant group 1 domain-containing protein/LEA group 1 domain-containing protein	3.18
At5g06760	Late embryogenesis abundant group 1 domain-containing protein/LEA group 1 domain-containing protein	3.17
At5g52300	Low-temperature-responsive 65-kD protein (LTI65)/desiccation-responsive protein 29B (RD29B)	3.08
At2g23110	Expressed protein	3.03
At3g15280	Expressed protein	2.92
At3g02480	ABA-responsive protein-related	2.87
At4g21020	Late embryogenesis abundant domain-containing protein/LEA domain–containing protein	2.72
At3g53040	Late embryogenesis abundant protein, putative/LEA protein, putative	2.66
At1g02700	Expressed protein	2.66
At5g01300	Phosphatidylethanolamine binding family protein	2.66
At1g03790	Zinc finger (CCCH-type) family protein	2.57
At4g31830	Expressed protein	2.49
At4g02280	Sucrose synthase, putative/sucrose-UDP glucosyltransferase, putative	2.43
At3g46660	UDP-glucoronosyl/UDP-glucosyl transferase family protein	2.38
At2g38465	Expressed protein	2.35
At5g05220	Expressed protein	2.33
At5g42290	Transcription activator-related	2.29
At3g11050	Ferritin, putative	2.18
At2g38905	Hydrophobic protein, putative/low temperature and salt-responsive protein, putative	2.12

unsaturated NAE content was significantly higher in ABA-treated seedlings at both 4 and 8 d ($P < 0.03$ and $P < 0.001$, respectively), such that the NAE composition of 8-d-old seedlings in ABA more resembled that of untreated seedlings at 4 d. Collectively, these results suggested that total NAE and ABA content are not influenced substantially by one another, although ABA treatment did have a significant impact on the relative proportion of the different NAE types. From these data we can conclude that 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); however, complex changes in NAE composition may contribute to, or be a result of, ABA-induced arrest.

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

To probe further the molecular mechanisms underlying NAE12:0 effects on seedling growth, we conducted global gene expression profiling using Affymetrix ATH1 whole-genome microarrays. Seeds were germinated and seedlings maintained for 4 d in liquid Murashige and Skoog 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 with seedlings grown without NAE12:0 (Figure 1A). Results were evaluated from three replicate microarrays for both NAE treated- and solvent-treated (0.05% DMSO) seedlings. With a false discovery rate (FDR) of 0.1 (as indicated by the Q value using extraction of differential gene expression; Storey and Tibshirani, 2006), 8124 genes were shown to be differentially expressed by NAE treatment. Among these 8124 genes, 548 also were selected using associative analysis (Dozmorov and Centola, 2003), where the Bonferroni correction P value threshold of 2.19298E-06 and the ratio threshold of two was applied (see Supplemental Table 1 online). These stringency conditions were applied to compare our results with prior published gene lists where 2- to 2.5-fold differences in expression were applied as selection thresholds (Nakabayashi et al., 2005; Li et al., 2006). Among the 548 genes in seedlings that were differentially regulated by NAE12:0, 280 were upregulated and 268 were downregulated (see Supplemental Table 1 online). This group of 548 NAE-regulated genes (out of the original 8124 genes identified) was most differentially expressed between solvent control and NAE12:0 treatment, and this gene list completely overlapped with selections made using significance analysis of microarrays (Tusher et al., 2001) with a FDR of 0.

The elevated levels of NAE and ABA in desiccated *Arabidopsis* seeds, the parallel time course of their depletion during seed germination, and their similar inhibitory effects on early seedling growth (Figure 1) suggest that both compounds may indeed interact to negatively influence the seed to seedling transition. Hence, we compared our NAE microarray data sets with other published whole-genome microarray studies in ABA-treated seedlings or *Arabidopsis* seeds. Interestingly, 22% of the genes upregulated in NAE-treated seedlings also were upregulated in ABA-treated seedlings (Li et al., 2006; see Supplemental Table

Table 2. Top 10 Motifs in the 1-kb Upstream Region of Genes Differentially Regulated by NAE12:0 and Occurrence of ABREs (CE, CE3, and ABRE)

	CE (CACGT)	CE3 (CGTGTC)	ABRE (ACGTG)	Oligomer	Absolute Number of This Oligomer in Query Set	Absolute Number in Genomic Set	Number of Sequences in Query Set Containing Oligomer	Number of Sequences (out of 31,407 in Genomic Set) Containing Oligomer	P Value from Binomial Distribution
NAE-upregulated genes									
1	1			A**CACGT**	235	11801	141/276	8521/31407	1.99E-17
2			1	**ACGTG**T	235	11801	141/276	8521/31407	1.99E-17
3			1	C**ACGTG**	244	11559	91/276	4693/31407	3.70E-14
4				GACACG	128	6361	92/276	5402/31407	3.88E-11
5		1		**CGTGTC**	128	6362	92/276	5403/31407	3.92E-11
6			1	T**ACGTG**	130	7158	97/276	6080/31407	3.28E-10
7	1			**CACGT**A	130	7161	97/276	6083/31407	3.36E-10
8			1	**ACGTG**G	159	8619	97/276	6619/31407	2.64E-08
9	1			C**CACGT**	159	8622	97/276	6621/31407	2.68E-08
10				TCTAGA	262	16682	101/276	7047/31407	3.61E-08
NAE-downregulated genes									
1				TATATA	1656	126893	227/265	20659/31407	1.45E-13
2				ATATAA	855	72817	251/265	25332/31407	2.58E-11
3				TTATAT	855	72823	251/265	25335/31407	2.64E-11
4				GTATAT	399	32733	202/265	18528/31407	1.56E-09
5				ATATAC	399	32742	202/265	18532/31407	1.59E-09
6				TGTATA	388	33249	202/265	18763/31407	6.22E-09
7				TATACA	388	33257	202/265	18764/31407	6.26E-09
8				AATAAT	809	77924	249/265	25832/31407	1.28E-08
9				ATTATT	809	77943	249/265	25834/31407	1.29E-08
10				ATATAT	1622	131275	217/265	21054/31407	2.67E-08

1 online). Moreover, compared with a well-characterized data set developed by Seki et al. (2002), many ABA-induced genes were found in our NAE upregulated gene list (see Supplemental Table 1 online). A number of genes in the NAE upregulated gene list (and ABA-seedling arrays) were annotated as ''embryo associated'' (e.g., late embryogenesis abundant genes, dehydrins, globulins, oleosins, and vicilins; Table 1; see Supplemental Table 1 online). 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, 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; see Supplemental Table 1 online). Overall these comparative 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.

