FUNCTIONAL CHARACTERIZATION OF PLANT FATTY ACID AMIDE HYDROLASES

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Fatty acid amide hydrolase (FAAH) terminates the endocannabinoid signaling pathway that regulates numerous neurobehavioral processes in animals by hydrolyzing a class of lipid mediators, N-acylethanolamines (NAEs). Recent identification of an Arabidopsis FAAH homologue (AtFAAH) and several studies, especially those using AtFAAH overexpressing and knock-out lines suggest that a FAAH-mediated pathway exists in plants for the metabolism of endogenous NAEs. Here, I provide evidence to support this concept by identifying candidate FAAH cDNA sequences in diverse plant species. NAE amidohydrolase assays confirmed that several of the proteins encoded by these cDNAs indeed catalyzed the hydrolysis of NAEs in vitro. Kinetic parameters, inhibition properties, and substrate specificities of the plant FAAH enzymes were very similar to those of mammalian FAAH. Five amino acid residues determined to be important for catalysis by rat FAAH were absolutely conserved within the plant FAAH sequences. Site-directed mutation of each of the five putative catalytic residues in AtFAAH abolished its hydrolytic activity when expressed in Escherichia coli. Contrary to overexpression of native AtFAAH in Arabidopsis that results in enhanced seedling growth, and in seedlings that were insensitive to exogenous NAE, overexpression of the inactive AtFAAH mutants showed no growth enhancement and no NAE tolerance. However, both active and inactive AtFAAH overexpressors displayed hypersensitivity to ABA, suggesting a function of the enzyme independent of its catalytic activity toward NAE substrates. Yeast two-hybrid screening identified Arg/Ser-rich zinc knuckle-containing protein as a candidate protein that physically and domain-specifically interacts with AtFAAH and its T-DNA knock-out Arabidopsis was
hypersensitive to ABA to a degree similar to AtFAAH overexpressors. Taken together, AtFAAH appears to have a bifurcating function, via NAE hydrolysis and protein-protein interaction, to control Arabidopsis growth and interaction with phytohormone signaling pathways. These studies help to functionally define the group of enzymes that metabolize NAEs in plants, and further will expand the knowledge-base of lipid metabolism and signaling for manipulation of various physiological processes important to plant growth and responses to environmental stress.
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Sang-Chul Kim
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COMPREHENSIVE LIST OF ABBREVIATIONS

2-AG, sn-2-arachidonoyl glycerol

ABA, abscisic acid

ABI, ABA-insensitive

AS, amidase signature

At-AMI1, Arabidopsis amidase 1

AtFAAH, Arabidopsis fatty acid amide hydrolase

BiFC, biomolecular fluorescence complementation

Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol

CB, cannabinoid

DDM, n-dodecyl-β-D-maltoside

DMSO, dimethyl sulfoxide

EDTA, ethylenediamine tetraacetic acid

ESI-MS/MS, electrospray ionization tandem mass spectrometry

EST, expressed sequence tag

FAAH, fatty acid amide hydrolase

FFA, free fatty acid

FRET, fluorescence resonance energy transfer

GFP, green fluorescence protein

HA, hemagglutinin

IPTG, isopropyl-β-D-thiogalactopyranoside

KO, knock-out
LOX, lipoxygenase
LW, leucine-tryptophane
LWH, leucine-tryptophane-histidine
LWHAdX, leucine-tryptophane-histidine-adenine-X-gal
MAFP, methyl arachidonoyl fluorophosphonate
NAAA, NAE acid amidase
NADA, N-arachidonoyl dopamine
NAE, N-acylethanolamine
NAPE, N-acylphosphatidylethanolamine
OE, overexpressor
PA, phosphatidic acid
PABP8, polyadenylate-binding protein 8
PAL, phenylalanine ammonia lyase
PC, phosphatidyl choline
PE, phosphatidyl ethanolamine
PLD, phospholipase D
PMSF, phenylmethylsulfonyl fluoride
PPAR, peroxisome proliferator receptor
RK, R307A mutant AtFAAH expressed in Arabidopsis AtFAAH knock-out
RRM, RNA recognition motif
RSZ33, Arg/Ser-rich zinc knuckle-containing protein 33
RT-PCR, reverse transcriptase-polymerase chain reaction
RW, R307A mutant AtFAAH expressed in Arabidopsis wild-type
SD, synthetic drop-out

