ALTERATIONS IN FATTY ACID AMIDE HYDROLASE (FAAH) TRANSCRIPT LEVELS AND ACTIVITY LEAD TO CHANGES IN THE ABIOTIC STRESS SUSCEPTIBILITY OF *Arabidopsis thaliana*

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N-Acylethanolamines (NAEs) are a class of bioactive lipids, and FAAH is one of the enzymes responsible for degrading NAEs in both plants and animals. In plants, FAAH appears to be closely associated with ABA, a phytohormone which has long been associated with plant stress responses, since the overexpression of FAAH in *Arabidopsis* results in ABA hypersensitivity. Therefore, it is reasonable to speculate that alterations in FAAH transcript levels will result in altered stress responses in plants. To investigate this hypothesis experiments were carried out in which wild type (WT), FAAH-overexpressing (OE), and T-DNA insertional FAAH knockouts of *Arabidopsis* (*faah*) were grown in MS media under stress conditions. The stress conditions tested included chilling stress, heavy metal stress induced by cadmium or copper, nutrient limitations induced by low phosphorus or low nitrogen, salt stress induced with NaCl, and osmotic stress induced with mannitol. The OE plants were consistently hypersensitive to all stress conditions in relation to wild type plants. Inactive FAAH overexpressors did not have the hypersensitivity to the salt and osmotic stress of the active OE plants and were instead tolerant to these stresses. FAAH2 (*faah2*) knockouts and FAAH 1 and 2 double knockouts (*faah*

1+*2*) were based on some root development parameters somewhat more tolerant than WT plants, but more sensitive in terms of shoot growth. Collectively the data suggests that FAAH activity may interact with stress-responsive pathways in plants, perhaps including pathways involving ABA.

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ABA .....Abscisic acid

ABI3..... Abscisic acid insensitive 3

AtHVA22b.....*Arabidopsis thalina Hordeum vulgare* A22b homolog

AS.....Amidase signature

BSTFA..... N,O-bis(trimethylsilyl)trifluoroacetamide

CMV .....Cauliflower mosaic virus

COS.....CV-1 (simian) in Origin, and carrying SV40 genetic material

COX..... Cyclooxygenase

DMSO..... Dimethyl sulfoxide

FAAH.....Fatty acid amide hydrolase

FAAHm..... Inactive FAAH

faah ..... FAAH 1 knockout

faah(2) ..... FAAH 2 knockout

faah(1+2) ..... FAAH 1 and FAAH 2 double knockout

GABA .....Gamma amino butyric acid

GC-MS .....Gas chromatography – mass spectrometry

IAA.....Indole acetic acid

JA .....Jasmonic acid

LEA..... Late embryogenesis abundant

LOX ..... Lipoxygenase

MS ..... Murashigee and Skoog

NAAA..... *N*-acylethanolamine-hydrolyzing acid amidase

NA ..... *N*-acylethanolamine

NAE 12:0 ..... *N*-lauroylethanolamine

NAE 14:0 ..... *N*-myristoylethanolamine

NAE 16:0 ..... *N*-palmitoylethanolamine

NAE 18:0 ..... *N*-stearoylethanolamine

NAE 18:1 ..... *N*-oleoylethanolamine

NAE 18:2 ..... *N*-linoleoylethanolamine

NAE 18:3 ..... *N*-linolenoylethanolamine

NAPE..... *N*-Acylphosphatidylethanolamine

ND ..... Not detectable

OE ..... FAAH-overexpressor

PLD ..... Phopholipase D

RD29b ..... Response to dehydration 29b

RT-PCR..... Real time – polymerase chain reaction

SA..... Salicylic acid

SK..... Inactive FAAH overexpressors in *faah* background

SPE ..... Solid phase extraction

SW..... Inactive FAAH overexpressors in WT background

SOS..... Salt overly sensitive

SYBR..... Synergy brands

TMS.....Trimethylsilyl

WT.....Wild type

CHAPTER 1

INTRODUCTION

N-Acylethanolamines (NAEs) are lipid components of plant and animal cell membranes and were first reported as constituents of soy lecithin and peanut meal (Kuehl et al., 1957). Despite their initial discovery in plant materials, their function in plants is not as well understood as it is in animals. In vertebrates, these lipids have been shown to participate in the endocannabinoid signaling system (Schmid and Berdyshev, 2000) which regulates various processes including neurotransmission (Wilson and Nicoll, 2002; Di Marzo, 2011) and immune responses (Berdyshev et al., 2000; Stella 2009), and embryo development (Paria and Dey, 2000). NAEs have also been associated with abiotic stress responses in animal cells including ischemia (Berger et al., 2004), exposure to heavy metals (Kondo et al., 1998), exposure to UV light, and nutrient deprivation (Berdyshev et al., 2000). Despite a lesser degree of understanding as to how these lipids function in plants, NAEs have been implicated in various processes in plants including germination (Shrestha et al., 2002), seedling development (Blancaflor et al., 2003; Teaster et al., 2007), and responses to pathogens (Tripathy et al., 1999; Tripathy et al., 2003; Kang et al., 2008). However, the function of NAEs in abiotic stress responses has received little attention.

N-Acylphosphatidylethanolamines (NAPEs), a minor phospholipid component in plant and animal cells, function as precursors for NAEs. In mammalian systems NAE formation occurs by the hydrolysis of NAPEs by a Ca^{2+}-dependent phospholipase D (PLD) (Schmid et al., 2002). The idea that NAEs are derived from NAPEs is supported by experiments with this mammalian PLD enzyme, which has been cloned and shown to have activity toward NAPEs but no other membrane phospholipids (Okamoto et al., 2005). The overexpression of this NAPE-PLD in COS-7 cells resulted in decreased NAPE levels and a corresponding increase in NAEs (Okamoto et al., 2005). Plant NAPEs also appear to be the precursors for NAE formation. The notion that NAPEs are precursors to NAEs in plants is supported by radiolabeling studies which indicate that NAEs produced by plants are a reflection of the *N*-acyl composition of their NAPE species (Chapman, 2000). However, an NAPE-selective PLD has yet to be identified in plants. In *Arabidopsis*, there are five PLD isoforms which are encoded by 12 genes (Wang, 2004). Two of these isoforms, PLDβ and PLDγ, may be involved in NAE formation in plants, since they have been shown to be capable of NAPE hydrolysis *in vitro*. Unlike the mammal NAPE-PLD, PLDβ and PLDγ in *Arabidopsis* were capable of hydrolyzing multiple phospholipid substrates. Therefore, the identification of a plant NAPE-PLD is still somewhat uncertain due to the presence of multiple enzymes and substrates.

NAEs have important signaling functions in animals. NAE 20:4, also known as anandamide, plays a key role in mammalian endocannabinoid signaling, and it is believed that the signaling function of this molecule is terminated upon hydrolysis into arachidonic acid and ethanolamine (De Fonseca et al., 2005; Farrel and Merkler 2008). This hydrolysis is mediated by the enzyme fatty acid amide hydrolase (FAAH). Cloning and characterization of rat FAAH (Cravatt et al., 1996) led to the identification of FAAH homologs in other mammalian species including mouse and human (Giang and Cravatt, 1997). Mammalian FAAH enzymes belong to a larger family of proteins containing a conserved amidase signature sequence (AS). However, unlike other proteins in the AS family, FAAH contains an *N*-terminal transmembrane domain which was predicted to function as a membrane localization sequence (McKinney and Cravatt, 2005). However, a truncated variant of the enzyme which lacks the first 30 residues at the N-terminus retains its association with the membrane (Bracey et al., 2002). Instead, interaction with the membrane is believed to occur though a hydrophobic “foot” in the protein represented by α-helix18 and α-helix19 in the rat enzyme (Bracey et al., 2002).

More recently, a different type of NAE-hydrolyzing enzyme was identified and named *N*-acylethanolamine-hydrolyzing acid amidase (NAAA) (Tsuboi et al., 2005). This enzyme, which is capable of hydrolyzing NAEs under acidic conditions, bears no homology to FAAH based on its primary structure. Instead this enzyme belongs to the choloylglycine hydrolase family and it shows

structural and functional similarity to acid ceramidase, which is itself capable of hydrolyzing *N*-lauroylethanolamine (Tsuboi et al., 2005).

The process of NAE inactivation appears to be functionally conserved between plants and animals. A FAAH homolog was recently identified in *Arabidopsis* based on the presence of an AS domain and conservation of catalytic residues. Despite a low amino acid sequence identity of 18.5% between the mammalian and the *Arabidopsis* FAAH over their entire length, the AS domain in the *Arabidopsis* FAAH was 60% identical to the AS domain of the mammalian FAAH (Shrestha et al., 2003). Also, like the mammalian FAAH, the *Arabidopsis* homolog contained a putative transmembrane domain at the N-terminus (Shrestha et al., 2003). However, FAAH from rice and *Medicago* were not predicted to have this transmembrane domain. *Arabidopsis* FAAH is capable of hydrolyzing a wide variety of NAEs including anandamide, which occurs in mammalian tissues but not in plants (Shrestha et al., 2003). An additional similarity between the *Arabidopsis* and the mammalian FAAH is that both are inhibited by methylarchidonyl fluorophosphonate (Shresta et al., 2006), an active-site directed FAAH inhibitor (Bracy et al., 2002).

Polyunsaturated NAEs may also be oxidized by lipoxygenase (LOX) in both plant and animal systems producing a variety of oxylipin metabolites. Oxylipins are the oxidation products from fatty acid metabolism which can function as signaling molecules. In mammalian systems, polyunsaturated NAEs can be oxidized into numerous types of signaling molecules including

leukotrienes, eicosanoid ethanolamides, and prostaglandins (De Petrocellis et al., 2004). Polyunsaturated NAEs in plants can also be oxidized by LOX or by cyclooxygenase (COX) enzymes into oxylipins (van der Stelt et al., 2000; Shrestha et al, 2002; Keereetaweep et al., 2010; and Kilaru et al., 2011), providing a possible mechanism by which NAEs may exert at least some of their effects in plants. For example, jasmonic acid (JA) and its methyl esters are an important class of oxylipins that function as signaling molecules during different stages of plant development (Turner et al., 2002). However, there is still not much information available on the roles of NAE oxylipins in plants, and additional work is needed to better understand their function in all eukaryotic organisms.

The involvement of NAEs in plant responses to biotic stress was first observed in tobacco suspensions cultures in response to fungal elicitors. One of these studies revealed that the fungal elicitor, xylanase, stimulated tobacco cell suspension cultures to release NAE 12:0 and NAE 14:0 into the culture medium (Chapman et al., 1998). Radiolabelling confirmed the origin of the released NAEs to be from NAPEs in the cell membranes. The release of NAEs was followed by a round of NAPE synthesis, perhaps to replace the NAPEs consumed in the production of the released NAEs. The NAEs only accumulated extracellularly, possibly indicating a signaling function for these molecules.

The short term alkalinization of the media by plant cell suspension cultures in response to various pathogen elicitors was first described in tomato cells (Felix et al., 1993). Despite the fact that the biological significance of this alkalinization

response remains unknown, it serves as a convenient marker for the perception of signal compounds. This alkalinization response is exhibited by tobacco cell suspension cultures when treated with xylanase (Baier et al., 1999). NAE14:0 does not affect the alkalinization response when added alone, but when added in combination with xylanase, the alkalinization response is attenuated in a concentration dependent manner. This inhibition occurred when xylanase was combined with most other NAEs, including the mammalian NAE anandamide (Tripathy et al., 1999). NAE 14:0 also inhibited the alkalinization induced by other pathogen elicitors in a time and concentration dependent manner (Tripathy et al., 1999).

A recent study suggests a link between NAE metabolism and plant immunity (Kang et al, 2008) but in a different manner. FAAH-overexpressing (OE) plants were shown to have an enhanced susceptibility to several pathogens along with a decrease in the transcript levels of numerous defense-related genes. This same study also revealed a connection among NAEs and the phytohormones salicylic acid (SA) and abscisic acid (ABA). It was determined that FAAH-overexpressing plants had lower levels of salicylic acid and ABA as well as lower transcripts levels for genes involved in SA biosynthesis and perception as compared to WT plants. These alterations in the OE plants may in part account for their enhanced susceptibility to pathogens, which supports a possible involvement of NAEs in plant defense responses.

There is evidence which strongly suggests the possible interaction of NAEs with ABA during germination and early seedling development in *Arabidopsis* (Teaster et al., 2007). The levels of both NAEs and ABA were shown to decline during germination. Also, ABA and NAE12:0 had an inhibitory effect on seedling growth within a similar developmental window. A result which more directly shows the possible interaction of NAEs with ABA is that a synergistic inhibition in seedling growth occurred when seedlings are grown in the combined presence of both ABA and NAE 12:0. The resulting inhibition was more severe than that caused by either ABA or NAE 12:0 alone (Teaster et al., 2007 and Cotter et al, 2011). Additional evidence for a possible interaction between ABA and NAEs comes from microarray studies of seedlings grown in the presence of NAE 12:0 which showed elevated transcript levels for several ABA-responsive genes (Teaster et al., 2007). Also, plants over-expressing FAAH, while tolerant to the inhibitory effects of NAE were hypersensitive to the growth-inhibiting effects of ABA (Teaster et al., 2007). The apparent connection between NAEs and ABA, a well characterized stress phytohormone, suggests the possible involvement of NAEs in plant stress responses. However, it is possible that NAEs also may carry out their functions through ABA-independent paths either alone or through their interactions with other phytohormones (Cotter et al., 2011).

There are data which suggest a possible link between NAEs and the phytohormone auxin. The microarray studies of NAE 12:0-treated seedlings not

only revealed changes in the transcript levels of numerous ABA-related genes, but also changes in the transcript levels in a considerable number of genes associated with auxin metabolism (Teaster et al., 2007). Interestingly, these genes are involved at various points along the auxin signaling pathway including synthesis, possible deactivation through glycosylation, and perception. Another possible connection between NAEs and auxin is through a group of proteins known as expansins. Expansins are proteins which are capable of loosening the cell wall and thus allowing plant growth through cell expansion, and auxin has been shown to increase the transcript levels of these genes (Cho and Cosgrove, 2004). Microarray data showed that NAE12:0 treatment altered the transcript levels of nine different expansins and expansin-like proteins, resulting in down regulation for most of them (Teaster et al., 2007). These data suggest that NAE may act antagonistically to auxin in the regulation of expansin gene expression. Indeed cell expansion was dramatically affected by either exogenous application of NAEs (Blancaflor, 2003; Motes et al., 2005) or up-regulation of endogenous NAE catabolism (Wang, 2006).

The apparent connection between NAEs and phytohormones, particularly ABA, suggests that NAEs may play a role in plant responses to abiotic stresses, such as osmotic stress resulting from drought or high salinity. Both drought and salt stress result in reduced water availability, and ABA plays an important role in the adaptive responses of plants to these conditions. Microarray data revealed that NAE12:0 altered the transcript levels of multiple ABA-regulated genes

(Teaster et al., 2007), and several of these genes are involved in responses to salt and osmotic stress, including *ABI3* (Cumming et al., 2007), *RD29b*, (Nakashima et al., 2005), and *AtHVA22b* (Chen et al., 2002).

NAEs also may be involved in plant stress responses in a more indirect manner, and that is through alterations in the phenylpropanoid pathway. A close examination of microarray data of NAE 12:0-treated seedlings, showed that the transcript levels of several genes involved in the phenylpropanoid pathway including phenylalanine ammonia lyase, chalcone synthase, and anthocyanin acyltransferase were altered (Teaster et al., 2007). Products from the phenylpropanoid pathway, such as anthocyanin, have antioxidant properties which may play an important role in counteracting the oxidative stress resulting from the reactive oxygen species which are generated during abiotic stress conditions, including salt and drought stress (Vanderauwera et al., 2005).

Both salt and osmotic stresses are known to negatively impact plant / water relations. However, other stress conditions can also result in stress from water deficit. For example, chilling stress (Markhart, 1984) and heavy metals, such as cadmium (Poschenrieder et al., 1989), can result in decreased root hydraulic conductance, leading to water deficit and subsequently osmotic stress. Nitrogen limitation can also negatively impact plant / water relations by decreasing root hydraulic conductance in addition to decreasing stomatal conductance, leading reduced water absorption at the roots (Stuart Chapin et al., 1988).

Salt stress and heavy metals also have the potential of impacting plant growth by ion toxicity. During salt stress Na^+ ions are capable of competing with K^+ for access to potassium channels in plant roots, which can lead to potassium deficiency. High salt concentrations also have the potential of disrupting the hydrophobic-electrostatic balance between the forces maintaining protein structure (Wyn Jones and Pollard, 1983). Cadmium (Gussarsson et al., 1996) and copper (Ouzounidou, 1994) exposure in plants can negatively influence the uptake Ca, K, and Mg by plant roots, leading to disruptions in the homeostasis of these ions.

Abiotic stress conditions, including chilling (Prassad et al., 1994), cadmium stress (Romer-Puertas et al., 2007), copper stress (Deckert, 2005) salt and osmotic stress (Borsani et al., 2001) can also result in the formation of reactive oxygen species (ROS). The ROS resulting from these stress conditions can then lead to oxidative stress and thereby negatively impacting plant growth.

The number of studies examining the function of NAE metabolism in plants, although increasing, are still limited. Despite this lack of attention, there is evidence which suggests that NAEs play an important role in plant development and biotic stress responses. To date no studies have been carried out to determine the possible roles of FAAH and NAEs in abiotic stress. There is overlap among the genes involved in abiotic stress responses with those that are ABA-regulated, and it has been shown that NAE metabolism and ABA signaling pathways interact (Teaster et al., 2007). Therefore, I hypothesized that altered

NAE metabolism will cause altered responses to abiotic stresses in *Arabidopsis* plants, and this thesis is directed toward testing this assertion and providing insights into the molecular mechanism(s) involved in these interactions.