The apparent overlap of a significant set of transcripts that were upregulated by both NAE12:0 and ABA was also supported by in silico examination of potential *cis*-regulatory elements in genes found in our microarray data set (Table 2). The 1-kb regions upstream of the NAE12:0 upregulated and downregulated genes were queried with the statistical motif analysis tool from The Arabidopsis Information Resource (TAIR; http://www.arabidopsis.org/tools/bulk/motiffinder/index.jsp). This search yielded multiple sequences in the upstream region of NAE12:0 upregulated 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 Pages, 1998). We found that the 1-kb upstream region in 31.5% of the NAE12:0 upregulated genes contained these ABRE motifs (Table 2; see Supplemental Table 1 online). Furthermore, a *cis*-element–based approach was recently employed to identify and experimentally validate an *Arabidopsis*-specific module as a predictor for ABA-responsive genes with the sequence ABRE and CE motifs (Zhang et al., 2005); again, the analyzed motifs were significantly well represented in the 1-kb upstream region of NAE12:0 upregulated genes (Table 2). Of the top 10 most significantly enriched motifs in the promoter regions of the NAE12:0 upregulated genes, eight were either known ABRE or CE/CE3 elements, whereas in the downregulated genes, TA-rich motifs were the most predominant (Table 2). The latter are not known to be associated with ABA-regulated genes, suggesting that additional pathways outside of ABA may operate in the NAE modulation of gene expression. Nonetheless, results from whole-genome microarray analysis clearly indicate that ABA-responsive gene expression is a major target in NAE-induced, growth-arrested seedlings.

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 RT-PCR during seed germination and seedling growth (Figure 3). Additionally, expression of ABI3 was profiled over the same time course since this gene product is known to participate in the regulation of several of the genes in the microarray data sets, 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 our comparison with published microarray data (Table 1; see Supplemental Table 1 online), 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*, and *RAB18*; data not shown). Some genes in the microarray list (see Supplemental Table 1 online) were not known to be ABA responsive, such as *EXPR3* (an expansin-related gene), and the temporal profile of *EXPR3* transcripts was altered

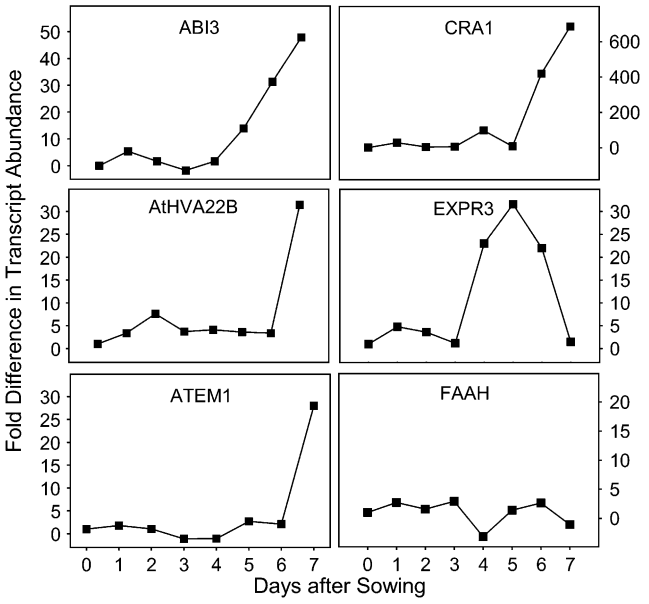

Figure 3. NAE-Modulated Changes in Transcript Abundance for Several ABA-Responsive (*ABI3*, *HVA22B*, *ATEM1*, and *CRA1*) and ABA-Nonresponsive (*EXPR3* and *FAAH*) Genes.

Transcript levels were quantified in total RNA extracts by real-time RT-PCR against 18S rRNA and plotted as fold difference (NAE treated versus untreated) using the ΔΔCT 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 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.

in NAE12:0-treated seedlings, albeit differently than the ABA-responsive transcripts. Transcript levels of FAAH, encoding an NAE hydrolase, increased during the normal course of seedling establishment (Wang et al., 2006) but were not affected by NAE12:0 (Figure 3). A comparison in the fold difference of four ABA-responsive genes induced by either ABA or NAE 12:0 after 4 and 7 d showed that transcript levels, quantified by real-time RT-PCR, generally were influenced to a similar extent by both compounds (Table 3), except that the transcripts appeared to be upregulated 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. FAAH transcripts were not modulated by either ABA or NAE (Figure 3, Table 3).

The transcription factor ABI3 is considered to be a key regulator of the seed-to-seedling transition (Nambara et al., 1994; Parcy et al., 1994; Parcy and Giraudat, 1997); therefore, we conducted additional gene expression studies with ABI3 during NAE12:0-induced growth inhibition (Figure 4). Interestingly, *ABI3* expression was associated inversely with NAE12:0-modulated growth. This relationship was demonstrated in a dramatic manner with FAAH overexpressors, which are tolerant to NAE12:0-induced growth arrest, and FAAH 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 4). Highest levels of *ABI3* transcripts were associated with the least growth (*faah* grown in NAE12:0); by contrast, the lowest ABI3 transcript levels were associated with the most robust growth (FAAH overexpressors). Furthermore, we 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 1D). 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 5). Taken together, these results suggest that similar to ABA (Lopez-Molina et al., 2002), arrest of seedling growth by NAE12:0 is mediated at least in part via *ABI3* gene expression.

Table 3. Comparison of Fold Changes in Transcript Abundance in NAE12:0- and ABA-Treated Seedlings at 4 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).