SK, S281A/282A mutant AtFAAH expressed in *Arabidopsis AtFAAH* knock-out

SW, S281A/282A mutant AtFAAH expressed in *Arabidopsis* wild-type

T-DNA, transfer DNA

THC, Δ⁹-tetrahydrocannabinol

TLC, thin layer chromatography

URB597, 3’-carbamoyl-biphenyl-3-yl-cyclohexylcarbamate

YPDA, yeast extract-peptone-dextrose-adenine hemisulfate

X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
CHAPTER 1
INTRODUCTION AND BACKGROUND*

N-Acylethanolamine (NAE)

N-Acylethanolamines (NAEs) consist of a fatty acid with various chain lengths and double bonds linked to ethanolamine via an amide bond (Fig. 1.1). They are a family of minor lipid constituents naturally present in a variety of organisms from fungi to plants and animals. Most of research on NAEs thus far has been in mammalian systems where these lipids exert physiological, behavioral and neurological roles as part of the endocannabinoid signaling system.

The Endocannabinoids Signaling System in Mammals

Cannabis sativa (marijuana) is well known for both its contribution to drug addiction, and its medicinal use in the relief of chronic pain. Although utilized for medicinal and recreational purposes for centuries, the molecular mechanisms underlying marijuana’s physiological effects remained unresolved until it was demonstrated that Δ²-tetrahydrocannabinol (THC; Fig. 1.1), an abundant secondary metabolite in the Cannabis plant, binds to a group of G-protein coupled receptors in the brain, the cannabinoid (CB) receptors (Di Marzo et al., 2007). The binding of THC to CB receptors mimics the activity of endogenous NAEs some of which are natural ligands of CB receptors (Devane et al., 1992). CB receptors together with an array of intracellular enzymes that catalyze the formation, breakdown and intracellular transport of endogenous CB receptor ligands, constitute an important signaling pathway in animal tissues, the endocannabinoid system, which includes the metabolism of NAEs (Mouslech and Valla, 2009;…

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Di Marzo, 2009). Since it was discovered that THC and endogenous NAEs are agonists of CB receptors, there has been an exponential increase in the number of studies on the endocannabinoid signaling system, which have implicated this pathway in the regulation of a broad range of physiological, behavioral and neurological functions in animals (Di Marzo et al., 2007; Wang and Ueda, 2009).

The most widely studied NAE in mammals is N-arachidonylethanolamine or anandamide (Fig. 1.1), shown to be an endogenous ligand of the CB1 receptors, the predominant CB receptor subtype associated with the central nervous system (Devane et al., 1992; Goodfellow and Glass, 2009). A second CB receptor (CB2) is expressed in immune and blood cells, and mostly linked to functions of peripheral systems (Wang and Ueda, 2009). In addition to anandamide, many structurally similar compounds were identified that could function as CB receptor agonists, including 2-arachidonoyl glycerol ether (noladin ether; Hanus et al., 2001), N-arachidonoyl dopamine (NADA; Bisogno et al., 2000), and virodhamine (Porter et al., 2002). Other endogenous NAEs including N-oleylethanolamine and N-palmitoylethanolamine also possess biological activity that could exert their physiological effects via CB-dependent or CB-independent pathways (Di Marzo et al., 2007; Wang and Ueda, 2009). Various NAE species are present in plants and fungi, suggesting that a signaling pathway similar to the mammalian endocannabinoid system might operate in other organisms (Chapman, 2004; Kilaru et al., 2007; Merkel et al., 2005) although there is no direct evidence that these organisms have the canonical endocannabinoid system per se.