CHAPTER 2

MATERIALS AND METHODS

Plant Materials and Growth Assays

All *Arabidopsis thaliana* (L.) seeds used in these experiments were in the Colombia background (col). The active FAAH over-expressing OE lines were developed at the Noble Foundation in Ardmore, OK by the laboratory of Dr. Blancaflor (Wang et al., 2006). In these plant lines, FAAH is constitutively expressed by the strong cauliflower mosaic virus (CaMV35S) promoter. The FAAH knockout lines (*faah*) were generated by the Salk Institute (Alonso et al., 2003), and obtained from the Arabidopsis Biological Resource Center (ABRC) at Ohio State University. Two T-DNA insertional knockouts for FAAH were identified, but only one with a disruption in intron 13 was utilized. In addition two lines were obtained in which site-directed, inactive FAAH mutants were expressed in the wild-type (col-0) or *faah* backgrounds. These FAAH mutant lines were generated by Dr. Li Kang in Dr. Kiran Mysore's laboratory at the Noble Foundation, Ardmore, OK.

For growth experiments in solid media, the seeds were surface-sterilized with 70% ethanol followed by commercial bleach for three minutes each and rinsed three times with sterile, ultrapure water (18.2 MOhm). The seeds were then stratified for three days at 4 °C in the dark under sterile conditions on filter paper saturated with ultrapure water. The seeds were then transferred to square

Petri plates with 0.5X Murashige and Skoog (MS) media containing 1% sucrose and solidified with 0.5% phytagel plus any additives necessary to produce the desired stress conditions. For the growth of plants exposed to chilling stress the media contained nothing else. For the salt-stress experiments, NaCl was added to the media in concentrations of 100 mM, 125 mM, 150 mM, or 175 mM. Osmotic stress was induced by the addition of mannitol to produce concentrations of 300 mM, 400 mM, or 500 mM. Heavy metal stress was produced by the addition of either cadmium chloride or copper chloride to the media. The cadmium chloride was added to produce Cd^{2+} concentrations of 3.5 µM or 7.0 µM, and the copper chloride was added to produce Cu^{2+} concentration of 20 µM, 40 µM, or 60 µM. The chloride concentration was balanced with potassium chloride in the control plates and in the experimental plates containing less than the maximum concentration of Cu^{2+} or Cd^{2+}. The media used for the experiments involving nutrient deficiencies, and their corresponding control plates, were solidified with agar in place of phytagel. The MS media used for the nitrogen deficiency experiments was modified so that all of the nitrogen was present in the form of potassium nitrate. The plates deficient in nitrogen had a portion of the potassium nitrate replaced with potassium chloride. The N concentration of the control plates was 30 mM and the N-deficient plates had a N concentration of 1 mM. The MS media used for the phosphorus deficiency experiments also was solidified with agar in place of phytagel. In order to produce phosphorus deficiency in this media, a portion of the potassium

phosphate in the MS media was replaced with potassium chloride. The P concentration of the control plates was 623 µM and the P concentration of P-deficient plates was 1 µM. For all plants, growth was quantified from photographs analyzed by Image J software.

Growth conditions were the same for all experiments in solid media. The plates were sealed with Parafilm^{TM} and placed at a 45° angle in a growth room at 21 °C. The light cycle was 16 hours of light alternating with 8 hours of darkness with a light intensity between 75 and 100 µE/m^2/s.

The seeds used for the salt stress experiments in liquid culture were handled differently. The seeds for this experiment were surface sterilized and stratified under the same conditions as described for the experiments in solid media except that the stratification was carried out in centrifuge tubes containing one ml of sterile deionized water. The seeds were then transferred to 250 ml flasks containing 90 ml of liquid 0.5X MS media with 1% sucrose. The flasks were placed on an orbital shaker at 60 rpm. The seeds were allowed to germinate for 2 days after which the flasks were supplemented with 10 ml of sterile deionized water or 10 ml of concentrated NaCl solution to produce a final concentration in the flasks of 175 mM NaCl. The seedlings were allowed to continue growing for 14 additional days before harvesting. The light intensity for the liquid culture experiments was between 40 and 50 µE/m^2/s.

The seedlings grown in solid media were harvested by gently pulling them out of the media, blotting them dry, and freezing them with liquid nitrogen. The

plants were then stored at -80° C until needed for analyses. Seedlings grown in liquid were harvested by filtration using a Buchner funnel lined with filter paper. The seedlings were then blotted dry, frozen with liquid nitrogen, and stored at -80° C.

Quantitative RT-PCR

RNA was extracted from tissues previously frozen at -80° C using the RNeasy Plant Mini Kit (Qiagen). The resulting RNA was analyzed for the transcript levels of selected genes by quantitative, RT-PCR using a Smart Cycler II (Cepheid) instrument in conjunction with the real time one step assay system (Takara Bio Inc) with SYBR^{TM} Green I. The following gene-specific primer sets were used: *ABI3*(At3g24650) (F) 5'-GAGCTGGCTCAGCTTCTGCTATG-3' (R) 5'-AGGCCAAAACCTGTAGCGCATGTTC-3', *AtHVA22b* (At5g62490) (F) 5'-CATCGCTGGACCTGCATTAC-3' (R) 5'-GGATATAATGGGATCCATTCGAGG-3', *AtFAAH* (At5g64440) (F) 5'-CCATCTCAAGAACCGGAGCATG-3' (R) 5'-GGTGTTGGAGGCTTGTCATAGC-3', *RD29b* (At5g52300) (F) 5'-CATAAAGGTGGAGAAGCTGGAGTA-3' (R) 5'-CCTCCAAATCTTGCCGGAGAATTC-3', (Teaster et al., 2007), *SOS1* (At2g01980) (F) 5'- GGATCTCTCGAATATGGAGCTAAA-3' (R) 5'-ACCTTCACAAGCGATCCAAG-3', *SOS2* (At5g35410) (F) 5'-ATGGAATTGAGGGCAGTTATG-3' (R) 5'-TTGAGTTCGCTACAGCCTCA-3', and *SOS3* (At5g24270) (F)5'-CTGCTTGCACGAAAGCCTTAT-3' (R) 5'-

ATTTGTCCGGTCCTTAGGTGTC-3'. All the primers were designed so that at least one in each set, either the forward or the reverse primer, would span an intron in order to amplify cDNA and not genomic DNA. Relative transcript levels in all the samples were normalized to 18S RNA as a constitutively expressed internal control, with primers (F)5'-TCCTAGTAAGCGCGAGTCATCA-3' and (R)5'-CGAACACTTCACCGGATCAT-3' (Dean Rider at al., 2003). Quantitative RT-PCR reactions were carried out in duplicate with 0.2 µg total RNA and 0.5 µL of 10 µM primers for each 25 µL reaction. The reaction mix was subjected to the following conditions: 42° C for 15 min, one cycle; 95° C for 2 min, one cycle; 94°C for 10 sec, 58 ° C for 25 sec (read cycle), and 72° C for 20 sec. The number of cycles and annealing temperatures were experimentally determined for each set of primers. RT-PCR products were examined by gel electrophoresis and by melting curve analysis from 60° C to 95 ° C at a rate of 0.2° C / sec to rule out anomalous amplification products. The $2^{-\Delta\Delta CT}$ cycle threshold (C_T) method was used to calculate the relative changes in transcript levels determined from quantitative RT-PCR (Livak and Schmittgen, 2001). The data were analyzed using the equation $\Delta\Delta C_T = (C_{T,Target} - C_{T,18s})_{Treated} - (C_{T,Target} - C_{T,18S})_{Not\ Treated}$. "Treated" refers to samples from plants exposed to stress conditions and "Not Treated" refers to the control samples. Q-RT-PCR reactions were performed on a minimum of three biological replicates.

Metabolite Quantification

NAEs and phytohormones were quantified by isotope-dilution mass spectrometry. NAEs were extracted using glass beads and a bead beater from 50 mg to 70 mg of fresh or frozen plant tissue with 70 °C 2-propanol in the presence of deuterated NAE standards (NAE 12:0, NAE 18:0, and NAE 20:4). Total lipids were extracted with a monophasic solution of chloroform/2-propanol/water (Chapman and Moore, 1993), and the NAEs were enriched by silica SPE solid-phase-extraction column fractionation or by normal-phase high-performance-liquid chromatography (HPLC) as described by Wang et al (2001). The NAEs were derivatized with *N*,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (Sigma) and quantified as trimethylsilyl (TMS)-esters by gas chromatography-gas spectroscopy GC-MS (Venables et al., 2005).

For phytohormone analysis, between 50 mg and 100 mg of frozen tissue were ground in 30 mM imidazole buffer pH 7.0 in 70% 2-propanol with glass beads in a bead beater. Deuterated ABA, JA, (both from CDN Isotopes) and IAA (Cambridge Isotopes), were added at 200 ng each as internal standards. Three additional 2-propanol extracts of the same tissue were combined and reduced under N_2 in a heating block at 70° C to a volume between 1 and 2 mL to remove the 2-propanol. Samples were stored at 20° C until they fractionated on SPE columns, fractionated by HPLC, and derivatized with diazomethane. Quantification of phytohormones was carried out by GC-MS as described by Wang et., al (2001). Diazomethane was prepared in an ice-cooled Wheaton

apparatus by dissolving 0.2 g diazald (Sigma) in 1 mL of carbitol (Fluka) and slowly adding 1 mL of 7 M NaOH to the resulting solution. The generated diazomethane gas was collected in 5 mL of ether for 30 minutes. Precautions were taken to avoid explosion and fire hazards by carrying out the preparation in a fume hood away from flames and other heat sources.

Chlorophyll content was quantified as described by Tait and Hik (2003). For this method 20 mg of plant tissue were macerated and incubated in 7 mL of DMSO for 7 hours in the dark at room temperature. The resulting extracts were adjusted to 10 ml and were centrifuged at 663 x g for 5 minutes at room temperature using a Beckman Model TJ-6 centrifuge. The absorbance of the extract was measured at 646 nm and 665 nm with a Genesys 5^{TM} spectrophotometer. Chlorophyll content was determined with the following equations, chlorophyll a = $12.19A_{665}$-$3.45A_{646}$ and chlorophyll b = $21.99A_{646}$-$5.32A_{665}$.

CHAPTER 3

RESULTS

FAAH-Overexpressors Display Some Sensitivity to Chilling Temperatures

Seedling growth was examined at different temperatures to test if perturbation in FAAH expression would influence growth. There were no significant differences in the primary root length of WT, *faah*, OE2, OE7, and OE10 when grown at 21 °C over the course of 7 days. (Figure 1A). When these plants were grown under chilling stress at 7 °C over the same length of time there was mild, but significant ($p < 0.05$), hypersensitivity of the OE2 plants to chilling in terms of their primary root length, which was less than that of the WT plants (Figure 1B). There were no significant differences in the primary root length of the *faah* plants or of the OE7 and OE10 plants in relation to the WT plants under these chilling stress conditions. These data suggest that FAAH overexpression may be associated with hypersensitivity to chilling stress, but this is not consistently observed in all FAAH overexpression lines.

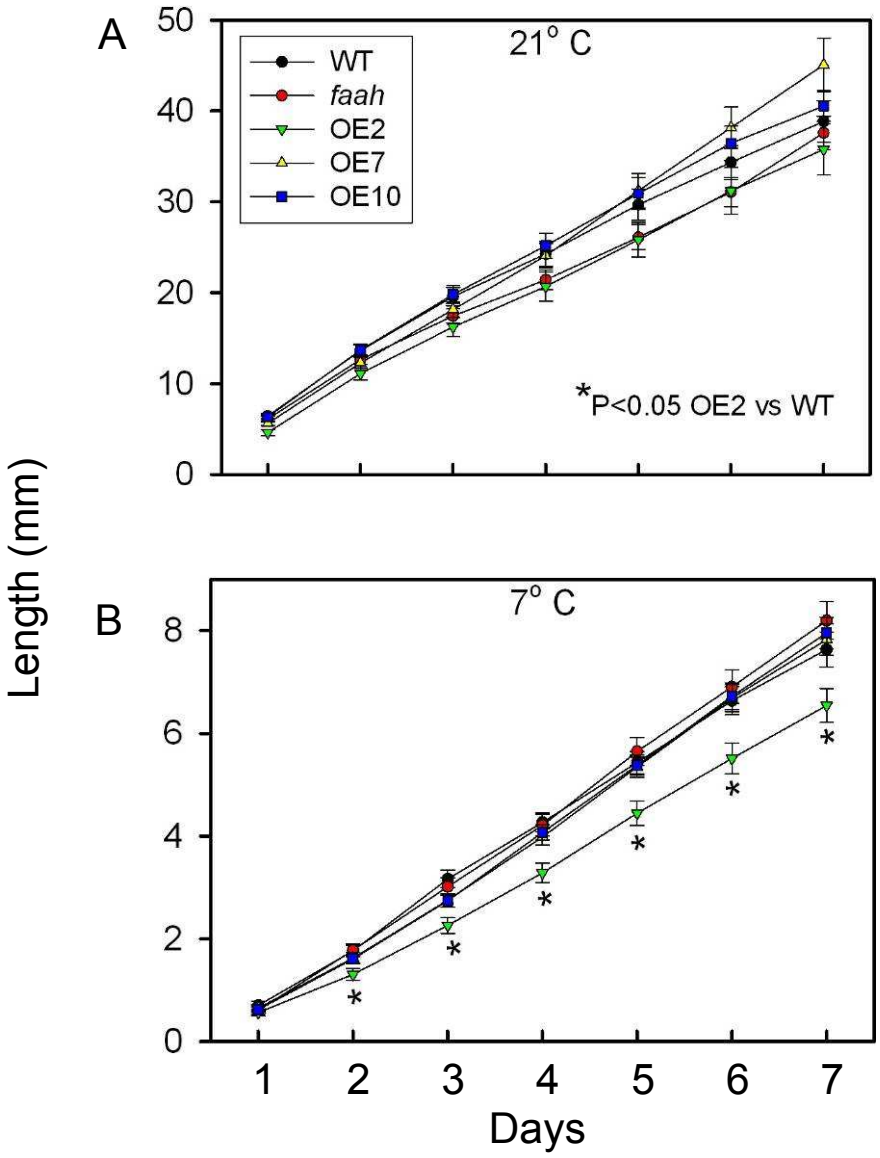


Figure 1. Primary root length in response to chilling stress.

(A) Primary root length over a 7 day time course at 21° C.

(B) Primary root length in the presence of chilling stress at 7° C over a 7 day time course.

FAAH-Overexpressors are Hypersensitive to Copper and Cadmium Toxicity

To test if FAAH expression influences growth responses in the presence of heavy metals, seedlings were grown in different concentrations of Cd^{2+} or Cu^{2+}. There were no significant differences in the primary root length of WT, *faah*, and OE2 plants in the absence of Cd^{2+} stress at day 4 (Figure 2A). However, at 3.5 μM Cd^{2+} the OE2 plants produced significantly shorter primary roots than the WT plants. The roots of the *faah* plants were not significantly different from those of WT plants. At 7 μM Cd^{2+} all of the plants were equally inhibited, showing no significant differences in their primary root length. The same patterns of sensitivity continued at day 6 (Figure 2B) with only the OE2 plants displaying a hypersensitivity to 3.5 μM Cd^{2+}.

After 6d there were no significant differences in the primary root length of WT, *faah*, OE2, and OE11 plants in the absence of Cu^{2+} stress (Figure 3A). However, at 20 μM and 40 μM Cu^{2+} the OE2 and OE11 plants produced significantly shorter primary roots than the WT plants. At 60 μM Cu^{2+} all of the plants were inhibited to a similar degree with no significant differences in their primary root length. After 10 d growth, the roots of all the plants tested continued to show no significant differences in the absence of Cu^{2+} stress, and the hypersensitivity of the OE2 and OE11 plants to 20 μM Cu^{2+} was no longer apparent while still remaining hypersensitive to 40 μM Cu^{2+} by producing shorter primary roots than the WT plants (Figure 3B). Also, at day 10 the OE11 plants began to show a hypersensitivity to 60 μM Cu^{2+} in relation to the WT. The

response of the *faah* plants was comparable to the WT plants in all the Cu^{2+} concentrations tested during the time course. Overall, these data suggest that FAAH overexpression is associated with hypersensitivity to heavy-metal stress. The lack of a considerable effect by disruption of AtFAAH (*faah* knockout) is likely due to compensation by a second FAAH that has been identified in Arabidopsis (Kim, Faure, Chapman and Blancaflor, unpublished results).

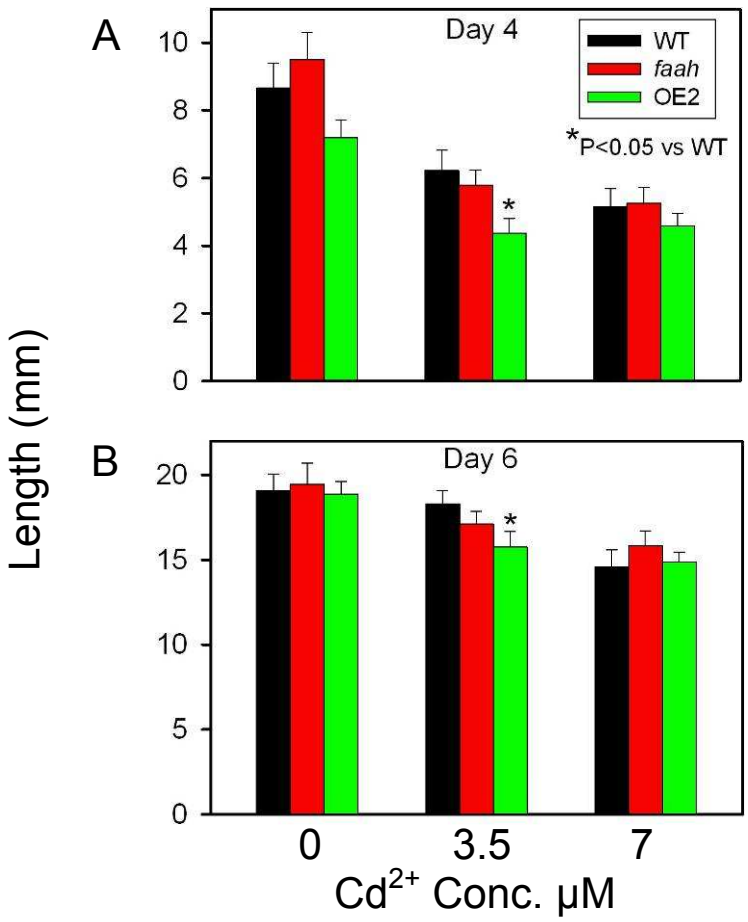


Figure 2. Primary root length in response to Cd^{2+} toxicity.