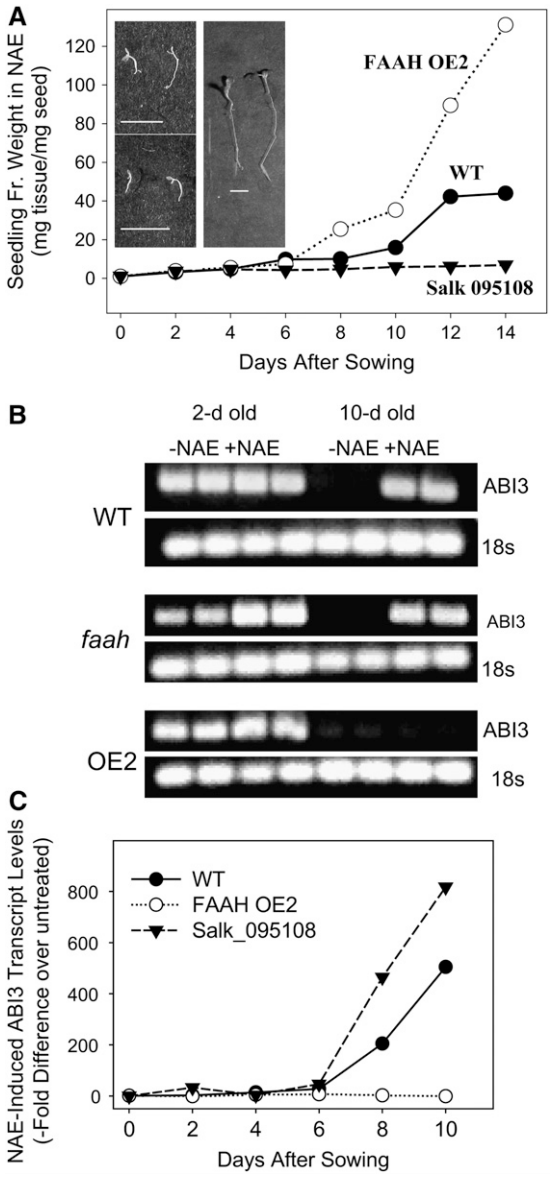

Figure 4. NAE-Induced *ABI3* Expression Is Inversely Associated with NAE-Regulated Seedling Growth.

(A) Growth of wild-type and FAAH-altered seedlings in response to exogenous NAE 12:0 (35 μM). Values for gain in fresh weight are from a representative experiment, and replicate experiments exhibited similar trends (i.e., FAAH overexpressors tolerate NAE12:0, FAAH knockouts do not, and the wild types are intermediate; Wang et al., 2006). Insets show seedlings of the wild type (top left), knockout (bottom left), or the FAAH overexpressor, OE2, grown in 35 μM NAE12:0 for 12 d. Bars = 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.

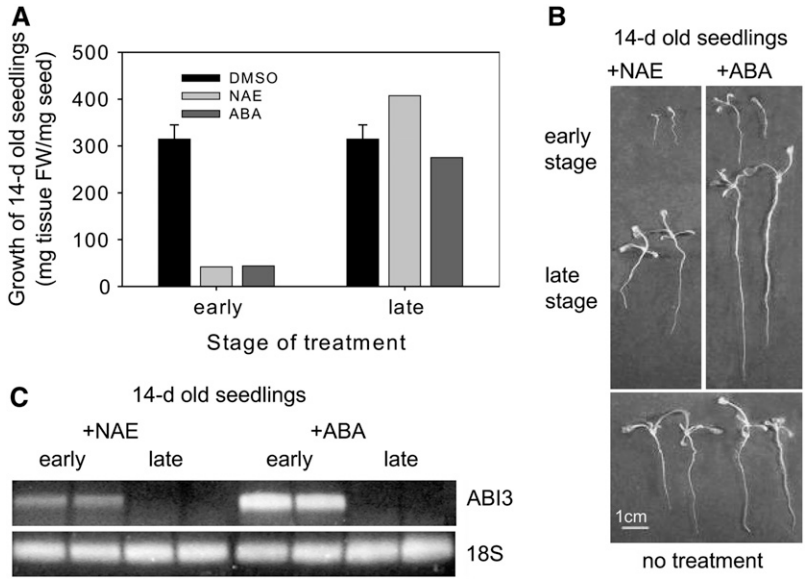

Figure 5. *ABI3* Expression Is Associated with Growth Arrest and Is Responsive to NAE 12:0 (35 μM) or ABA (0.5 μM) Treatment Only within the NAE/ABA-Sensitive Interval (up to 6 d).

(A) and **(B)** Treatment of 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 d of growth. A representative agarose gel of duplicate samples following semiquantitative RT-PCR with either *ABI3*- or 18S rRNA gene-specific primers.

ABI Mutants Display Partial Tolerance to NAE12:0

The FAAH overexpressor lines, while able to grow well in NAE12:0 (Figure 6A), were hypersensitive to ABA (Figure 6B). This was quantified by ABA inhibition of radicle emergence 3 d after imbibition (Figure 6C) or by ABA inhibition of cotyledon expansion at 7d (Figure 6D). On the other hand, ABI mutant lines (*abi1-1*, *abi2-1*, and *abi3-1*) exhibited partial tolerance to NAE, showing a pronounced shift in the dose–response curves for seedling growth quantified as cotyledon area (for *abi1-1* and *abi2-1*) or radicle length (for *abi3-1*; Figures 7A to 7C). These results suggest that seedling growth inhibition by NAE is mediated in part by an intact ABA signaling pathway.

NAE12:0 and ABA Have Synergistic Effects on Seed Germination and Seedling Growth

Perhaps the most compelling evidence for an interaction between ABA and NAE comes from experiments where *Arabidopsis* seeds were germinated in both of these compounds at the same time. Low micromolar concentrations of NAE12:0 or submicromolar concentrations of ABA added individually had only a minor impact on seedling growth; however, when applied together, these compounds had a profound inhibitory effect on seedling growth (Figures 8A to 8C), indicating a synergistic interaction between NAE12:0 and ABA. In other words, it appeared that NAE12:0 could potentiate the activity of ABA and vice versa. The synergistic inhibition of seedling growth by NAE and ABA was fully prevented in the seedlings of ABI mutants, *abi1-1* and *abi2-1*, and partially prevented in *abi3-1* and *abi5-1* (Figures 8A and 8C). Interestingly, there appears to be some stage-specific nature to the synergistic effects of ABA and NAE12:0 since *abi1-1*, *abi3-1*, and *abi5-1*, but not *abi2-1*, displayed a reversal of the delay in seed germination induced by both NAE12:0 and ABA (see Supplemental Figure 1 online). However, the prevention of the synergistic effect of ABA and NAE12:0 for *abi2-1* was observed in primary root length of older (i.e., 10-d-old) seedlings and at lower NAE12:0 concentrations (Figure 8C). NAE-tolerant seedlings (FAAH overexpressors; see Figure 6), on the other hand, which were hypersensitive to ABA alone, remained consistently hypersensitive to ABA despite the presence of NAE12:0 in the growth medium (Figures 8B and 8C). These effects on seedling growth were evident when seedlings were grown in liquid (Figures 8A and 8B) or on solid (Figure 8C) medium. Overall, these results continue to support our hypothesis that NAE action in seedlings depends, in part, upon a functional ABA signaling pathway.