Structure and Occurrence of N-Acylethanolamines in Plants

The acyl chain length and degree of saturation of NAEs vary depending upon the tissue,
developmental stage, and pathological condition of the plant (Chapman, 2004; Kilaru et al., 2007). NAE are named based on the number of double bonds and carbons in their acyl chain. For example, anandamide, which has 20 carbons and 4 double bonds in its acyl chain, is often referred to as NAE20:4 (Fig. 1.1). Plant NAEs were first identified in processed, seed-derived products at concentrations several fold higher than typically found in vegetative plant tissues (Chapman et al., 1999). The NAE types identified in seeds were composed of 12 to 18 carbons with 0 to 3 double bonds, with NAE12:0, NAE16:0, NAE18:1, and NAE18:2 being the most abundant. The major types of NAEs identified in seeds are similar to those that occur in animal tissues, except for NAE12:0 being unique to plants and NAE20:4, which has only been detected in animals.

Biological Functions of N-Acylethanolamines in Plants

NAEs impact a number of important biological processes in plants indicating that these compounds could play fundamental roles in plant physiology (summarized in Table 1.1).

**NAE in Plant-Microbe Interactions**

One of the first indications that NAE could play a significant role in plants was the observation that these compounds accumulated in the growth medium of tobacco suspension cells and in leaves within 10 min of applying the fungal elicitor, xylanase (Chapman et al., 1998). These results suggested that NAE accumulation in plants might function in defense against microbial pathogens and play analogous roles to the cytoprotective activity attributed to NAEs in animal tissues subjected to stress. One mechanism by which NAE could facilitate pathogen defense could be through the induction of defense related genes. Indeed, exogenous NAE14:0
application triggered the expression of phenylalanine ammonia lyase (\textit{PAL2}; Tripathy et al., 1999), which had been previously implicated in plant defense against pathogens (Dixon et al., 2002). The activation of \textit{PAL2} expression was blocked by antagonists of mammalian CB receptors (Tripathy et al., 2003), suggesting that NAE, like in animals, might participate in signal transduction events leading to plant defense responses through a CB-like receptor. Moreover, the ectopic overexpression of a plant fatty acid amid hydrolase (FAAH), an enzyme that degrades NAE, renders \textit{Arabidopsis} plants more susceptible to both host and non-host bacterial pathogens (Kang et al., 2008). The enhanced susceptibility of FAAH overexpressing plants to bacterial pathogens was associated with the down-regulation of several defense related transcripts (Kang et al., 2008). NAE might influence plant-microbe interactions by modulating the levels of other lipids that impact plant responses to pathogens, such as phosphatidic acid, by affecting the activity of enzymes involved in their biosynthesis. Indeed, NAE can specifically inhibit phospholipase D alpha (PLD\(\alpha\)) activity in several plant species \textit{in vitro} and selectively inhibit abscisic acid (ABA)-induced stomatal closure that normally involves PLD\(\alpha\) activity, thereby providing a mechanism in which phosphatidic acid levels in plant cells are regulated when localized NAE concentrations rise (Austin-Brown and Chapman, 2002).

\textit{NAE in Seedling Development and Hormone Signaling}

NAEs are present in substantial amounts in desiccated seeds, and their levels decline after imbibition and germination to barely detectable levels (Chapman et al., 1999), suggesting that these lipids may have a role in the regulation of seed germination and normal seedling development. In fact, NAE12:0 and NAE18:2 have been shown to have potent growth inhibiting properties when supplied exogenously to \textit{Arabidopsis thaliana} seedlings (Blancaflor et al., 2003;
Wang et al., 2006). One mechanism by which NAEs may impact seedling growth is by disrupting the organization of the cytoskeleton and endomembrane system, indicated by the observation that exogenous application of NAE12:0 to *Arabidopsis* resulted in bundled actin networks in hypocotyls and disrupted microtubule arrangement and endomembrane reorganization in roots (Blancaflor et al., 2003; Motes et al., 2005). The abundance of NAEs in desiccated seeds and their rapid depletion during seed germination (Chapman et al., 1999) suggest that these compounds might be negative regulators of normal seedling growth. Thus, the rapid catabolism of NAE during germination could be essential for the transition from seeds to post-germinative seedling growth, and their depletion could serve as a signal to reorganize the cytoskeleton and endomembrane system and support normal cellular functions that are necessary for early seedling development.