(A) Primary root length at day 4 of exposure to 0mM, 3.5 mM, or 7 mM Cd^{2+}.

(B) Primary root length at day 6 of exposure to 0mM, 3.5 mM, or 7 mM Cd^{2+}.

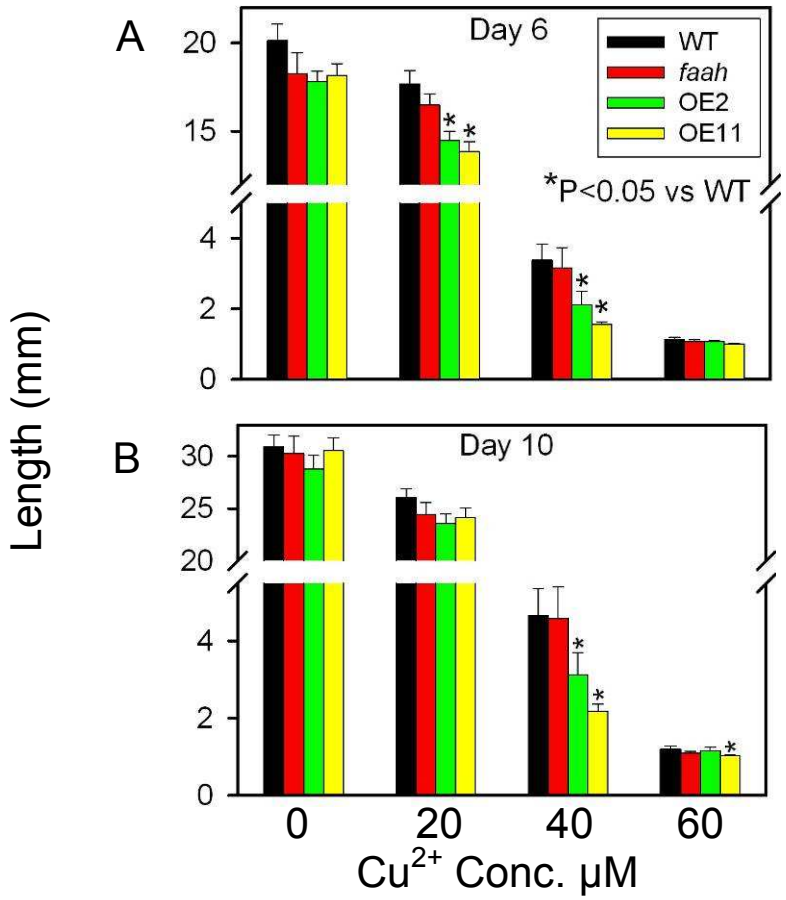


Figure 3. Primary root length in response to Cu^{2+} toxicity.

(A) Primary root length at day 6 of growth in 0mM, 20 mM, 40 mM, or 60 mM Cu^{2+}.

(B) Primary root length at day 10 of gorwth in the presence of 0 mM, 20 mM, 40 mM, or 60 mM Cu^{2+}.

FAAH-Overexpressors Are Hypersensitive to Phosphorus and Nitrogen Limitation

Seedlings were grown under N or P limitation to determine if perturbations in FAAH transcript levels influenced their growth under these conditions. There were no significant differences in the lateral root production among the WT, *faah*, OE2 and OE11 plants under conditions of standard P concentration of 623 μM in half strength MS media over a time course from day 8 to day 14 (Figure 4A). As expected, there was stimulation in the production of lateral roots under conditions of P limitation at a concentration of 1 μM towards the end of the time course (Figure 4B), as has been shown by others (Calderón et al., 2005). However, the OE2 and OE11 plants did not increase their lateral root numbers to the same degree as the WT under conditions of P deficiency. The *faah* plants produced similar numbers of lateral roots as the WT plants both under standard and limited P concentrations.

Under conditions of standard N concentration for half strength MS media at 30 mM there were no significant differences in the number of lateral roots per plant or in lateral root density among the different FAAH genotypes (Figure 5A). The only significant difference observed was in primary root length in which the OE plants had somewhat longer primary roots than the WT plants. However, under N deficiency the OE plants produced fewer lateral roots and had a lower lateral root density than the WT plants (Figure 5B). Also, the difference in primary root length of the OE plants was no longer significantly different from the

WT plants under conditions of N limitation. The *faah* plants did not show any significant differences from the WT plants in their root development either in the presence or absence of N limitation. Overall, these data suggest that FAAH overexpression is associated with hypersensitivity to nutrient-stress. The lack of a considerable effect by disruption of AtFAAH is likely due to compensation by a second FAAH that has been identified in *Arabidopsis* (Kim, Faure, Chapman and Blancaflor, unpublished results).

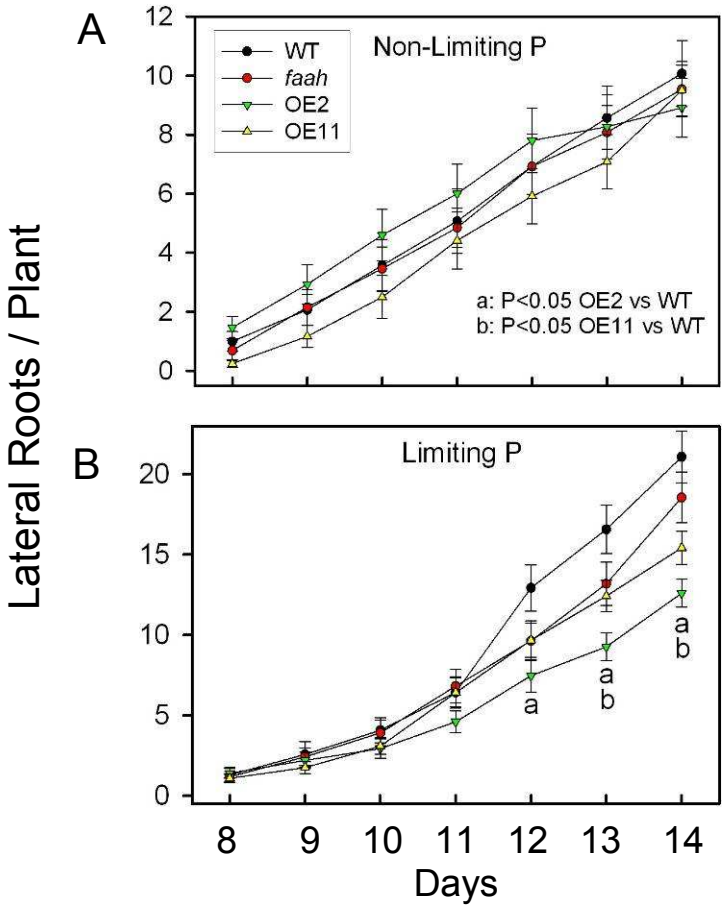


Figure 4. Lateral root production under P limitation.

(A) Lateral root production in the presence of P levels corresponding to 0.5X MS media (623 µM P) over a 7 day time course from day 8 to day 14.

(B) Lateral root production under conditions of limiting P (1 µM) over the same 7 day time course.

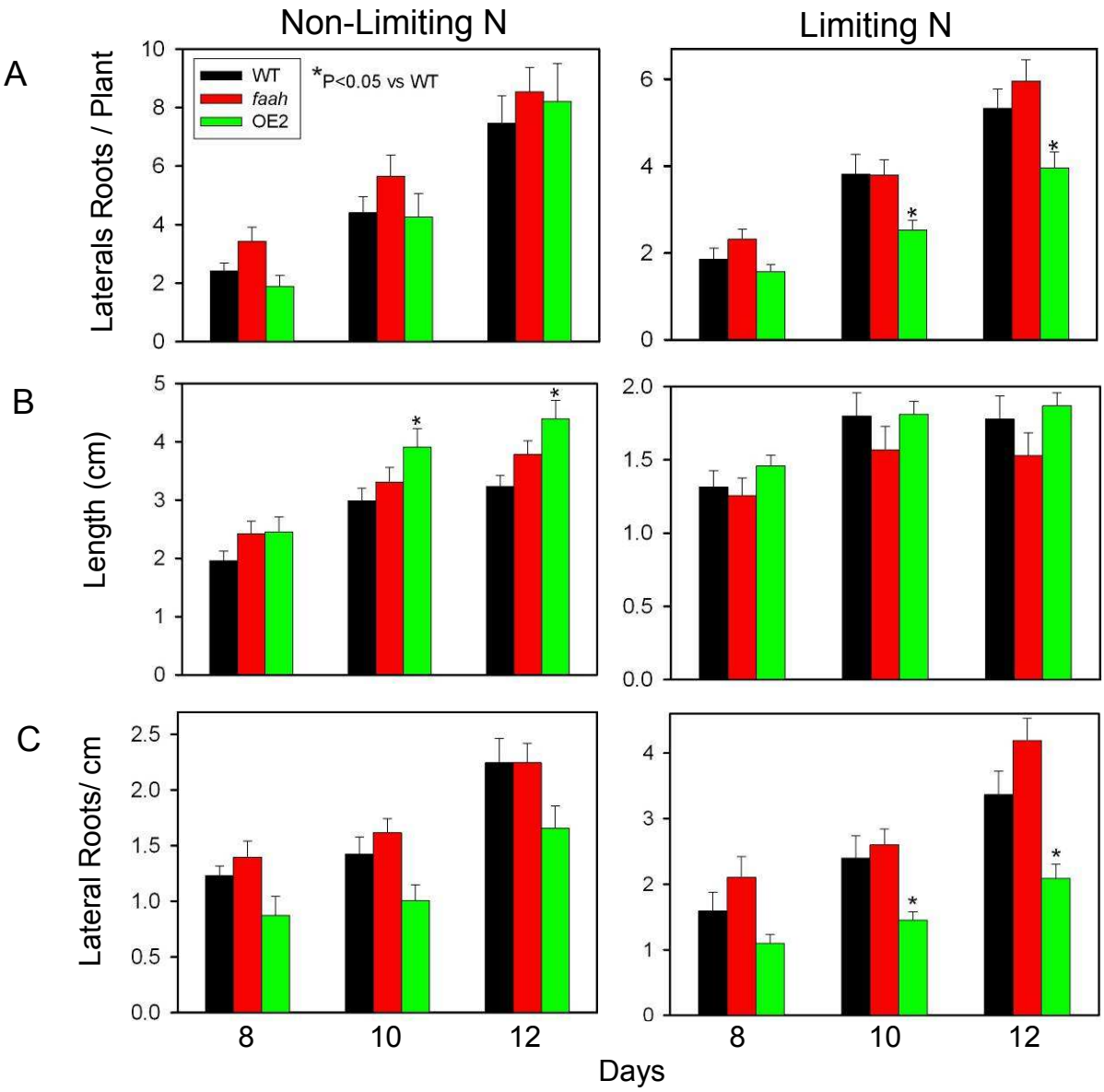


Figure 5. Root development under conditions of non-limiting N (30 mM) or limiting N (1 mM).

(A) Lateral root production per plant.

(B) Primary root length.

(C) Lateral root density per cm of primary root.

FAAH-Overexpressors Are Hypersensitive to Salt Stress in Liquid Culture

Seedlings were grown in liquid culture in the presence of salt stress to determine if alterations in FAAH transcript levels influenced their growth under these conditions. The growth of the OE plants was clearly hypersensitive to salt stress when grown in liquid MS media (Figure. 6). In the absence of salt stress it is the OE plants which grow most robustly (Figure 6A and Figure 6B); however, they were most severely affected after 14 days in 175 mM NaCl by producing the least amount of tissue mass. There was no significant difference in growth (quantified as accumulated fresh weight) between the WT and the *faah* plants. The lack of a considerable effect by disruption of AtFAAH is likely due to compensation by a second FAAH that has been identified in Arabidopsis (Kim, Faure, Chapman and Blancaflor, unpublished results).

To test if growth differences in FAAH OE were associated with alterations in phytohormone levels, IAA, ABA, and JA levels were quantified. The OE plants indeed showed differences in their phytohormone levels in response to salt stress. There were no significant differences in the levels of JA in the WT, *faah*, and OE plants in the absence of NaCl (Figure 7). After 14 days at 175 mM NaCl there were also no significant differences in the JA levels of the WT, *faah*, and OE plants (Figure 7A). However, the OE plants showed a significant increase in JA levels in the presence of 175 mM NaCl in relation to their JA levels in the absence of salt stress. A similar trend was observed for ABA. The OE plants showed a significant increase of ABA in the presence of salt stress in relation to

their ABA levels in the absence of NaCl (Figure 7B). Despite the differences in seedling growth, there were no differences in the IAA levels among any of the genotypes tested either in the presence or absence of salt stress (Figure 7C), although the amounts in samples varied greatly, making comparisons difficult.

To see if changes in endogenous NAE content were associated with growth differences in FAAH, OE, NAE types were quantified by GC-MS. There were differences in NAE levels among the different genotypes both in the presence and the absence of salt stress. In the absence of salt stress the *faah* plants had higher levels of the unsaturated NAEs 18:2 and 18:3 than the WT plants (Figure 8A). However, in the presence of 175 mM NaCl these differences were insignificant (Figure 8B). The salt-stressed OE plants had significantly higher levels of the saturated NAEs 14:0, 16:0, and 18:0 when compared to the untreated OE plants. The salt-stressed *faah* plants had elevated levels of the saturated NAEs 14:0 and 16:0 in the presence of salt stress in relation to the *faah* plants in the absence of NaCl. Also, the NAE 16:0 levels of the stressed *faah* plants were higher than those of the stressed WT plants.

The OE seedlings appeared to be bleached to a greater degree than the WT and *faah* plants after the 14 days of salt stress in liquid media, and this was quantified by measuring chlorophyll levels. Chlorophyll measurements revealed that the OE plants had lower levels of total chlorophyll and chlorophyll b than the WT and *faah* plants both in the presence and in the absence of salt stress (Figure 9A). Exposure to salt stress was associated with chlorophyll depletion in

all the genotypes. However, the OE plants showed a greater percentage reduction in chlorophyll b than the WT or *faah* plants (Figure 9B), again indicating a greater sensitivity to salt stress than WT or *faah*.

To test if classic molecular responses to salt-stress in *Arabidopsis* seedlings were altered in FAAH overexpressors, transcript levels of several known salt-responsive genes were quantified by QRT-PCR. Some of these were found to be up-regulated in NAE treated seedlings by microarray analysis (Teaster et al, 2007). The FAAH OE plants had altered transcript levels of some, but not all stress-responsive genes relative to the WT plants in response to salt stress (Table 1). Transcript levels for *ABI3* and *AtHVA22b* were undetectable in all of the plant lines in the absence of NaCl. Thus, it was not possible to calculate a fold-change in transcript levels for these genes by QRT-PCR in response to salt stress (so these levels were estimated visually by semi-quantitative RT-PCR, below). On the other hand, transcript levels for *RD29b* and the *SOS* genes were present in all of the plant lines tested in the absence of salt stress, making their quantification possible by QRT-PCR in response to NaCl. The greatest increase in *RD29b* transcripts took place in the OE plants showing a more than 500- fold increase compared to untreated WT plants. There were no significant differences in the transcript levels of *RD29b* between the WT and *faah* plants, both of which showed a marked induction (96- and 108-fold, respectively), albeit not as great as in FAAH OE. Similar to *RD29b*, the *SOS*1, *SOS2*, and *SOS3* genes had detectable transcripts in the absence of salt stress, making it

possible to calculate a fold change in transcript levels in response to NaCl. These genes are part of a salt responsive mechanism to remove excess Na^+ ions from plant cells (Zhu, 2002). During salt stress rapid, transient increases in cytosolic Ca^{2+} take place, and SOS3 is one of the targets of the Ca^{2+} ions. When Ca^{2+} binds to SOS3 it causes a conformational change in this protein which allows it to interact with and activate SOS2. SOS2 is a protein kinase capable of phosphorylating SOS1. SOS1 is a Na^+ / H^+ antiporter at the plasma membrane which is activated upon phosphorylation resulting in the efflux of excess Na^+ ions. As expected, the transcript levels for the *SOS* genes were elevated in the presence of salt stress in all the plants tested, but there were no significant differences in these transcript levels among the different FAAH genotypes.