NAE18:2 was the most abundant NAE species in *Arabidopsis* desiccated seeds and it also negatively affected seedling growth (Wang et al., 2006). Therefore, we tested if NAE18:2, like NAE12:0, when applied together with ABA, had a synergistic effect on seedling growth. When applied alone, NAE18:2 at 10 μM inhibited hypocotyl elongation and primary root growth but did not affect cotyledon area (see Supplemental Figure 2 online). The inhibition of primary root growth by NAE18:2 was accompanied by the extensive formation of lateral roots (see Supplemental Figure 2 online, inset). On the other hand, when ABA was applied alone (0.05 μM), there was a slight reduction in cotyledon

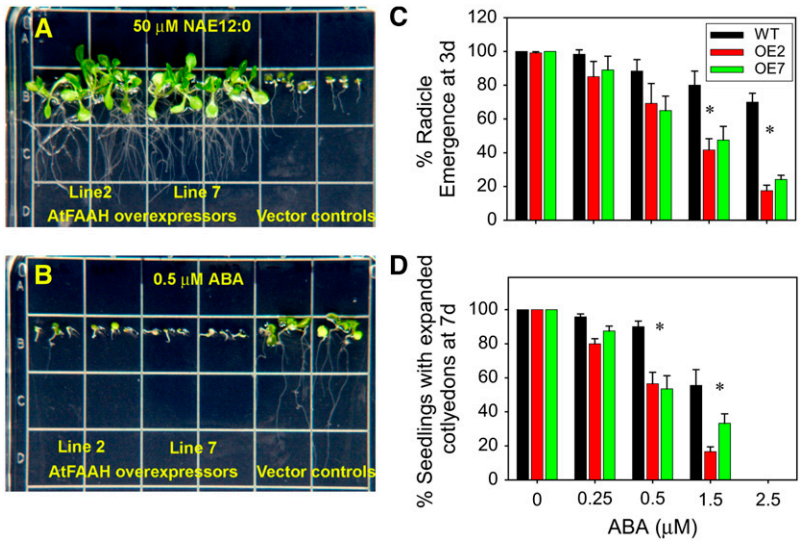

Figure 6. FAAH Overexpressors Are Insensitive to NAE but Appear to Be Hypersensitive to Exogenous ABA.

Representative seedling images of 14-d-old seedlings germinated and grown in 50 μM NAE12:0 **(A)** or 0.5 μM ABA **(B)**. Two FAAH-overexpressing lines (OE2 and OE7) are shown. Radicle emergence after 3 d **(C)** or cotyledon expansion after 7 d **(D)** also showed ABA hypersensitivity for FAAH overexpressors compared with the wild type, particularly at higher ABA concentrations. Values plotted are means and SE of six replicate experiments, each experiment consisting of 25 individuals. Asterisks indicate $P < 0.001$ versus the wild type.

area, but there was no impact on hypocotyl and root length. When NAE18:2 and ABA were applied together, cotyledon expansion and hypocotyl and root length were inhibited (see Supplemental Figure 2 online). However, unlike the combination of NAE12:0 and ABA, which caused dramatic growth inhibitory effects on seedlings (Figure 8), the combination of NAE18:2 and ABA appeared to be a result of the additive effects of each compound rather than a synergistic interaction (see Supplemental Figure 2 online). Hence, the interaction of NAE metabolism and ABA signaling in seedling development is considerably complex and may be conferred by differences in NAE type, tissue type, and developmental stage.

DISCUSSION

Exogenous NAE application inhibits *Arabidopsis* seedling growth in a profound manner (Figure 1). Severe morphological and cellular defects accompany 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 is (are) unclear. 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.

Exogenous ABA treatment of seedlings, or the application of desiccation stress, induces seedling growth arrest comparable to NAE12:0, and this growth arrest may be mediated by ABA (Lopez-Molina et al., 2001, 2002). Seedlings exhibit ABA-induced growth arrest and are desiccation tolerant only within a narrow window of early postgerminative 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 in seedlings (Figure 2) to allow the seed-to-seedling transition. The sensitivity of the tissues to either ABA or NAE changed with progression of seedling development (Figures 1 and 2). This change can be marked at the molecular level by the occurrence and elevation of ABI3 transcript levels (Figures 3 to 5). We 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 (or both) of these compounds.

Several lines of evidence argue that NAE impacts seedling growth, in part, via the activation (or delayed decay) of ABA-related processes. First, whole-genome microarray studies show that the transcript levels of a number of upregulated genes in *Arabidopsis* seedlings treated with ABA (Li et al., 2006) are also elevated in NAE12:0-treated seedlings. In addition, genes that were enriched in desiccated *Arabidopsis* seeds but whose expression is typically depleted within hours of seed imbibition remained elevated in NAE12:0-treated seedlings (Table 1; see Supplemental Table 1 online; Nakabayashi et al., 2005). Many of the genes upregulated by both NAE12:0 and ABA treatment encoded for proteins involved in desiccation tolerance (e.g., dehydrins), seed storage reserves, and late embryogenesis abundant proteins (Table 1). The elevated transcript levels of genes enriched in desiccated seeds correlates well with the higher endogenous levels of NAE and ABA (Figure 1A, Table 1; Nakabayashi et al., 2005). The involvement of ABA in the NAE

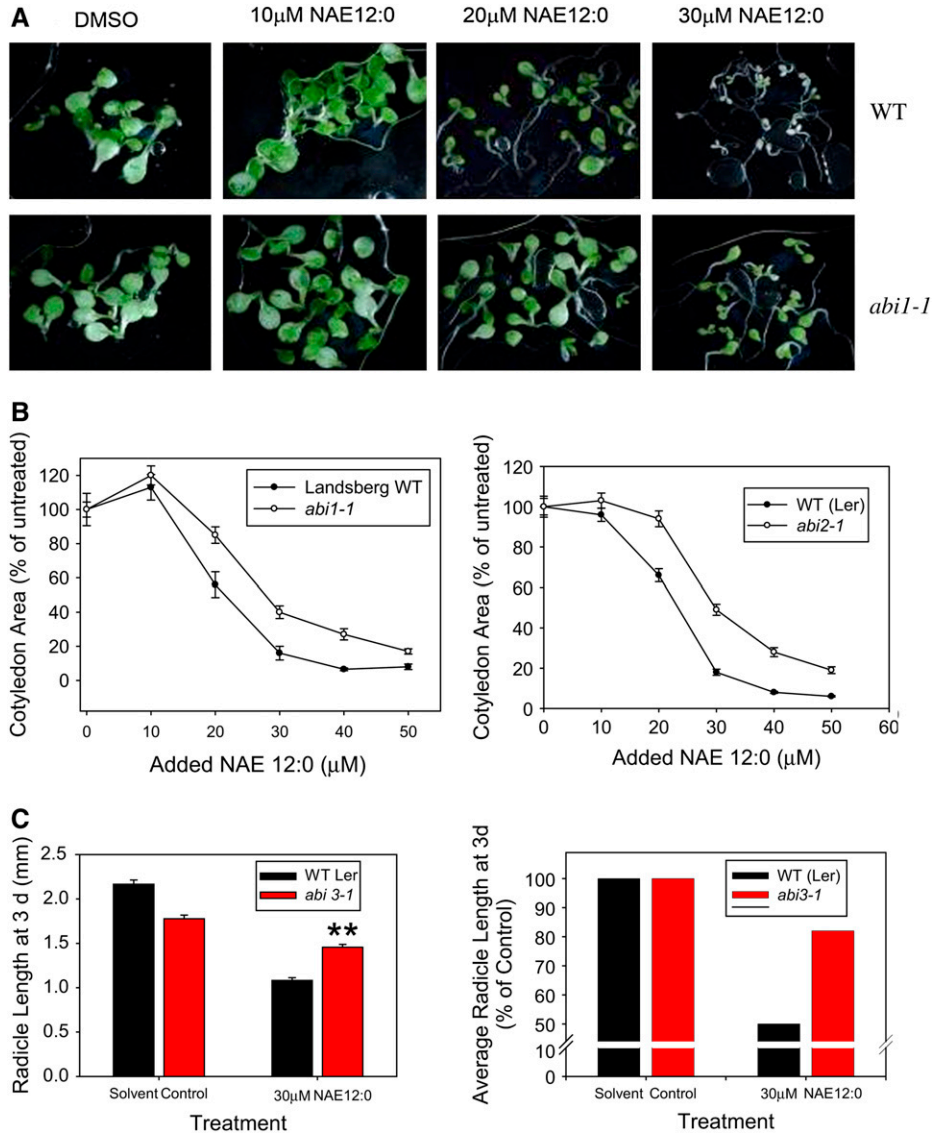

Figure 7. ABI Mutants Exhibit Increased Tolerance toward Exogenous NAE12:0.

(A) Representative images of 6-d-old seedlings at increasing concentrations of NAE12:0 show that *abi* mutants (e.g., *abi1-1*) are larger than the wild type (Landsberg *erecta*).

(B) Cotyledon area, quantified for 45 to 65 seedlings and plotted as a percentage of untreated controls, revealed a shift in the dose–response curve for NAE12:0-induced growth inhibition for *abi1-1* and *abi2-1* relative to the wild type. Both *abi1-1* and *abi2-1* were significantly more tolerant to NAE than the wild type (e.g., $P < 0.0001$ at 20, 30, and 40 μM).

(C) Quantification of root length of 3-d-old seedlings showed that *abi3-1* was significantly more tolerant (**; $P < 0.0000001$) to NAE 12:0 than the wild type plotted as either absolute length (left) or as relative length to untreated controls (right). $n = \sim$300 to 400 (3-d-old seedlings).

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 (Table 2). Thus, from our microarray work, the elevated levels of NAE and ABA delay the normal development of seedlings by maintaining the expression, or resumption of expression, of genes that are normally associated with the ungerminated, desiccation-tolerant state. For seed germination and cell expansion associated with postgerminative seedling growth to occur normally, the expression of these embryo-associated genes has to be downregulated, and this occurs in parallel with the normal depletion of both NAE and ABA.

A second set of experimental results supporting our hypothesis that NAE mediates its inhibitory effect on early seedling development via ABA signaling comes from our analysis of *ABI3* expression. ABI3 is considered to be a key transcriptional