Relative transcript levels also were also analyzed qualitative RT-PCR to visualize relative band intensities (Figure 10). The transcript levels of *ABI3* and *AtHVA22b*, which could not be quantified by qRT-PCR, showed different transcript levels by gel electrophoresis. In the absence of salt stress, these genes were undetectable. However, in the presence of NaCl both of these genes show detectable transcripts, with the OE plants showing the greatest levels of *AtHVA22b* after 25 PCR cycles based on band intensity. The WT and OE plants show similar levels of *ABI3*, while the levels for the *faah* plants are somewhat lower after 45 PCR cycles. Transcripts for *RD29b* and the *SOS* genes were also analyzed by gel electrophoresis. The transcript levels for *RD29b* in the absence of salt stress were similar in all the plants tested. However, in the

presence of 175 mM NaCl, the OE plants showed a greater band intensity for *RD29b* corresponding to higher transcript levels than either the WT or *faah* plants after 23 PCR cycles. Transcript levels for the *SOS1* genes were similar based on band intensities in the absence of salt stress for the WT and *faah* plants, while those of the OE plants seemed somewhat higher based on band intensity. In the presence of 175 mM NaCl, the levels of *SOS1* transcripts were all very similar for all three genotypes tested after 30 PCR cycles. The *SOS2* transcript levels were similar in the absence of salt stress between the *faah* and OE plants, but lower for the WT plants. In the presence of salt stress the levels of *SOS2* transcripts were similar in all the plants tested after 33 PCR cycles. The levels of *SOS3* were similar in all the plant lines in the lack of salt stress, but in the presence of NaCl the levels of SOS3 transcripts were somewhat higher in the *faah* and OE plants than in the WT plants after 30 PCR cycles. Taken together, these results indicate that the molecular responses of FAAH OE, at least for some salt-responsive genes, are exaggerated, but the well characterized SOS system does not appear to be differentially regulated in these FAAH over-expressors. These results are consistent with FAAH OE plants having a heightened sensitivity to salt stress.

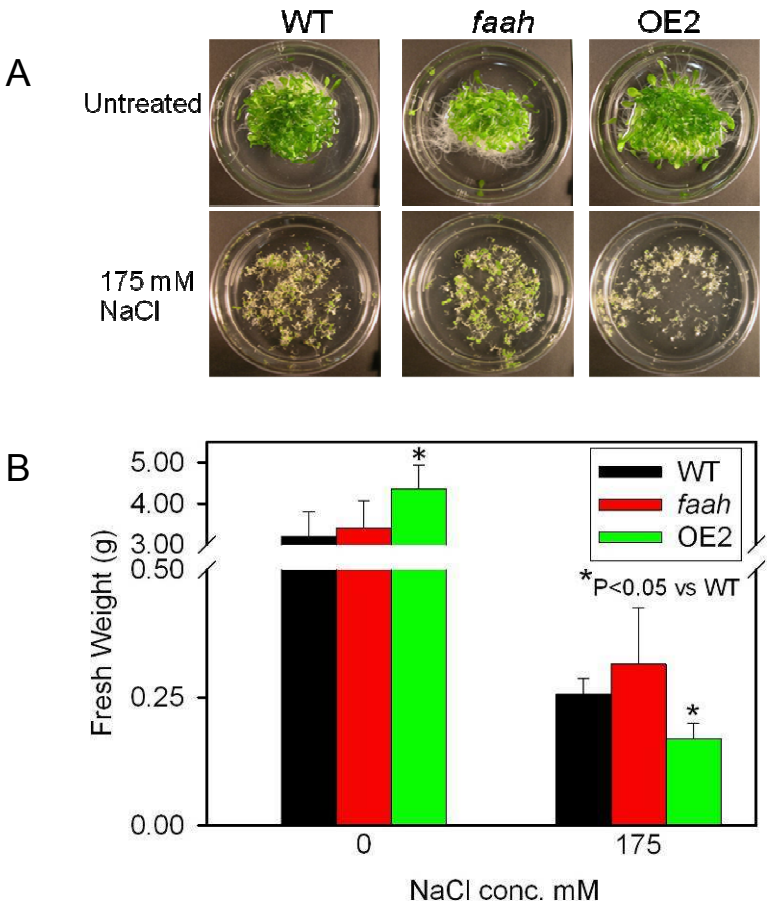


Figure 6. Growth for 14 days in liquid culture in the presence or absence of salt stress.

(A) The appearance of plants grown in 0 mM or 175 mM NaCl.

(B) The tissue mass of plants grown in 0 mM or 175 mM NaCl.

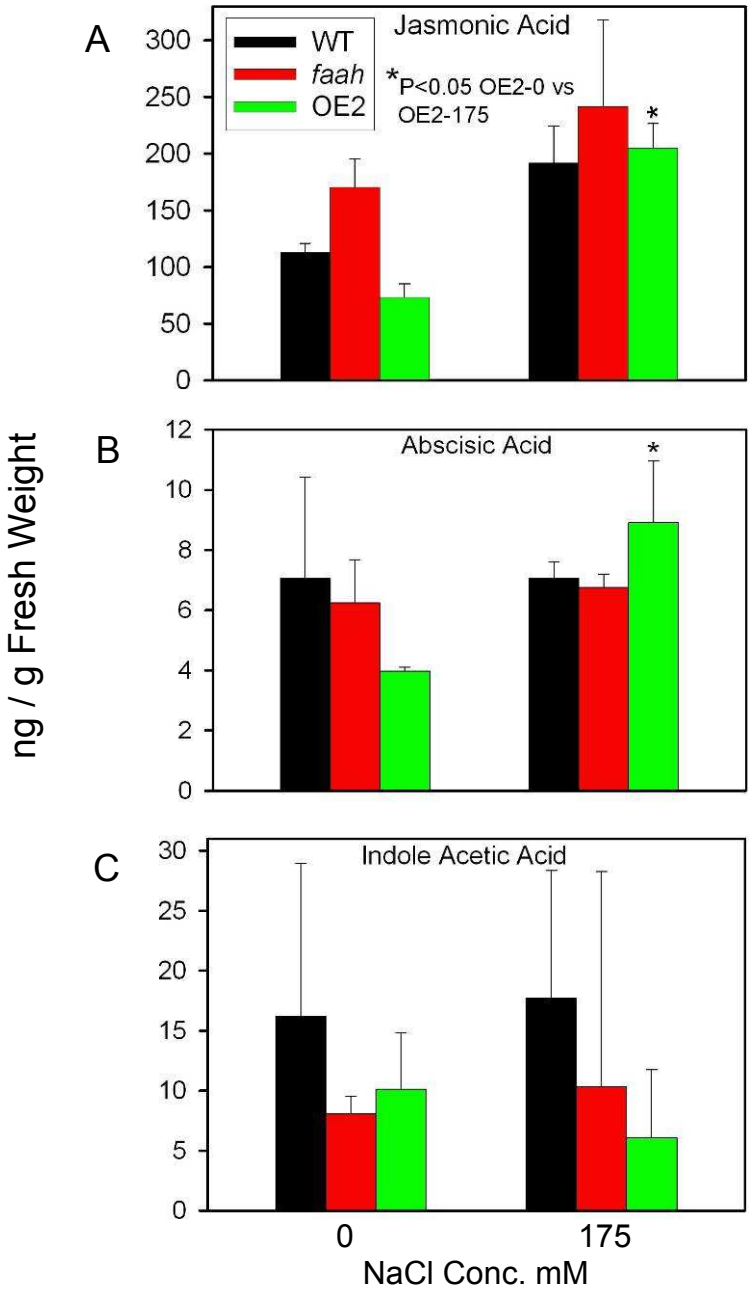


Figure 7. Phytohormone levels in response to 14 days of salt stress.

(A) Jasmonic acid levels.

(B) Abscisic acid levels.

(C) Indole acetic acid levels.

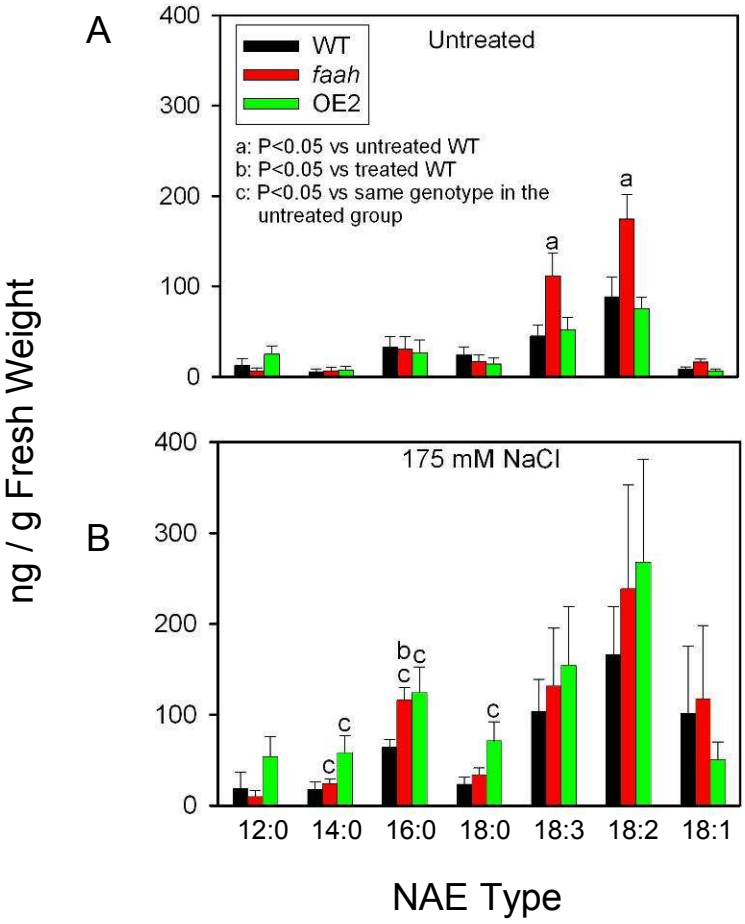


Figure 8. NAE levels in response to 14 days of salt stress.

(A) NAE levels of plants grown in 0 mM NaCl.

(B) NAE levels in plants grown in 175 mM NaCl.

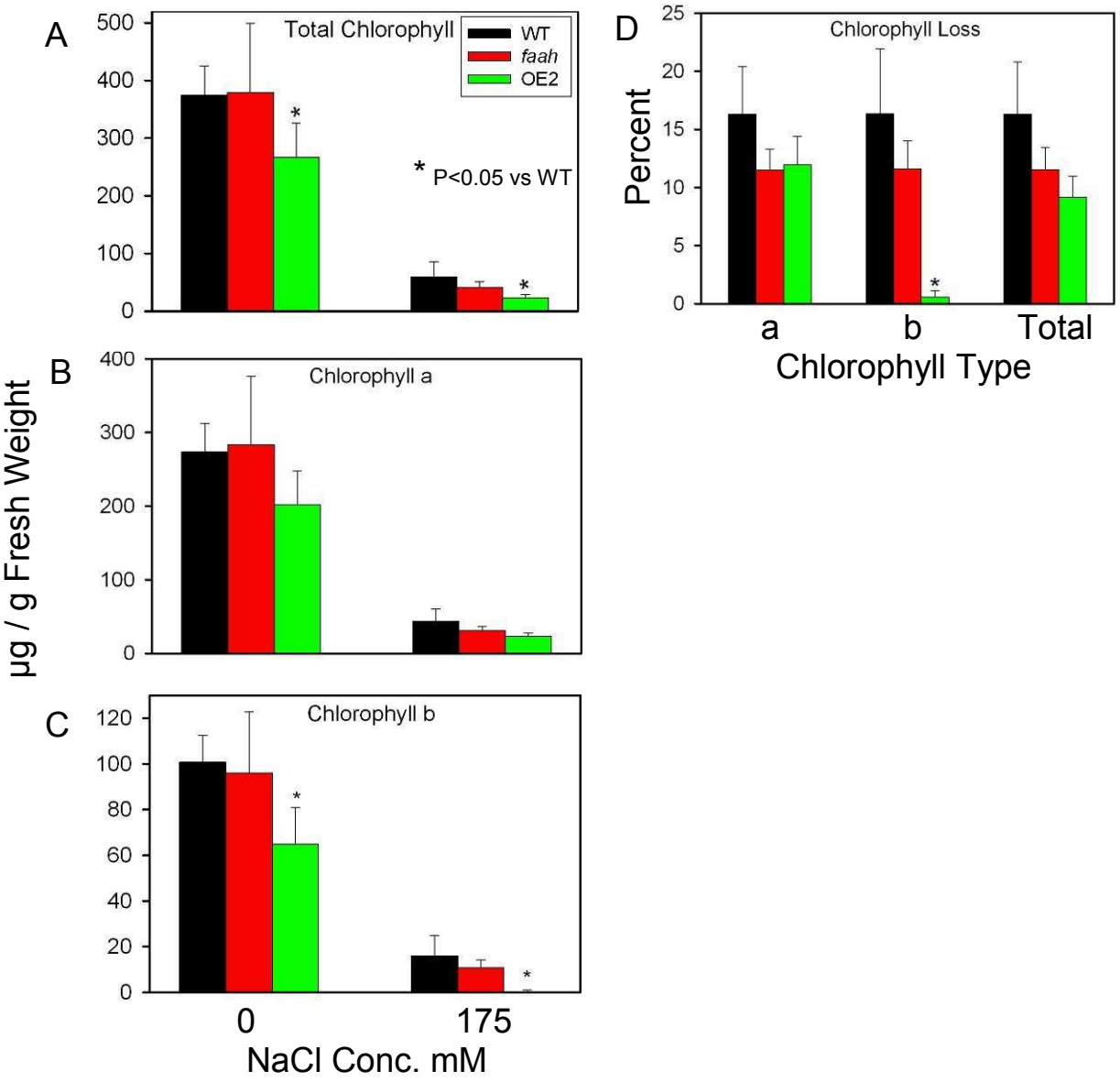


Figure 9. Chlorophyll levels in response to 14 days of salt stress.

(A) Total chlorophyll levels in 0 mM or 175 mM NaCl.

(B) Chlorophyll a levels.

(C) Chlorophyll b levels.

(D) Percent loss of chlorophyll a, chlorophyll b, and total cholorophyll as a result of salt stress with 175 mM NaCl.

Table 1. Fold-change in the transcript levels of *ABI3*, *AtHVA22b*, *RD29b*, and three *SOS* genes in response to 175 mM NaCl in relation to untreated WT		
Gene	Plant Line	Fold Change
ABI3	WT	ND in untreated
	faah	ND in untreated
	OE2	ND in untreated
AtHVA22b	WT	ND in untreated
	faah	ND in untreated
	OE2	ND in untreated
RD29b	WT	96 ± 12
	faah	110 ± 50
	OE2	570 ± 180
SOS1	WT	5.0 ± 1.7
	faah	9.0 ± 4.2
	OE2	8.0 ± 2.7
SOS2	WT	6.0 ± 0.82
	faah	8.0 ± 3.0
	OE2	9.0 ± 2.7
SOS3	WT	12 ± 5.5
	faah	19 ± 4.7
	OE2	18 ± 6.5

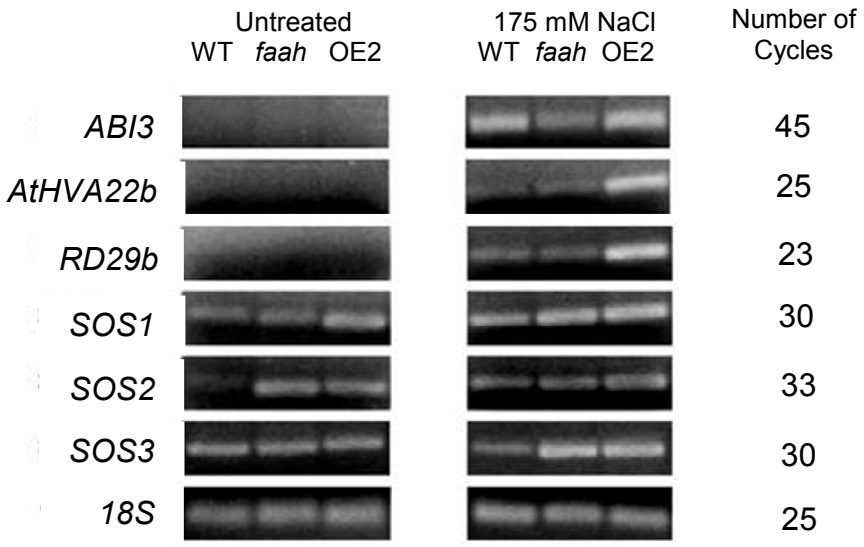


Figure 10. Relative band intensities of three ABA-responsive genes and three genes from the SOS pathway after the indicated number of PCR cycles.

FAAH Activity Appears to be Important in Determining the Sensitivity of *Arabidopsis* Plants to Salt Stress and Osmotic Stress

An active FAAH OE and two inactive FAAH (FAAHm) OE lines were grown in solid MS media to assess the effect of FAAH activity on salt and osmotic stress tolerance. The two inactive FAAHm OE lines overexpress a site-directed mutant form of FAAH in which serine 281 and serine 282 were replaced with alanine, rendering the enzyme inactive (Kang et al, 2009). One of these mutants overexpresses the inactive FAAHm in the WT background (SW) and the other in the *faah* background (SK). The characterization of FAAHm plants and their hypersensitivity toward ABA and NAE were published elsewhere (Kim et al., 2010). The growth of these plants is inhibited by both NAE and ABA. By contrast the overexpression of native FAAH in either the WT or *faah* background confers tolerance toward NAE (because FAAH is able to hydrolyze exogenous NAE), but also hypersensitivity toward ABA. These previous results indicated that some actions of FAAH (enhanced growth) are dependent upon FAAH activity, whereas other actions of FAAH (hypersensitivity toward ABA and non-host pathogens) were dependent upon protein, but not enzymatic activity. Here, the active FAAH OE plants were consistently hypersensitive to both salt and osmotic stress, while the inactive FAAHm OE plants did not show this hypersensitivity and in some cases showed some degree of tolerance to these stresses (Figures. 11- 15). So, collectively, these results suggest that the enzymatic activity of FAAH is, in part, responsible for the sensitivity of plants to

salt and osmotic stresses, and it implies that hydrolysis of NAEs may be involved in abiotic stress responses in *Arabidopsis* seedlings. Details of these experimental results follow.