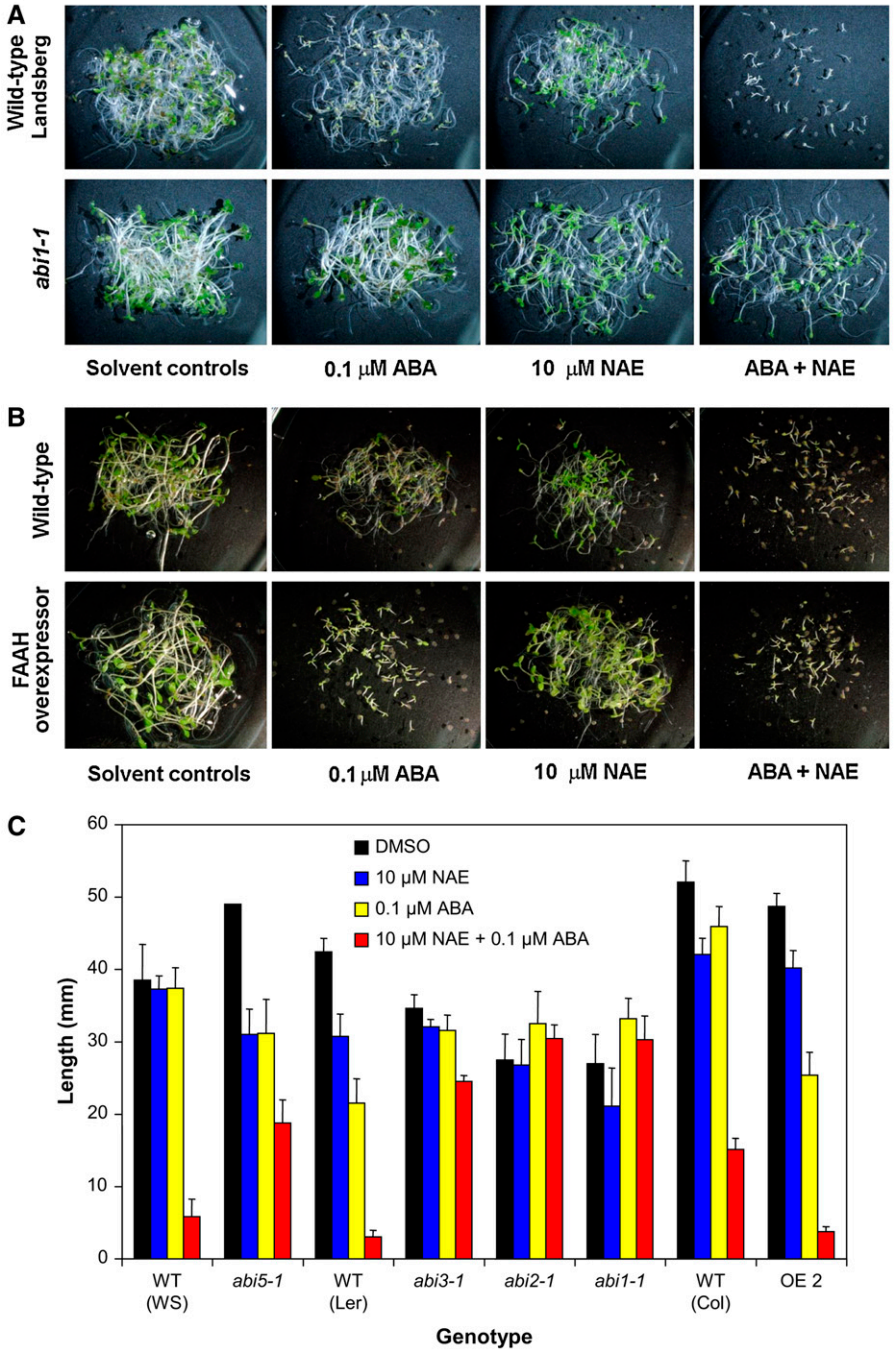

Figure 8. ABA and NAE12:0 Have Synergistic Negative Effects on Seedling Growth in Liquid Media, but This Synergism Is Prevented in *abi* Mutants.

(A) Low levels of NAE12:0 (10 μM) potentiate the inhibitory effects of low levels of ABA (0.1 μM) (both Landsberg *erecta* and Columbia ecotypes, first and third series of images). *abi1-1* is insensitive to the synergistic inhibition.

(B) NAE-tolerant seedlings (FAAH overexpressor) are hypersensitive to ABA, and this hypersentivitiy is not rescued by exogenous NAE12:0.

(C) Quantification of the synergism between NAE12:0 and ABA by measuring primary root lengths of 10-d-old seedlings grown on solid media (bottom histogram). ABA-insensitive mutants (*abi1-1*, *abi2-1*, *abi3-1*, and *abi5-1*) did not exhibit the synergistic inhibition of root growth, indicating that NAE action requires an intact ABA-signaling pathway.

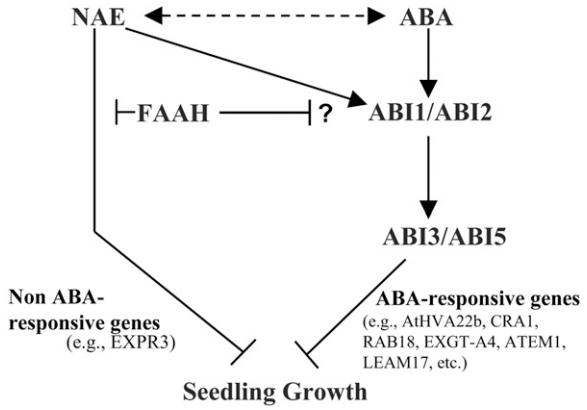

Figure 9. Diagram Showing the Hypothetical Action of Elevated Levels of NAE on Seedling Growth and Its Relationship to ABA Action.

NAE arrests seedling growth via a pathway dependent upon *ABI1*, *ABI2*, *ABI3*, and *ABI5*, and this is associated with upregulation of ABA-responsive genes, such as At *HVA22b*, *CRA1*, *RAB18*, *EXGT-A4*, *ATEM1*, and *LEAM17*. This suppression of seedling growth is reminiscent of the ABA-induced secondary dormancy program discovered by Chua and coworkers (Lopez-Molina et al., 2002), which is operable in young seedlings. We 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 metabolism. Independent of ABA signaling, NAE12:0 can influence gene expression in seeds and seedlings and suppress seedling growth. Ectopic overexpression of FAAH can reverse the growth suppression by NAE, but the interaction with ABA signaling in FAAH overexpressors is complex since FAAH overexpressors are hypersensitive to ABA. By contrast, the situation with *abi* mutants is more straightforward because they exhibited partial tolerance to NAE12:0 in addition to their ABA-tolerant phenotypes.

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 NAE12:0 resulted in higher steady state levels of *ABI3* transcripts, which was particularly evident in older seedlings (e.g., 7 d old; Figure 3, Table 1) with an ~50-fold greater abundance of *ABI3* transcripts compared with untreated seedlings. The inverse relationship between *ABI3* transcript levels occurred in the same time frame for NAE12:0- and ABA-modulated growth (Figures 4 and 5).

ABA levels remained essentially unchanged in 8-d-old, NAE12:0-arrested seedlings (cf. with untreated seedlings in Figure 2), suggesting that the elevation of ABA-responsive transcripts (see Supplemental Table 1 online; Figure 3, Table 1) was more likely a direct result of NAE12:0-induced *ABI3* transcript levels. The higher levels of *ABI3* transcripts, particularly in 7-d-old seedlings, likely led to increased expression of other ABA-responsive genes, such as *ATEM1*, At *HVA22B*, *CRA1* (Figure 3), and several others (e.g., *RD22* and *RD29B*, Table 1; see Supplemental Table 1 online). A common feature of these ABA-responsive genes is the occurrence of ABRE elements upstream of the genes that confer ABA/ABI3-dependent expression (reviewed in Finkelstein et al., 2002). Although it remains to be examined whether NAE acts through these ABRE regulatory elements, the observation that a number of NAE12:0 upregulated genes contain ABA-responsive motifs in their promoter region (Table 2) is a strong indication 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.

Finally, the synergistic inhibitory effect of ABA and NAE12:0 on seedling growth when seedlings were subjected to concentrations of NAE12:0 and ABA that individually had little or no effect further reinforced the notion that the two metabolites interact in regulating early seedling growth (Figure 8). This effect was prevented in *abi1-1*, *abi3-1*, and *abi5-1* mutants, while in *abi2-1* mutants, the effect was only observed in older seedlings. Moreover, *abi1-1*, *abi2-1*, and *abi3-1* mutant lines were partially tolerant toward the growth inhibitory effects of exogenous NAE12:0 (Figure 7). The synergistic effect of NAE12:0 and ABA on seedling growth indicates that each metabolite may potentiate each other's effects during seed germination and early seedling establishment (Figure 8). This is further suggested indirectly by the similar time course of ABA and NAE depletion during germination and the similar developmental window wherein both compounds exert their growth inhibiting properties on seedling growth (Figure 1). However, our data indicate that a more complex level of interaction between ABA and NAE might operate in regulating seedling development. Because FAAH overexpressors, which have lower endogenous NAE levels (Wang et al., 2006), were hypersensitive to ABA, the effect of exogenous NAE might differ from that of endogenous NAE (Figures 6, 8B, and 8C). It is possible that manipulation of endogenous NAE levels leads to other yet to be determined changes in seed/seedling physiology, including increased flux through this pathway, that render FAAH overexpressors hypersensitive to ABA. For instance, other metabolites that impact ABA sensitivity, such as PA, might be altered in FAAH overexpressors. It was shown recently that increased PA levels in *Arabidopsis* lipid-phosphate phosphatase knockouts led to enhanced ABA sensitivity in germination tests (Katagiri et al., 2005). Since NAE12:0 has been shown previously to inhibit PLD-α activity (Austin-Brown and Chapman, 2002), it is possible that PA, which is a downstream product of PLD signaling, is elevated in FAAH overexpressors. The lower levels of endogenous NAEs in FAAH overexpressors could lead to elevated PLD-α activity and result in increased PA production and ABA hypersensitivity. It is also worth noting that another yet to be characterized amidase that is closely related to FAAH was highly expressed in seeds of the *ABA-hypersensitive germination1* mutant, which is disrupted in a protein phosphatase 2C (Nishimura et al., 2007).

Another level of complexity in regard to the interaction between ABA and NAE is the observation that NAE18:2, the most abundant NAE species in seeds, did not act synergistically with ABA. The inhibitory effect of NAE18:2 and ABA on seedlings when applied together was additive rather than synergistic (see Supplemental Figure 2 online). This indicates that different species of NAE could differentially impact the ABA signaling pathway. NAE12:0 and other short-chain saturated NAEs might be

more important in mediating ABA effects in early seedling development compared with NAE18:2 and other long-chain unsaturated NAEs. In this regard, it is worth mentioning that FAAH overexpressors are less tolerant to NAE18:2 compared with NAE12:0 (Wang et al., 2006). It is possible that the breakdown products of NAE18:2 resulting from the overexpression of FAAH (e.g., 18:2 free fatty acid) may be a more potent partner of ABA in negatively regulating seedling growth. Detailed studies on how different NAE types and their corresponding free fatty acid derivatives impact ABA responses in seeds and seedlings will be necessary to gain a complete understanding of NAE–ABA interaction in plants. For the future, quantification of metabolites in seeds and seedlings of NAE-treated and FAAH-altered plants should allow us to determine why ABA sensitivity is heightened by either elevated exogenous NAE or lower endogenous NAE.

The biochemical and genetic evidence suggests that NAE and ABA interact to arrest early seedling development, and Figure 9 provides a summary developed from the results presented here. During the normal course of germination and seedling growth, levels of these metabolites drop, and the tissue sensitivity to these regulators declines in a commensurate manner (Figure 1). However, if the levels of either ABA or NAE are elevated in early seedling development, there is an arrest in seedling growth (Figure 1). NAE-arrested growth is not mediated by the coincident elevation of ABA levels and vice versa, although the relative proportion of NAE types was altered considerably in ABA-treated seedlings (Figure 2). Nonetheless, NAE-arrested growth involves a functional ABA signaling pathway, including ABI1, ABI2, ABI3, and ABI5 (Figures 7 and 8; see Supplemental Figure 1 online), and is associated with increased transcript levels of well-characterized ABA-responsive genes (Figure 3, Tables 1 to 3; see Supplemental Table 1 online). On the other hand, expression of non-ABA-responsive genes was modulated by NAE as well (see Supplemental Table 1 online; Figure 3), and at higher concentrations of NAE, seedling growth is likely to be arrested by mechanisms outside the ABA signaling pathway. Hence, we postulate two parallel pathways of NAE-induced seedling growth arrest: one that is ABA dependent and another that is ABA independent. Because overexpression of FAAH leads to tolerance of seedling development to NAE12:0, FAAH might be considered a suppressor of seedling growth arrest. Some of this tolerance to NAE12:0 in FAAH-overexpressing seedlings likely results from lowered levels of *ABI3* expression (Figure 4 and 5) since a reduction in this transcription factor is important in the seed-to-seedling transition. However, *ABI3*-independent mechanisms cannot be ruled out. Future plans include the production of double mutants and in overexpressing FAAH in the *abi3-1* mutant backgrounds. Coupled with the quantification of ABA and NAE levels and the association with relevant growth phenotypes, a more detailed and definitive understanding of the link between NAE metabolism and ABA signaling in plants will likely be revealed.

NAE appears to act downstream from steady state ABA levels (Figure 2) and possibly upstream from ABI1 (Figures 7 to 9). ABI1 activity is inhibited by the lipid mediator PA, a product of PLD activity (Zhang et al., 2004). A complex scheme for the G protein regulation of PLD activity in guard cells (in response to ABA) was proposed recently (Mishra et al., 2006), and several of these same G protein subunits appear to function in ABA-mediated regulation of seedling development (Chen et al., 2006; Pandey et al., 2006). In particular, the G protein α-subunit (GPA1) appears to have opposite responses to ABA in seedlings and guard cells (Assmann, 2005). Interestingly, this is precisely the case with NAEs. NAEs were shown to inhibit PLD-α1 activity in vitro and to block ABA-induced stomatal closure in vivo (Austin-Brown and Chapman, 2002); here, NAEs arrest seedling growth via the activation of the ABA signaling pathway. Although it is only speculation at this point, NAEs may influence the interaction of GPA1 with PLD-α1 or the newly discovered G protein–coupled receptor for ABA (GCR2; Liu et al., 2007), both of which act upstream from ABI1 and downstream from ABA. In any case, we conclude that NAE metabolism regulates seedling growth through modulation of the ABA signaling pathway, in part by the transcriptional activation of *ABI3* and ABA-responsive gene products. Future studies will be aimed at delineating the precise target of NAE action, whether by binding to a NAE receptor, the regulation of PLD-α1 and PA levels, or through a yet to be discovered mechanism.