The growth of the active FAAH OE plants was consistently less robust than that of the other FAAH genotypes based on their appearance in the presence of salt stress at 100 mM, 125 mM, and 150 mM NaCl as well as in the presence of osmotic stress induced with 300 mM, 400, mM, and 500 mM mannitol (Figure 11). Under these same conditions the inactive FAAHm OE genotypes, SW and SK, consistently produced more abundant growth than the OE plants. At first glance the growth of the *faah*, SW, and SK plants seemed comparable to that of the WT plants. However, on closer inspection of the roots and shoots of all the plants tested, the inactive FAAHm OE plants often produced more abundant growth than the WT plants. The *faah* plants also showed greater stress tolerance than the WT plants in some circumstances, particularly in the presence of osmotic stress.

One of the seedling growth parameters tested was the production of lateral roots (Figure 12). Under salt stress at 100 mM NaCl, the SW and SK plants produced more lateral roots than the OE and *faah* plants respectively and both produced more lateral roots than the WT plants at all time points examined (Figure 12A). At 125 mM and 150 mM NaCl there were no significant differences in lateral root production among any of the FAAH genotypes, except for a slight decrease in the SK plants in relation to the WT plants at 150 mM NaCl at day 12.

The results for lateral root production in the presence of osmotic stress were more pronounced (Figure 12B). At 300 mM mannitol, the elaboration of lateral roots of the SW and SK plants were similar to those in the presence of salt stress at 100 mM NaCl in that the SW and SK plants out-performed the FAAH OE and *faah* plants respectively and they both out-performed the WT plants. Also, in 300 mM mannitol the FAAH OE plants produced fewer lateral roots than the WT plants. At 400 mM mannitol, the FAAH OE plants were more sensitive than the WT or SW plants by consistently producing fewer lateral roots. Also, at 400mM mannitol the *faah* and SK plants were more tolerant than the WT plants since they produced a greater quantity of lateral roots. At 500 mM mannitol the FAAH OE plants produced fewer lateral roots than the WT or SW plants in 2 out of the 3 days in which root counts were carried out. The *faah* and SK consistently out-performed the WT plants, and the SK plants out-performed the *faah* plants on day 20. Interestingly the *faah* knockout plants appeared to be more tolerant than WT plants to osmotic stress in 400 mM mannitol (20 d) and in 500 mM mannitol (all time points).

Seedling growth was quantified in terms of primary root elongation (Figure 13). The FAAH OE plants consistently developed longer primary roots than other FAAH genotypes in all the NaCl concentrations tested (Figure 13A). The primary root length of the SW and *faah* plants was comparable to that of the WT plants. The root lengths of the SK plants were more variable, but they were either comparable to that of the WT and *faah* plants or somewhat shorter, and all were

shorter than FAAH OE seedlings. In the presence of osmotic stress the FAAH OE plants also had longer primary roots than any of the other plants at all time points, except at the highest mannitol concentration of 500 mM (Figure 13B). At 500 mM mannitol the primary root length of the FAAH OE plants is comparable to that of the WT plants on days 16 and 20 and is somewhat less on day 12. The SW plants almost always had longer primary roots than the WT plants and longer primary roots than the FAAH OE plants at 500 mM mannitol. The *faah* plants consistently had longer primary roots than the WT plants at 400 mM and 500 mM mannitol. The SK plants had longer primary roots than the WT plants at 300 mM and 500 mM mannitol, while they remained comparable to the WT at 400 mM mannitol. In all cases, the phenotypes of FAAHm OE seedlings were the reverse of FAAH OE regardless of background, indicating that FAAH activity is responsible, in part, for hypersensitivity to osmotic stress.

To combine the parameters of root development, I calculated lateral root density (the number of lateral roots, per unit of root length) get an overall picture of seedling root development in response to abiotic stress (Figure 14). In general this accentuated the differences between FAAH OE and FAAHm OE genotypes. Similar to lateral root production per plant, most of the differences among the FAAH genotypes for lateral root density occurred at the lowest NaCl concentration tested (Figure 14A). At this salt concentration the FAAH OE plants had a lower lateral root density than WT, SW, SK or *faah* genotypes. Similar trends were noted at higher salt concentrations. Under conditions of osmotic

stress, the FAAH OE plants consistently under-performed the other plant lines in terms of lateral root density, while the SW, *faah*, and SK plants were at least comparable to the WT in their lateral root production, but often out-performed the WT (Figure 14B).

The shoots of the various FAAH lines also displayed differences in response to salt stress (Figure 15). At 100 mM NaCl the SW plants produced more shoot growth (estimated as area) than the FAAH OE plants while the SK plants produced more shoot growth than either the *faah* or WT plants (Figure 15A). All of the genotypes were similarly affected by 125 mM NaCl since there were no significant differences in the shoot area among any of the plant lines. The FAAH OE plants were more sensitive to 150 mM NaCl than the other plants lines since they produced the smallest shoots. However, at this salt concentration, the SW plants were not affected to the same degree and showed more shoot growth than the FAAH OE plants. Under osmotic stress conditions the FAAH OE plants were consistently affected to a greater degree than the WT plants (Figure 15B). The FAAH OE plants produced the least shoot growth in all the mannitol concentrations. The SW, *faah* and SK plants were more tolerant than the WT plants at 300 mM and 500 mM mannitol. The SW genotype showed more shoot growth than the FAAH OE plants in all the mannitol concentrations tested, and the SK plants showed comparable or more shoot growth than the *faah* background. These results of shoot growth estimates were consistent with

my evaluation of root development and support the conclusion that FAAH activity is responsible, in part, for the hypersensitivity to abiotic stress.

To test how classic molecular responses to stress in *Arabidopsis* seedlings were altered in FAAH overexpressors, transcript levels of several known stress-responsive genes were evaluated by RT-PCR (similar to experiments with liquid-grown seedlings, above). Transcript levels of *RD29b*, which is known to be induced by both salt and osmotic stress (Msanne et al., 2011), were measured by qRT-PCR, where as transcript levels for *ABI3* and *AtHVA22b* (not detectable in untreated tissues) were compared by visualized RT-PCR products by agarose gel electrophoresis. There were indeed differences in the transcript levels of these ABA-responsive genes in the presence of salt stress and osmotic stress. The transcript levels for *RD29b* increased in salt (150 mM) and in mannitol (400 mM and 500 mM) the most in FAAH OE (Table 2). On the other hand, transcript levels were least induced in FAAHm OE, with WT in between these extremes. *ABI3* and *AtHVA22b* transcripts were not detectable in the absence of salt or osmotic stress making calculations of the fold change by QRT-PCR not possible. However, differences in the transcript levels for these genes were detectable by agarose gel electrophoresis both in salt and osmotic stress after carrying out RT-PCR for the indicated numbers of cycles (Figure 16). In the presence of salt stress at 100 mM or 125 mM NaCl no induction of *ABI3* or *AtHVA22b* was detected after 55 cycles or 35 cycles respectively (Figure 16A). The induction of both genes was detected at 150 mM NaCl after 50 cycles for

ABI3 and 35 cycles for *AtHVA22b*. The band intensities were similar for the WT and OE plants, and considerably fainter for the *faah* plants, indicating a lower level of transcripts for the *faah* plants. The lowest transcript levels for *ABI3* and *AtHVA22b* occurred in the SW and SK plants where bands were extremely faint, or barely detectable. Also, no induction of these genes was detectable at 300 mM mannitol after 55 cycles for *ABI3* and 35 cycles for *AtHVA22b* (Figure 16B). However, the transcripts for these genes became detectable at 400 mM mannitol after 52 cycles for *ABI3* and 29 cycles for *AtHVA22b*, with the greatest transcript levels occurring in the FAAH OE plants based on relative band intensities. At 500 mM mannitol the transcripts were elevated to a comparable degree in all the plant lines after 50 cycles for *ABI3* and 25 cycles for *AtHVA22b*. Under both salt and osmotic stress the reference gene 18S had similar levels of transcript in all the plant lines at all the NaCl and mannitol concentrations tested. Taken together, these results were similar to experiments with liquid grown seedlings, where the molecular responses to abiotic stress by FAAH OE were exaggerated relative to WT, and here this seemed to be reversed in genotypes overexpressing the inactive FAAHm.

The differential physiological and molecular responses to abiotic stresses in genotypes with active or inactive forms of FAAH suggests that there might be differences in NAE composition in these lines as well. The genotypes were analyzed for total levels of NAE in response to salt or osmotic stress (Figure 17). The untreated control plants did not show any significant differences in NAE

levels among the different genotypes. However, in 100 mM NaCl, the FAAH OE plants showed lower levels of NAE than the WT plants, while the levels of the SW plants were similar to those of the WT. The *faah* plants showed higher NAE levels, while those of the SK plants were similar. At higher salt concentrations (e.g. 125 mM NaCl) there were no significant differences in NAE levels in any of the plant lines. At 300 mM mannitol the SK plants displayed higher NAE levels than the WT plants. At 400 mM mannitol more differences in NAE levels were discernable. The FAAHm OE plants SW and SK both had NAE levels which were higher than the FAAH OE. The NAE levels of the *faah* plants were higher than those of the WT, while those of the SK plants were lower than those of the *faah* plants. Although these changes were perhaps not as dramatic as might be anticipated, generally speaking there appeared to be alterations in endogenous NAE content as might be predicted from expressing active or inactive forms of FAAH. These changes were also more discernable under stress conditions where there were clear physiological differences in stress responses (i.e., 100 mM NaCl and 400 mM mannitol).

NAE compositions were compared for these genotypes in response to different stresses. No significant differences in the individual NAE types were observed in the absence of salt stress for the various FAAH genotypes (Figure 18A). However, under moderate salt stress at 100 mM NaCl differences in the NAE content became noticeable (Figure 18B). Under these conditions the FAAH OE plants had lower levels of NAEs 12:0, 14:0, 18:1, 18:2, and 18:3 than the WT

plants. The FAAHm OE and SW plants had higher levels of NAE than the FAAH OE plants, except for NAEs 18:0 and 18:2. The levels of all the NAEs types in the SW plants were similar to those of the WT plants. The *faah* plants had higher levels of NAE 12:0 and 18:0 than the WT plants. The levels of all other NAE types in the *faah* plants were comparable to those of the WT plants. The SK plants had greater quantities of NAEs 16:0, 18:0, and 18:1 compared to the WT plants, and lower levels of NAE 12:0. At higher salt concentrations (125 mM NaCl) there were no detectable differences in the NAE compositions of the various FAAH genotypes (Figure 18C), similar to comparing the total NAE content.

In the absence of osmotic stress there were no significant differences in the NAE composition of the various genotypes tested (Figure 19A). In the presence of moderate osmotic stress with 300 mM mannitol differences in the NAE levels among the various plant lines occurred (Figure 19B). At this mannitol concentration the FAAH OE plants had lower levels of NAEs 14:0, 16:0, 18:0, and 18:3 than the WT plants. The SW plants had NAE levels which were similar to those of the WT plants, and higher levels than the FAAH OE plants of NAEs 14:0 and 18:3. The *faah* plants had NAE levels similar to those of the WT plants for all the NAE species tested. The SK plants had higher levels of NAE 12:0 than the WT plants. At 400 mM mannitol more differences in NAE levels were observed (Figure 19C). Under this level of osmotic stress the OE plants had lower levels of NAEs 12:0 and 18:3, and higher levels of NAE 18:0 than the WT

plants. The SW plants had higher levels of NAEs 12:0, 14:0, and 18:3, and lower levels of NAEs 18:0 and 18:1, than the OE plants. Under these same conditions the *faah* plants had higher levels of NAEs 12:0, 16:0, 18:1, and 18:3 than the WT plants. The SK plants had higher levels of NAEs 18:0 and 18:1, and lower levels of NAEs 14:0 and 18:3, than the WT plants. The SK plants also had higher levels of NAEs 18:0 and 18:2, and lower levels of NAEs 12:0, 14:0, 16:0, and 18:3 than the *faah* plants. The patterns of NAE compositional changes are complex, and it is difficult to associate a particular NAE type with the differences observed in physiological responses. Future experiments to identify localized changes in NAE composition over shorter time periods may be required to uncover the types of NAEs that are most important to salt or osmotic stresses. Nonetheless it appears that changes in total NAE content measured in whole seedling extracts are in general agreement with differences in FAAH activities in these plants and these differences are associated with physiological responses to abiotic stresses.

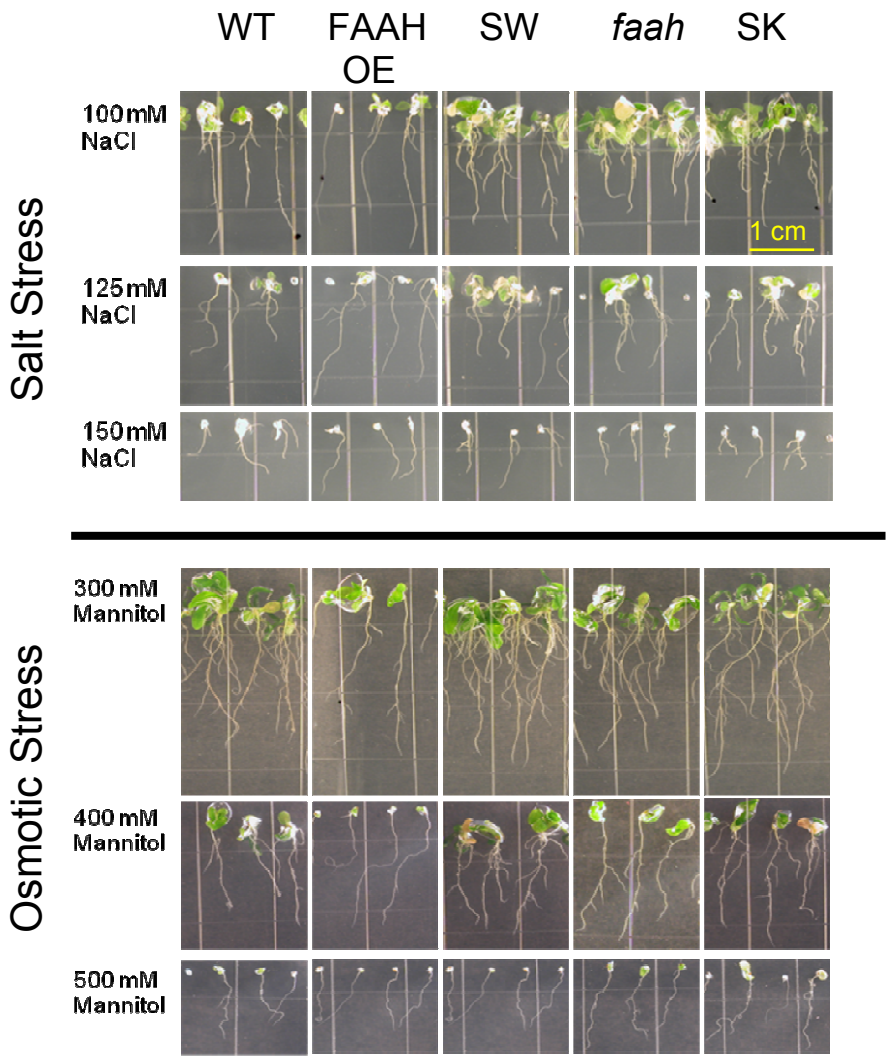
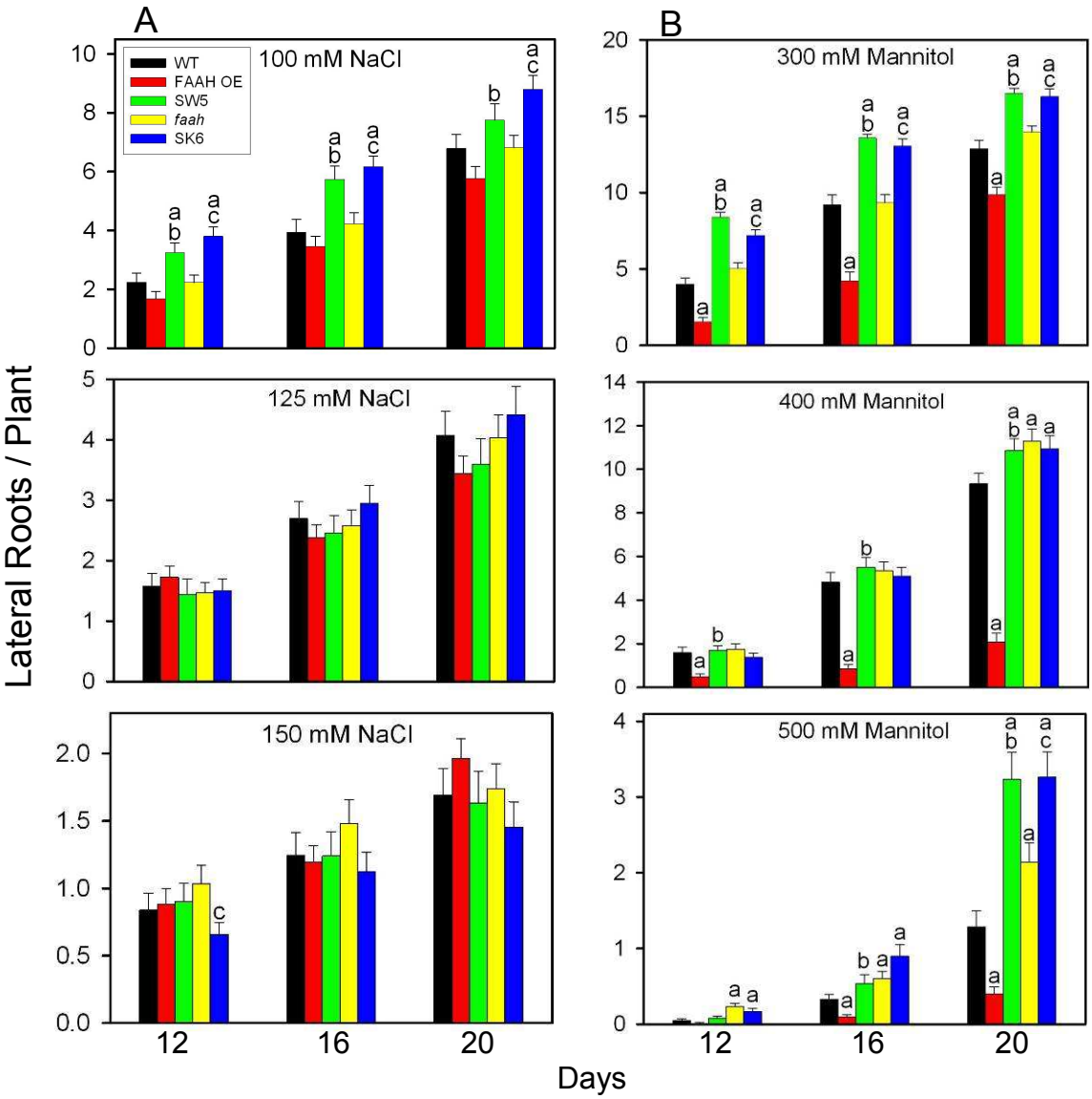


Figure 11. Growth in response to salt or osmotic stress in solid media.

(A) The appearance of plants after 20 days of growth in the presence of salt stress at the indicated NaCl concentrations.

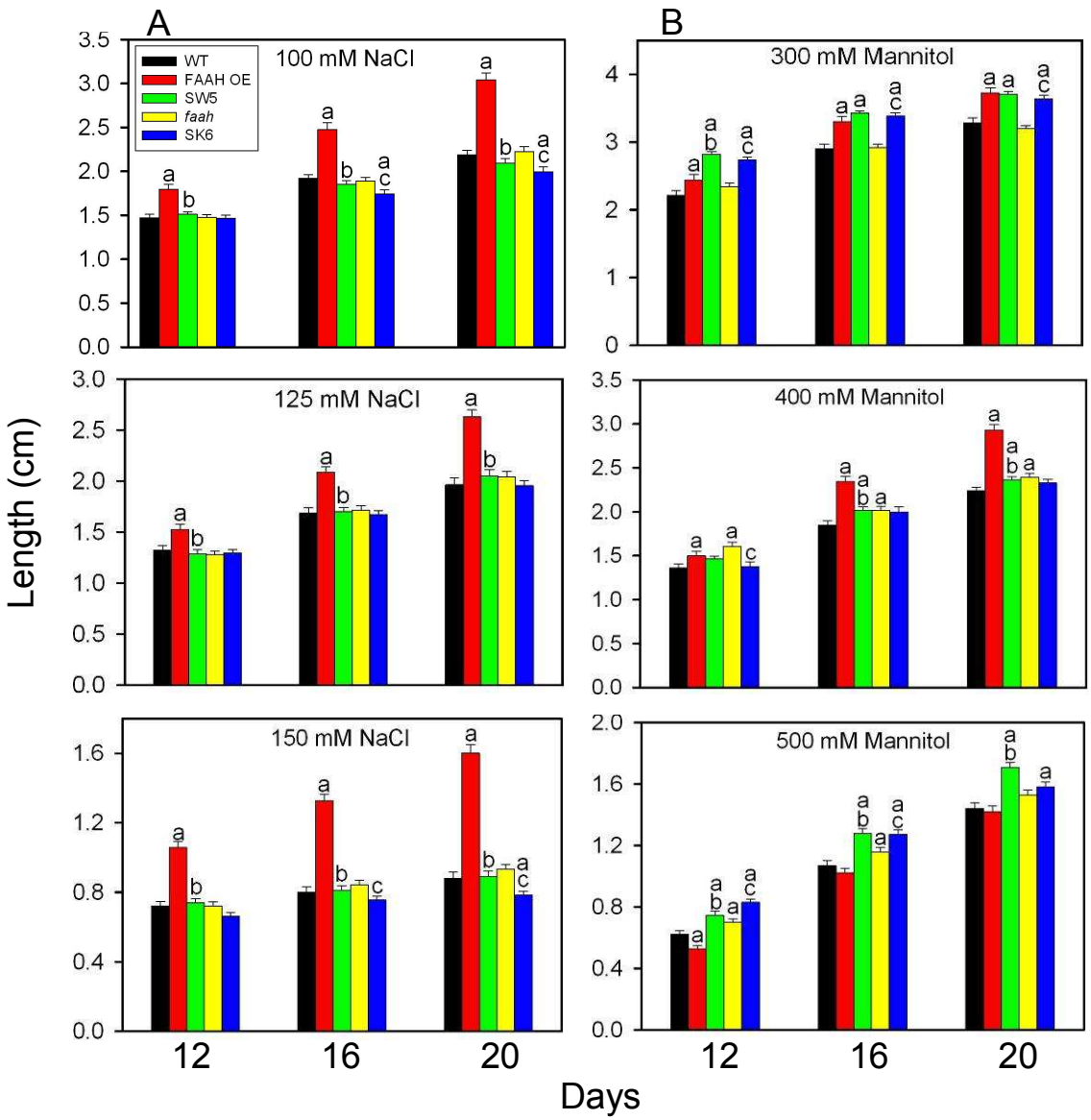
(B) The appearance of plants after 20 days of osmotic stress induced with the indicated concentrations of mannitol.



a: P<0.05 vs WT, b: P<0.05 vs FAAH OE, c: P<0.05 vs *faah*

Figure 12. Lateral root production in the presence of salt or osmotic stress. **(A)** Lateral root production on days 12, 16, and 20 of NaCl exposure at the indicated concentrations.

(B) Lateral root production on days 12, 16, and 20 in the presence of osmotic stress induced with the indicated mannitol concentrations.

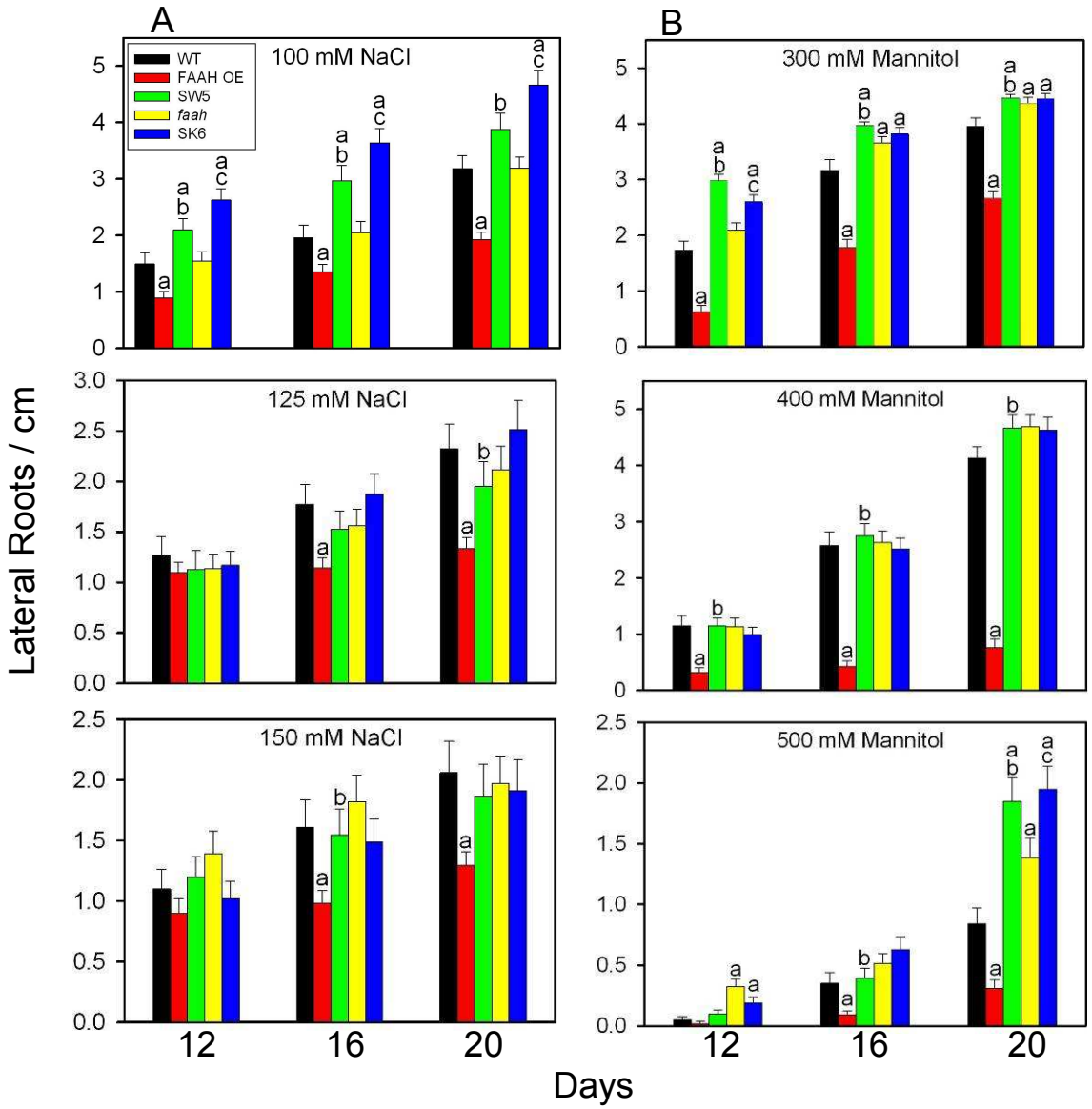


a: P<0.05 vs WT, b: P<0.05 vs FAAH OE, c: P<0.05 vs *faah*

Figure13. Primary root length in the presence of salt or osmotic stress.

(A) Primary root length on days 12, 16, and 20 of salt stress at the indicated NaCl concentrations.

(B) Primary root length on days 12, 16, and 20 of osmotic stress induced with the indicated mannitol concentrations.

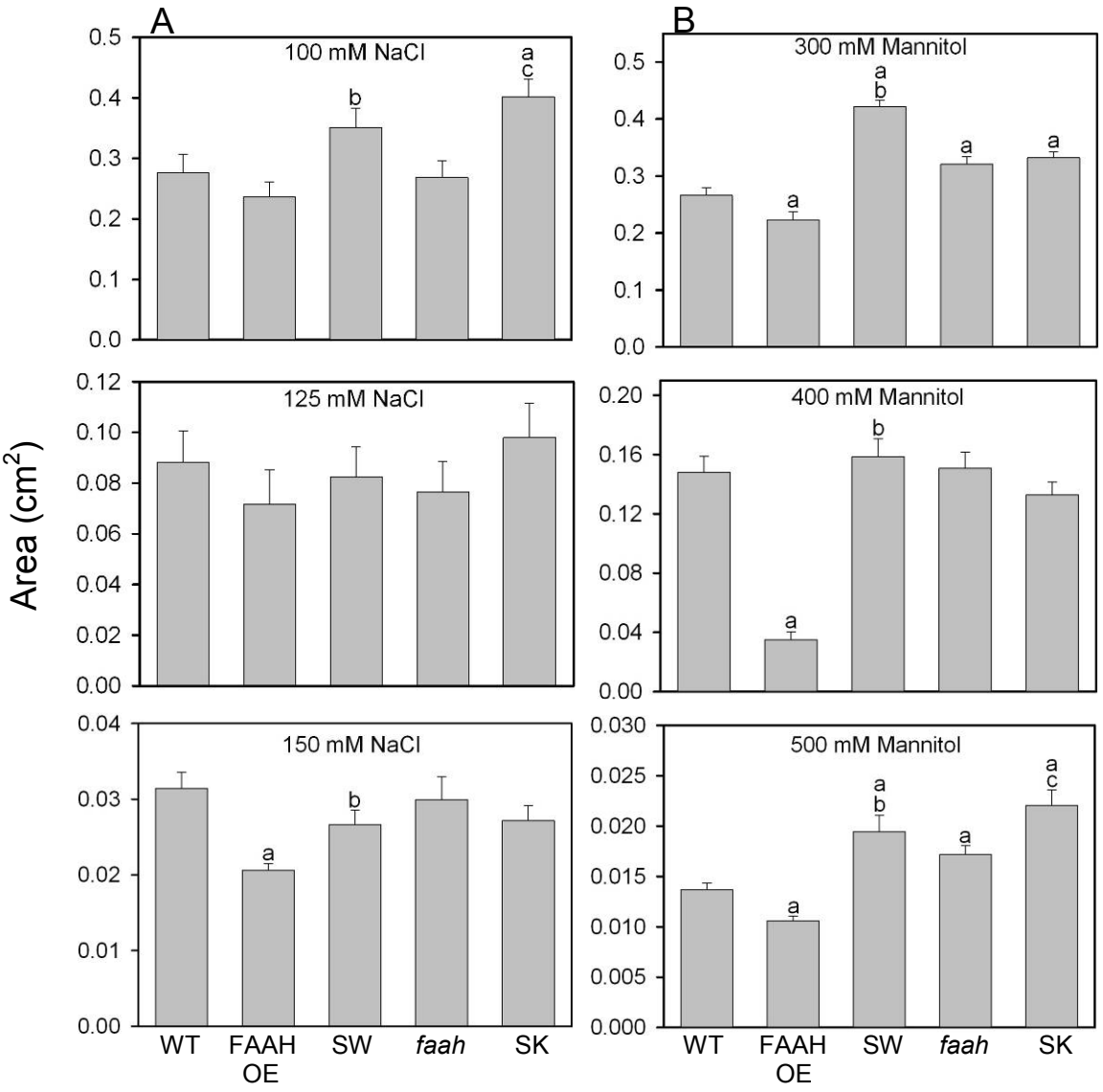


a: P<0.05 vs WT, b: P<0.05 vs FAAH OE, c: P<0.05 vs *faah*

Figure 14. Lateral root density in the presence of salt and osmotic stress.

(A) Lateral root density in number of lateral roots per cm of primary root in the presence of salt stress at the NaCl concentrations indicated.

(B) Lateral root density in number of lateral roots per cm of primary root in the presence of osmotic stress induced with the indicated concentrations of mannitol.



a: P<0.05 vs WT, b: P<0.05 vs FAAH OE, c: P<0.05 vs *faah*

Figure 15. Shoot area in the presence of salt or osmotic stress at day 20.

(A) Shoot area in the presence of salt stress at the NaCl concentrations indicated.

(B) Shoot area in the presence of osmotic stress induced with the indicated mannitol concentrations.

Table 2. Fold-change in *RD29b* transcript levels in response to salt and mannitol-induced osmotic stress in relation to unstressed WT					
Treatment	WT	FAAH OE	SW	*faah*	SK
No Treatment	1 ± 0.0	1.9 ± 0.82	1.8 ± 0.71	0.70 ± 0.09	1.3 ± 0.60
100 mM NaCl	1.0 ± 0.08	0.74 ± 0.08	0.62 ± 0.07	0.5 ± 0.01	0.88 ± 0.32
125 mM NaCl	1.1 ± 0.23	1.4 ± 0.74	1.3 ± 0.10	3.7 ± 2.2	1.1 ± 0.35
150 mM NaCl	13 ± 2.9	23 ± 3.5	2.5 ± 1.6	5.5 ± 0.40	3.2 ± 1.0
300 mM Mannitol	3.5 ± 2.9	1.3 ± 0.56	1.2 ± 0.62	1.2 ± 0.52	1.1 ± 0.56
400 mM Mannitol	7.8 ± 0.70	28 ± 5.5	1.3 ± 0.14	1.5 ± 0.18	1.1 ± 0.07
500 mM Mannitol	190 ± 100	170 ± 19	94 ± 40	280 ± 110	200 ± 110

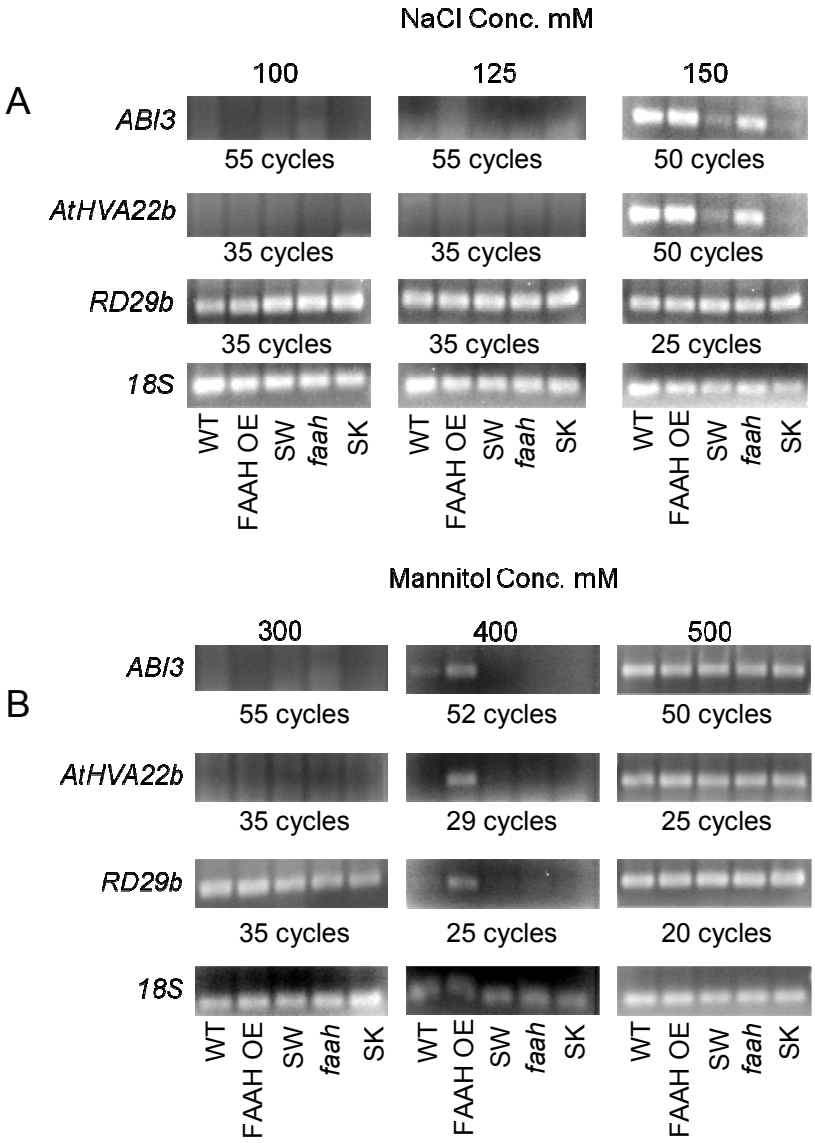


Figure 16. Relative band intensities in agarose gels corresponding to transcript levels of *ABI3*, *AtHVA22b*, and *RD29b* in response to salt or osmotic stress. **(A)** Relative transcript levels of *ABI3*, *AtHVA22b*, and *RD29b* in response to salt stress at 100 mM, 125mM, and 150 mM NaCl after the indicated number of RT-PCR cycles.