METHODS

Plant Materials and Growth Assays

The ABI mutants *abi1-1*, *abi2-1*, *abi3-1*, and *abi5-1*, corresponding ecotypes, and *faah* T-DNA insertion mutant (Salk 095108) were obtained from the ABRC. Plants were propagated in soil for seed production. For germination and growth experiments, seeds were first surface-sterilized with 95% ethanol followed by 33% bleach for 3 min each and rinsed several times with sterile, deionized water. Seeds were stratified for 1 to 3 d at 4°C in the dark and grown in liquid or solid nutrient media (0.5× Murashige and Skoog salts containing 1% sucrose) as described previously (Wang et al., 2006). Germination and growth proceeded in a controlled environment room with a 16-h-light/8-h-dark cycle (60 $\mu mol \cdot m^{-2} \cdot s^{-1}$) at 20 to 22°C. Seedlings grown on plates were oriented ~60° from vertical to facilitate reproducible measurements of root elongation, whereas liquid cultured seedlings were incubated with shaking (75 rpm) for overall measurements of 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. Growth was quantified as fresh weight and/or seedling size (cotyledon area, hypocotyl length, and primary root length) as previously described (Wang et al., 2006).

Gene Chip Microarray Experiments and Data Analysis

Total RNA was isolated from seed and seedling samples according to Dunn et al. (1988) or Vicient and Delseny (1999). RNA was quantified and evaluated for purity by UV spectroscopy and agarose gel electrophoresis (Krieg, 1996). Prior to microarray analysis, the integrity of the RNA was also checked on an Agilent Bioanalyzer 2100. Ten micrograms of total RNA was used as template for amplification.

Probe labeling, chip hybridization, and scanning were performed according to the manufacturer's instructions (Affymetrix). Three biological replicates per treatment were hybridized independently to the Affymetrix GeneChip(r) ATH1 Genome Array representing 24,000 *Arabidopsis* genes.

For each sample, the CEL file was exported from the Genechip Operating System program (Affymetrix). All six CEL files were imported into robust multichip average and normalized as described by Irizarry

et al. (2003). The presence/absence call for each probe set was obtained from dCHIP (Li and Wong, 2001). Differentially expressed genes in NAE12:0-treated seedlings were selected using associative analysis as described by Dozmorov and Centola (2003). Type I family-wise error rate was reduced using a Bonferroni-corrected P value threshold of 0.05/N, where N represents number of probe sets present on the chip. The gene selections were further confirmed by significance analysis of microarrays (Tusher et al., 2001). The FDR was monitored and controlled by calculating the Q value (FDR) using extraction of differential gene expression (http://www.biostat.washington.edu/software/jstorey/edge/) (Storey and Tibshirani, 2003; Leek et al., 2006). Genes that showed the most difference in transcript levels (more than twofold or greater, Q value < 0.1 and P value < 2.19298E-6) between NAE 12:0-treated and DMSO control samples were selected for further consideration. For promoter analysis, the up- and downregulated genes were analyzed using the Motif Analysis on the TAIR website (http://www.arabidopsis.org/tools/bulk/motiffinder/index.jsp), and the top 10 most significant motifs were presented.

Quantitative RT-PCR

Verification of microarray results and quantification of transcripts by quantitative RT-PCR were performed with a Smart Cycler II (Cepheid) instrument using a real-time one-step assay system (Takara Bio) with SYBR Green I. The following gene-specific primer pairs were used: *ABI3* (At3g24650) (F) 5′-GAGCTGGCTCAGCTTCTGCTATG-3′ and (R) 5′-AGGCCAAAACCTGTAGCGCATGTTC-3′; *ATEM1* (At3g51810) (F) 5′-CTGAAGGAAGAAGCAAGGGAG-3′ and (R) 5′- TCCATCGTACTGAGTCCTCCTTTAC-3′; *EXPR3* (At2g18660) (F) 5′-CCTACACTAGGTCTGCGTG-3′ and (R) 5′-GATAACCCGAAAAGCGT-3′; *EXGT*-A4 (At5g13870) (F) 5′-CTCTGCCTCACGTTTCTGATTTTGG-3′ and (R) 5′-GAAAGCCAGTGCCAGTGTACTTGTC-3′; *CRA1* (At5g44120) (F) 5′-CACCATTGCGTTTTGACGGAAGATC-3′ and (R) 5′-GATGACAACCGTGGAAACATTGTCC-3′; At *HVA22B* (At5g62490) (F) 5′-CATCGCTGGACCTGCATTA C-3′ and (R) 5′-GGATATAATGGGATCCATTCGAGG-3′; *FAAH* (At5g64440) (F) 5′-CCATCTCAAGAACCGGAGCATG-3′ and (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 of total RNA and 0.5 μL of 10 μM gene-specific primers in each 25 μL reaction. The reaction mix was subjected to the following RT-PCR conditions: 42°C for 15 min, one cycle; 95°C for 2 min, one cycle; 94°C for 10 s, 58°C for 25 s (read cycle), 72°C for 20 s. The number of cycles and annealing temperature were experimentally determined for each set of gene-specific primers. RT-PCR products were examined by gel electrophoresis and by melting curve analysis (60 to 95°C at 0.2°C/s) to rule out anomalous amplification products. The $2^{-\Delta\Delta CT}$ cycle threshold (C_T) method was used to calculate relative changes in transcript levels determined from quantitative real-time RT-PCR (Livak and Schmittgen, 2001). The data were analyzed using the equation 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 ~50 to 250 mg of 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 (derivatized in BSTFA; Sigma-Aldrich) by gas chromatography–mass spectrometry (Venables et al., 2005).

For ABA analysis, ~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°C to ~1 to 2 mL, removing the 2-propanol. Samples were stored at −20°C until fractionation by HPLC and quantification by gas chromatography–mass spectrometry as described previously (Wang et al., 2001).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Germination in *Arabidopsis* (L*er* and Ws Ecotypes) Treated with ABA and NAE12:0.

Supplemental Figure 2. Effects of NAE18:2 and ABA on *Arabidopsis* Seedling Growth.

Supplemental Table 1. Differentially Expressed Genes That Responded to 35 μM NAE12:0 Treatment in 4-d-Old *Arabidopsis* Seedlings.

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