(B) Relative transcript levels of *ABI3*, *AtHVA22b*, and *RD29b* in response to osmotic stress induced with 300 mM, 400 mM, or 500 mM mannitol after the indicated number of RT-PCR cycles. In both cases18S was always run for 25 cycles.

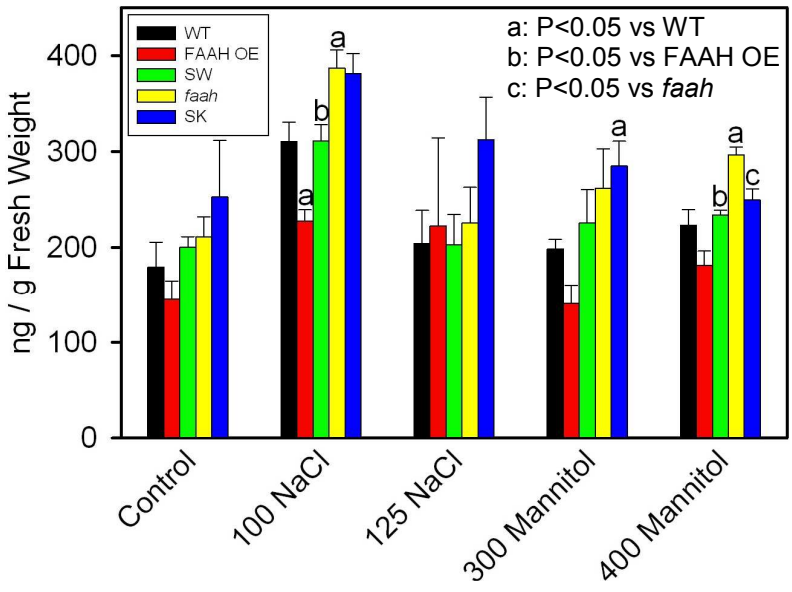


Figure 17. Levels of total NAE in response to salt or osmotic stress.

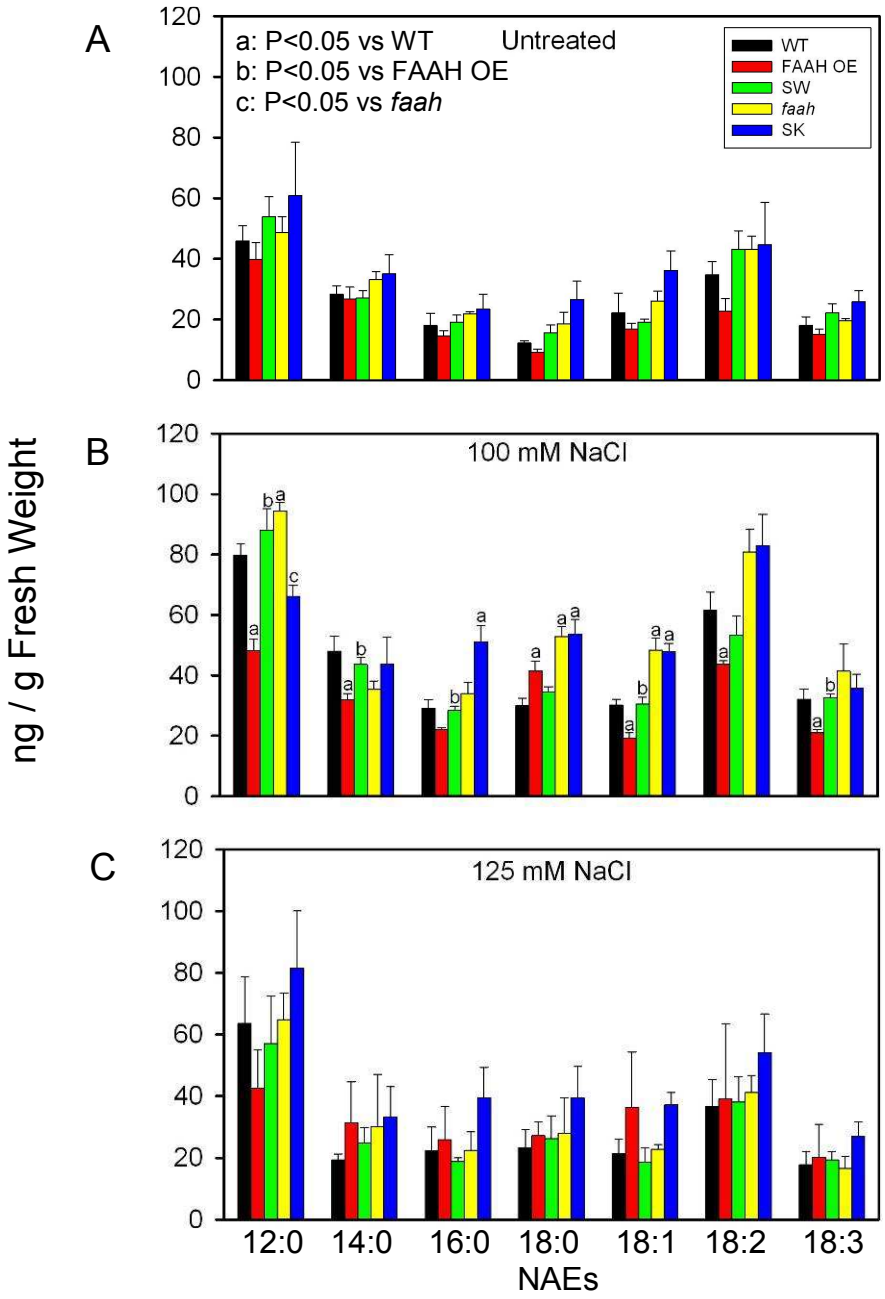


Figure 18. NAE profiles after 20 days of salt stress.

(A) NAE levels under untreated conditions.

(B) 100 mM NaCl.

(C) 125 mM NaCl.

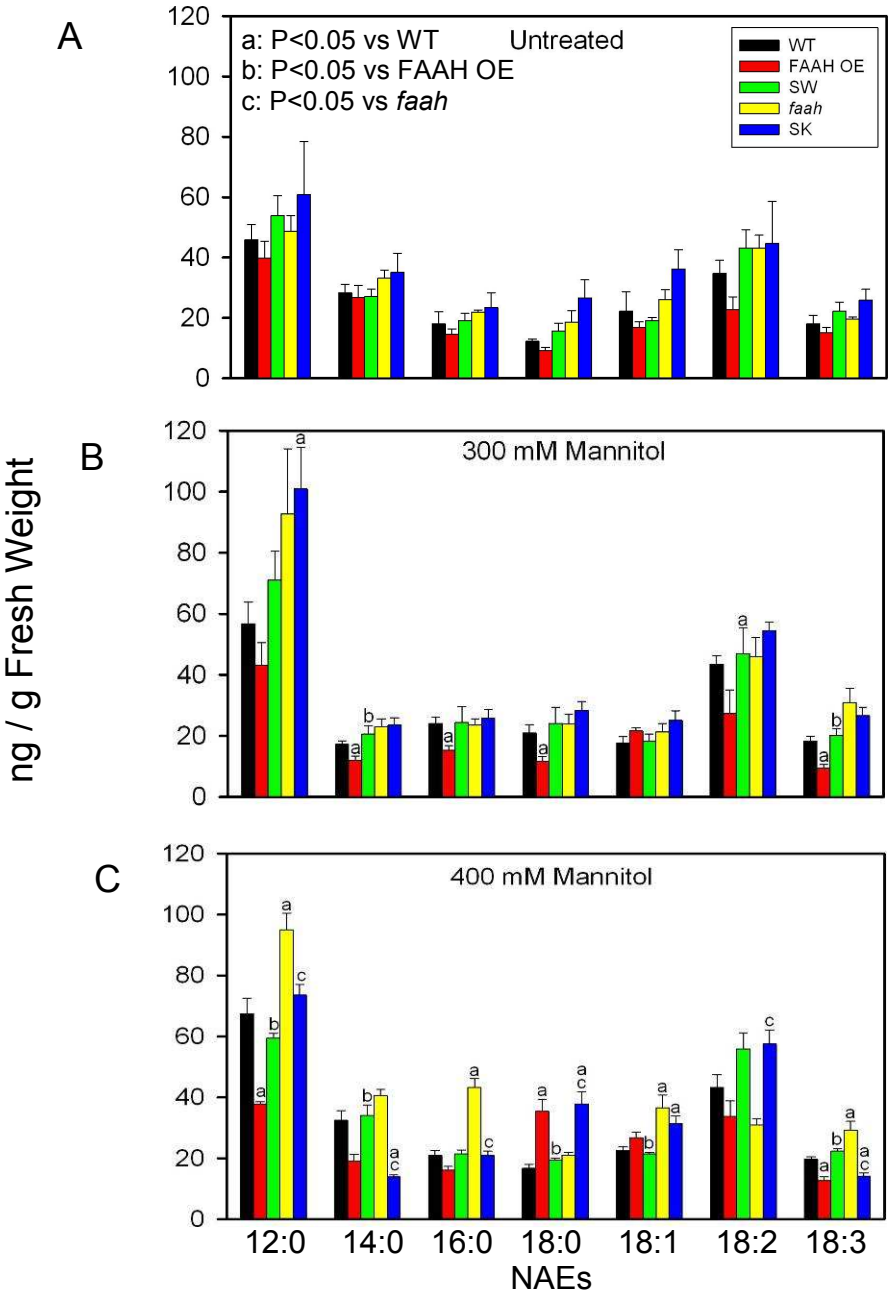


Figure 19. NAE profiles after 20 days of osmotic stress.

(A) NAE levels under untreated conditions.

(B) 300 mM mannitol.

(C) 400 mM mannitol.

Knockouts of a Prospective FAAH2 and Double Knockouts of FAAH 1 and 2 Respond Differently to Osmotic Stress than WT or *faah* Plants

In most cases in my experiments, phenotypes of *faah* knockout plants were similar to those of WT plants and we presume that this is due to redundancy, perhaps by a second FAAH gene. Others in the lab have been investigating this area and I have tested two other FAAH knockout genotypes for their ability to grow under osmotic stress conditions induced with 400 mM mannitol (Figure 20). These were the FAAH2 knockout (*faah(2)*) (At5g07360) and the FAAH1 and FAAH2 double knockout (*faah(1+2)*). Lateral root production was similar in the WT, *faah*, and *faah2* plants on days 12, 16, and 20 of mannitol treatment (Figure 20A). The *faah(1+2)* plants produced more lateral roots than the WT plants on day 12 of the time course. Primary root length was affected by a lesser degree by mannitol in the *faah(2)* and *faah(1+2)* plants, producing roots which were somewhat longer than those of the WT plants on days 12 and 16 of the time course (Figure 20B). The *faah(1+2)* plants formed more lateral roots than the WT plants on a per plant or per unit length basis, but only at early times in seedling growth (Figure 20C). In contrast to the signs of stress tolerance observed with the roots of the *faah2* and *faah1+2* plants, the shoots of the *faah2* plants showed signs of possible stress susceptibility by forming dramatically less shoot area than WT plants throughout the time course (Figure 20D), suggesting that FAAH1 and FAAH2 proteins may not have entirely overlapping functions.

Future work is required to tease apart the functions of FAAH1 and the prospective FAAH2 in abiotic stress.

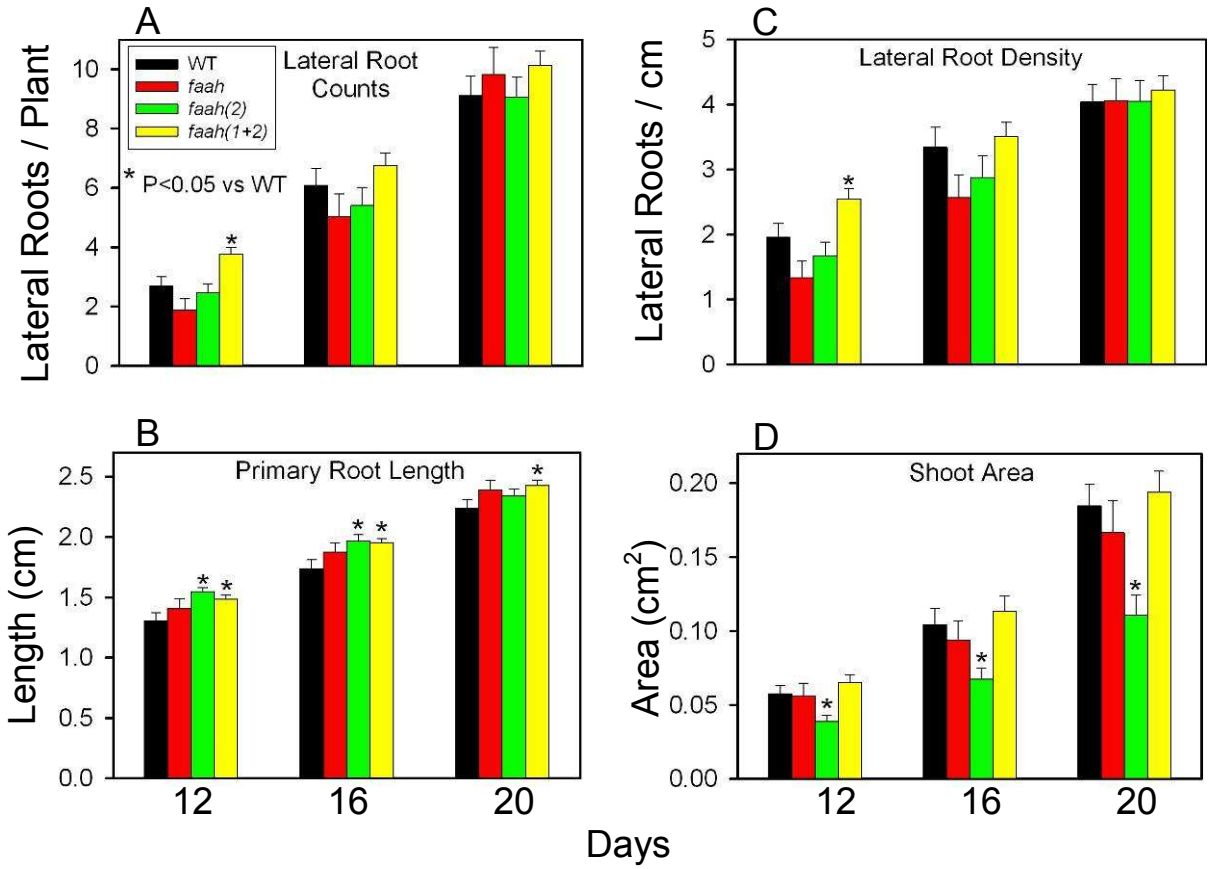


Figure 20. Growth of *faah*, *faah (2)*, and *faah (1+2)* after 20 days in the presence of osmotic stress induced with 400 mM mannitol.

(A) Lateral root production per plant.

(B) Primary root length.

(C) Lateral root density in lateral roots per cm of primary root.

(D) Shoot area.

CHAPTER 4

DISCUSSION

FAAH-overexpressors were consistently hypersensitive to abiotic stress conditions including salt stress, osmotic stress, chilling, heavy metal exposure, and nutrient limitation, while inactive FAAHm overexpressors were tolerant to salt and osmotic stress. One common factor among several of these stress conditions is the involvement of the phytohormone ABA. ABA is closely associated with responses involving water deprivation, and many of the above mentioned stress conditions can negatively influence plant-water relations. However, there may be other mechanisms accounting for the hypersensitivity of the FAAH OE plants and the tolerance of the FAAHm OE plants, SW and SK.

Only one of the FAAH OE lines tested, FAAH OE2, was hypersensitive to chilling stress (Figure 1). However, it is possible that FAAH OE2 is not the only line hypersensitive to chilling, but it is rather the most sensitive of the three, and this experiment was not able to resolve these differences clearly. Perhaps experiments with cooler temperatures or at different light intensities might reveal differences between other FAAH OE lines in relation to the WT and *faah* plants.

The reason for the hypersensitivity could be that cold stress can result in decreased root hydraulic conductance resulting in osmotic stress (Markhart, 1984). The levels of the phytohormone ABA rise in response to osmotic stress, and the FAAH OE plants have been shown to have a hypersensitivity to the

growth-inhibiting effects of this hormone (Teaster et al 2007). A difference in ABA translocation could in part explain the hypersensitivity of FAAH OE plants in terms of root development, but further experiments will be required to clarify phenotypic differences and understand the mechanisms of cold sensitivity in the FAAH OE lines.

The FAAH OE plants showed small but significant differential sensitivity to heavy metals- Cu and Cd (Figure 2). A connection between cadmium toxicity and NAEs has been demonstrated in animal systems (Kondo et al, 1998). The levels of NAEs in rat testes were observed to increase dramatically in the presence of $CdCl_2$. A possible reason for this accumulation of NAEs may be that NAEs have a protective role in injured tissues (Schmid et al, 1990). It has been proposed that NAEs carry out their protective functions in vertebrates by the following mechanisms (i) *N*-acylethanolamines stabilize mitochondrial membranes, thereby regulating the release of Ca^{2+} from the mitochondrial Ca^{2+} store sites (Epps et al., 1982) (ii) *N*-acylethanolamines enhance the rate of Ca^{2+} flux across the sarcoplasmic reticulum (Epps et al., 1982) and (iii) *N*-acylethanolamines inhibit lipid peroxidation (Parinandi and Schmid, 1998). It is possible that NAEs may carry out similar functions in plants in response to cadmium toxicity, and in the FAAH OE plants the accumulation of these potentially protective compounds is being inhibited through FAAH-mediated hydrolysis, resulting in hypersensitivity of the FAAH OE plants. Cadmium also targets plant-water relations (Poschenrieder et al., 1989), which may be

especially problematic for the FAAH OE plants. Cadmium has been shown to cause increased stomatal resistance along with decreased water content in the leaves of bean plants resulting in water deficit. Water deficit is known to trigger increases in ABA levels, a phytohormone to which the FAAH OE plants are hypersensitive. If a similar process is occurring in *Arabidopsis*, a cadmium-associated increase in ABA could also be contributing generally to the decreased root growth of the FAAH OE plants. Future experiments to measure the levels of phytohormones in Cd-treated plants may shed light on the relationship between ABA and FAAH-mediated hypersensitivity to Cd.

The FAAH OE lines also were more sensitive than WT or *faah* to copper (Figure 3), not surprising since copper can negatively affect plant growth by mechanisms similar to those of cadmium. Both cadmium (Romer-Puertas et al., 2007) and copper toxicity (Deckert, 2005) can lead to the formation of reactive oxygen species (ROS) in plants leading to oxidative damage of cellular components. FAAH OE plants may be compromised in their ability to deal with ROS if they are less able to accumulate NAEs and benefit from their ability to inhibit lipid peroxidation (Parinandi and Schmid, 1998). NAEs have been shown directly to inhibit lipid peroxidation by LOX isoforms (Keereetaweep et al., 2010), but they may also be able to play a role in protecting lipids from peroxidation by ROS.

The reduced ability of FAAH OE plants to accumulate NAEs, particularly NAE 12:0, may further interfere with their ability to counteract ROS by interfering

with the phenylpropanoid pathway, which is responsible for the production of compounds with antioxidant properties. Treatment of *Arabidopsis* plants with exogenously applied NAE 12:0 resulted in the up-regulation of various genes involved in the phenylpropanoid pathway, including phenylalanine ammonia lyase, chalcone synthase, and chalcone isoflavone isomerase (Teaster et al., 2007), which suggests that NAE 12:0 may be involved in up-regulating the phenylpropanoid pathway. If FAAH OE plants are indeed compromised in the ability to accumulate NAE 12:0, they may not be capable of properly regulating this pathway in response to heavy metals and other stresses, possibly resulting in a decreased production of protective compounds such as anthocyanins. Similarly to cadmium, copper toxicity can also lead to increases in the levels of ABA (Zengin and Kirbag, 2007), possibly contributing to the hypersensitivity of the FAAH OE plants to the growth-inhibiting effects of this hormone.

The roots of FAAH OE plants showed an increased sensitivity to phosphorus deficiency (Figure 4), another stress condition known to alter ABA levels in plants (Jeschke et al., 1997). Phosphorus deficiency was found to greatly enhance the levels of ABA synthesis in castor bean roots, resulting in a transient increase in the ABA levels of roots with most of the hormone subsequently being deposited into the xylem and transported to the leaves. If a similar situation is occurring in *Arabidopsis*, even if the increase in ABA is transient, it could be at least partly responsible for the increased inhibition of lateral root formation in the FAAH OE plants which was observed under P

deficiency. ABA has been shown to decrease lateral root production in *Arabidopsis* (De Smet et al., 2003). Since the FAAH OE plants are more sensitive to ABA than the other plant lines (Teaster et al., 2007), it could be at least in part why the FAAH OE lines produced fewer lateral roots than the other plants tested.

Nitrogen deficiency also caused significantly greater growth reductions in the FAAH OE plants than in the WT plants in terms of lateral roots produced and in lateral root density (Figure 5). This may be similar to the situation with these plants under P deficiency. Similar to phosphorus deficiency, nitrogen deficiency also can stimulate ABA synthesis in plant roots (Schraut et al., 2005). Since the FAAH OE plants are hypersensitive to ABA (Teaster et al., 2007) and since ABA has been shown to reduce lateral root production in *Arabidopsis* (De Smet et al., 2003), a greater reduction of lateral roots would be expected for the OE plants under N deficiency. Under conditions of N deficiency there may also be another mechanism taking place contributing to the greater inhibition of lateral roots observed for the OE plants. NAE 12:0 was found to increase the transcript levels of *At1g08090* and *At5g60770* (Teaster et al., 2007), suggesting a possible role for NAE 12:0 in their regulation. These genes are involved in high affinity nitrate transport (Cerezo et al., 2001), and *At1g08090* is also involved in lateral root formation in response to nitrogen limitation (Remans et al., 2006). If the roots of FAAH OE plants are compromised in their ability to accumulate NAE 12:0, it is possible that they are also compromised in their ability to initiate the transcription

of *At1g08090*, resulting in fewer lateral roots in the FAAH OE plants compared to the WT.

The FAAH OE plants were hypersensitive to salt stress when grown in liquid medium (Figure 6). The reason for this hypersensitivity may be found by their levels of ABA and JA in response to salt stress (Figure 7). While the levels of these hormones were not significantly different between the WT and FAAH OE plants either in the presence or absence of salt stress, the FAAH OE plants did undergo a significant increase in the levels of these hormones in the presence of 175 mM NaCl. This suggests that it may not be the absolute levels of these hormones which may affect the growth of the FAAH OE plants in the presence of salt stress, but rather the relative changes in the levels of these hormones. The levels of IAA were also analyzed in these plants, but no significant differences were found either in the presence or absence of salt stress. With the root growth phenotypes, IAA might be expected to have been different among these genotypes, but these seedlings were in liquid culture with constant motion, essentially subjecting the plants to agravitropic conditions. Since IAA is involved in gravitropic responses, the growth conditions in the liquid media could have lead to altered levels of IAA, which may have masked any differences in IAA levels due to the salt stress had they occurred. Future experiments with seedlings grown in solid media may show differences in auxin in FAAH OE roots in response to salt stress.

The levels of NAEs in response to salt and osmotic stress were also analyzed (Figure 8). The results obtained from salt stress in liquid culture were not as informative as it might have been expected since there were few differences in NAE levels which may have been related to the hypersensitivity of the OE plants in response to salt stress. These differences involved NAEs 18:0 and 16:0 in the OE plants, suggesting that these NAEs may play a role in the hypersensitivity of the OE plants. However, the experiments in solid media were much more informative about the NAE levels in response to salt and osmotic stress. In these experiments total NAE levels decreased in the OE plants in response to salt stress (100 mM NaCl) (Figure 17). Also, total NAE levels remained higher in one or both of the FAAHm plants than in the WT or FAAHm plants in response to salt stress (100 mM NaCl) or osmotic stress (300 mM and 400 mM mannitol). These findings suggest that overall higher NAE levels may serve a protective function under stress conditions since OE plants were generally hypersensitive to stress while the FAAHm plants were generally tolerant. It also became apparent that the levels of NAEs 12:0 and 14:0 were modulated in a manner that closely paralled the total NAE levels when individual NAE species were compared in the presence of salt (Figure 18) and osmotic stress (Figure 19). This is an interesting finding since both NAE 12:0 (Teaster et al., 2007) and NAE 14:0 (Tripathy et al., 1999) have been implicated with the regulation of genes involved in plant stress responses. Differences in the levels of other NAEs were also observed, and it is possible that these other NAEs may

play a role in plant abiotic stress responses. Unfortunately, while studies which implicate NAEs 12:0 and 14:0 with plant stress responses are limited, the function of other NAEs under stress conditions in plants remains to be explored. Future studies are needed to better understand the function of all NAEs in plants under stress conditions.

The transcript levels of selected genes known to be responsive to ABA and or salt stress were also analyzed for the salt stress experiments in liquid culture, and under these conditions the FAAH OE plants had higher transcript levels for *ABI3*, *AtHVA22b*, and *RD29b* (table 1; Figure 10). These results may suggest an enhanced sensitivity to ABA during salt stress. This sensitivity to ABA may be due to an enhanced ability for ABA to interact with promoters or other components controlling the transcription of these genes in the FAAH OE plants. However, it was only the FAAH OE plants that had a significant increase of ABA levels at the time they were analyzed in the presence of salt stress in liquid culture. Had the WT and *faah* plants also experienced a similar increase in ABA, they may have also showed a similar increase in the transcript levels of these genes and possibly a similar reduction in tissue mass. Therefore, it seems that the increase in transcript levels for these genes in the FAAH OE plants, and perhaps their hypersensitivity to salt stress, may be related not just to a greater sensitivity to ABA, but perhaps also to increased ABA levels resulting from increased synthesis and or reduced ABA catabolism in the FAAH OE plants during salt stress.

Another set of genes which was analyzed consisted of *SOS1*, *SOS2*, and *SOS3*. The transcript levels of all three of these genes were elevated in response to salt stress in the WT, *faah*, and FAAH OE plants to a comparable degree (table 1). Therefore, it is unlikely that any of these *SOS* genes may have anything to do with the enhanced sensitivity of the FAAH OE plants to salt stress.

The greater sensitivity of the FAAH OE plants to salt stress and osmotic stress was also evident when grown in solid MS media (Figure 11). Part of the reason why FAAH OE plants were consistently more sensitive to salt and osmotic stress than WT plants may be due in part to their levels of ABA. If there was a similar increase in the ABA levels of the FAAH OE plants as there was in the presence of salt stress in liquid culture, it could account for the greater growth inhibition of these plants when compared to the WT and *faah* plants.

The transcript levels of *ABI3*, *AtHVA22b*, and *RD29b* were analyzed after growth in solid media (table 2, Figure 16). Under the salt and mannitol concentrations in which these genes were induced they were consistently higher in the FAAH OE plants. Also, the growth of these FAAH OE plants was inhibited to a greater degree than that of the other plants. The function of *AtHVA22b* (Chen et al, 2002) and *RD29b* (Msanne et al., 2011) during stress responses remains unknown. These genes are ABA-responsive, with ABA responsive elements in their promoters, and their greater transcript levels in the FAAH OE plants during the stress conditions strongly suggests a role of altered ABA signaling, perhaps involving non-catalytic interactions with FAAH, contributing to

the hypersensitivity of the FAAH OE plants. Additional support for this hypothesis seems to come from Kim et al. (2009) in which inactive FAAH overexpressors were found to lose their tolerance to the growth-inhibiting effects of NAE 12:0 while still retaining their hypersensitivity to ABA. However, when these same inactive FAAH overexpressors where grown under osmotic stress or salt stress they have not only lost the stress hypersensitivity of the FAAH OE plants but are somewhat stress tolerant, particularly in mannitol-induced osmotic stress. This seems to indicate that unlike in ABA treatments alone there is a FAAH catalytic component involved in salt and osmotic stress hypersensitivity. This could be taking place in part by local alterations of NAE12:0 levels in specific cells within the plants which may be influencing the transcription of stress-related genes. According to the microarray study of the effects of NAE 12:0 on *Arabidopsis* by Teaster et al (2007), a number of stress-related genes are modulated by NAE 12:0 besides *ABI3*, *AtHVA22b*, and *RD29b*.

One of these genes is the proline transporter 2 (*At3g55740*). This gene codes for an amino acid transport protein with an affinity not only for proline but also glycine betaine, and gamma amino butyric acid (GABA) (Lehmann et al., 2011). Proline, glycine betaine, and perhaps GABA (Shelp et al., 1999) can function as compatible solutes during water deprivation in plants. However, these compounds may also have a function in protecting plants from oxidative damage during abiotic stress since these compounds, particularly GABA, are effective scavengers of hydroxyl radicals. If NAE 12:0 levels are reduced in a

specific location within the plant or at a specific developmental period, the ability of the plant to initiate transcription of this gene may be compromised, perhaps contributing to greater stress susceptibility of the FAAH OE plants.

Chitinases are known to be important in biotic stress responses in plants, particularly in response to fungal pathogens. However, these enzymes may also play a role in abiotic stresses including salt and osmotic stress (Takenaka et al., 2009). How chitinases function in abiotic stress responses is not clear, but it has been proposed that during abiotic stress that these enzymes may target carbohydrates, other than chitin, containing *N*-acetylglucosamine residues. The product(s) of this reaction may then act as signals leading to the generation of glycolipids, and the glycolipids may then act as signal molecules that function in the induction of proteins involved in protection from abiotic stress. The microarray study by Teaster et al. (2007) revealed that the transcript levels of two chitinases, At2g43590 and At5g24090, were modulated by NAE 12:0. The reduced levels of NAE 12:0 levels in the FAAH OE plants in the presence of stress conditions, could lead to reduced expression of these chitinases, possibly contributing to increased stress susceptibility.

NAE 12:0 was also found to modulate the expression of numerous late embryogenesis abundant proteins (LEAs) and heat shock proteins (Teaster et al., 2007). Both salinity and drought stress can lead to the denaturation and dysfunction of many proteins, and LEAs and heat shock proteins help protect plants from stress by controlling the proper folding of various proteins (Basia and

Altman, 2005). If levels of NAE 12:0 are reduced in certain tissues at certain times in the FAAH OE plants, it could be at least partly responsible for their hypersensitivity to salt and drought stress due to a reduction in the transcript levels of LEA and heat shock proteins.

FAAH has a broad substrate specificity with both amidase and esterase activities, and may thus be capable of hydrolyzing metabolites other than NAEs, which may play roles in plant stress responses. Another class of protective compounds produced by plants in response to salinity and drought stress are polyamines. Polyamines may modulate Ca^{2+} signaling involved in defense pathways (Yamaguchi et al, 2006) and function as antioxidants protecting the plants from oxidative damage (Bors et al., 1989). In the FAAH OE plants it could mean greater polyamine depletion, due to heightened amidase activity, than in the WT plants resulting in reduced signaling of defense pathways and in increased oxidative damage to the OE plants leading to reduced growth. Another possibility which may explain the increase in ABA of the FAAH OE plants in salt stress is the esterase activity of FAAH. ABA can be deactivated through glycosylation in which ABA is linked to a glucose moiety by an ester linkage. FAAH may be capable of releasing active ABA by breaking the ester linkage with glucose resulting in elevated ABA levels in the FAAH OE plants leading to a greater extent of growth inhibition.

The *faah* plants were somewhat stress tolerant in relation to the WT plants, particularly to salt and osmotic stress. This degree of tolerance

sometimes observed in the *faah* plants might be due to their ability to retain higher levels of NAE, possibly due to decreased NAE hydrolysis, during stress conditions which may provide them with a protective effect. A protective function of NAEs has been reported in animal tissues (Schmid et al, 1990), and a similar protective function may also occur in plants. A similar trend of subtle but yet significant signs of stress tolerance were also observed in the *faah(2)* and *faah(1+2)* plants in the presence of osmotic stress. It is possible that the *faah(2)* and *faah(1+2)* may also have somewhat higher levels of NAEs providing them with a degree of protection similar to the *faah* plants.

There was a significant amount of salt and osmotic stress tolerance in the SW and SK plants. A possible explanation for this may be that despite the inability of the inactive FAAH to hydrolyze substrates, it may still be capable of interacting with them. This may make it possible for the inactive FAAH to sequester some potentially protective compounds, including NAEs and possibly polyamines, and protect them from degradation by other enzymes, perhaps resulting in an accumulation of these protective compounds. This ability to sequester substrates may also help the plants to maintain more ideal ratios of conjugated and non-conjugated hormones contributing to plant protection but not excessive growth inhibition, by sequestering conjugated hormones and shielding them from dissociation from their glucose moiety, and thus keeping them in an inactive state.

Despite the various results pointing to the stress hypersensitivity of the FAAH OE plants, there was one area where they seemed to have a certain degree of tolerance, and that was in primary root elongation in the presence of salt and osmotic stress. A possible reason for this may be the ABA hypersensitivity of the FAAH OE plants. ABA has been shown to inhibit lateral root formation in *Arabidopsis* (DeSmet et al, 2003). The ABA hypersensitivity of the FAAH OE plants could result in a greater inhibition of lateral roots than in the other FAAH genotypes. The greater primary root elongation of the FAAH OE plants could be a compensatory response in order to increase their root surface area in order to increase their access to water under the water deficit conditions imposed by either the salt stress or osmotic stress. In the case of salt stress increased root surface area could also be beneficial in accessing nutrients since salt stress can result in potassium deficiency, resulting from the competition of sodium ions for access to potassium channels in roots (Zhu, 2002).

NAEs have well important roles in animal systems, and the same appears to be the case in plants. NAEs have been observed to have roles in plant development as well as in responses to both biotic and abiotic stress conditions. Lipids and their metabolism have been often overlooked in their possible roles in plant stress tolerance and susceptibility. However, NAEs and their metabolism may provide valuable targets for plant improvement. NAEs have been observed to have protective functions in animal, and similar protective functions may occur in plants. NAE 12:0 is closely associated with ABA, a phytohormone involved in

multiple plant stress responses. This association may provide a mechanism by which to modulate ABA levels resulting in increased stress tolerance. Also the overexpression of inactive FAAH may provide another possible mechanism by which to produce plants with enhanced abiotic stress tolerance.

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