In addition, NAEs might participate in the regulation of the seed to seedling transition through its interaction with plant hormones. For example, abscisic acid (ABA), which also has been demonstrated to affect cytoskeletal organization (Olinevich and Khokhlova, 2003), inhibits the growth of *Arabidopsis* seedlings within a similar developmental window as NAEs (i.e. within 6 days after germination; Teaster et al., 2007). Like NAE, ABA is rapidly depleted during seed germination and the combined application of both compounds to *Arabidopsis* seeds produced a synergistic reduction in germination and seedling growth (Teaster et al., 2007). Moreover, NAE-treated seedlings had elevated transcripts for a number of ABA-responsive genes, which was attributed in part to regulating the expression of ABA-insensitive 3 (ABI3), a key transcription factor for many ABA-responsive genes (Teaster et al., 2007). These observations imply that normal seedling establishment requires the coordinated depletion of both NAE and ABA. These studies also suggest that both metabolites could function together in modulating the growth of
very young seedlings when conditions are not favorable for establishment.

Enzymatic Regulation of $N$-Acylethanolamine Levels in Plants

While biosynthetic and hydrolytic pathways of NAEs are highly conserved between animal and plant systems, there are some notable differences in terms of biochemical properties of the enzymes that metabolize NAE. Notably, $N$-acylphosphatidylethanolamine (NAPE), the immediate lipid precursor of NAE, is synthesized through substantially different pathways between animals and plants. Pathways for the formation and hydrolysis of NAE in animal and plant systems are illustrated in Fig. 1.2.

Hydrolysis and Oxidation of NAE

The hydrolysis of NAEs to free fatty acids and ethanolamine is accomplished by the action of an amidase with broad substrate specificity, designated as the fatty acid amide hydrolase (FAAH; Fig. 1.2; Cravatt and Lichtman, 2002), whose structural and functional properties are thoroughly described in the next section. Alternatively, NAEs with two or more double bonds can be oxidized to produce NAE oxylipins, and in plants this oxidation was demonstrated to occur via the lipoxygenase (LOX) pathway in cottonseed microsomes (Shrestha et al., 2002). Thus, polyunsaturated NAEs are believed to be metabolized by two competing pathways: FAAH and LOX pathways. However, the relative contribution of these two pathways to NAE depletion during seed germination and normal seedling development remains to be determined. The oxidation of polyunsaturated fatty acids leads to important fatty acid signaling compounds in both animals (e.g. prostanoids) and plants (e.g. jasmonic acids), and so oxidation of polyunsaturated species of NAEs may lead to novel compounds with new biological activities.
Biosynthesis of NAE

It is generally accepted that in both animal and plant tissues NAEs are produced from a group of unusual membrane phospholipids called $N$-acylphosphatidylethanolamines (NAPE) by a PLD-type hydrolysis with concomitant release of phosphatidic acid (Fig. 1.2). A novel PLD with a NAPE-specific activity (NAPE-PLD) was recently cloned from mammals and was responsible for the formation of NAE from NAPE in vivo (Okamoto et al., 2004; Okamoto et al., 2005). Among the five isoforms of plant PLDs, only two recombinant Arabidopsis enzymes, PLD$\beta$ and PLD$\gamma$, have been reported to hydrolyze NAPE to NAE in vitro, although at that time only three of the twelve PLD genes in Arabidopsis had been cloned and no examples of PLD$\delta$ or PLD$\zeta$ were tested (Pappan et al., 1998). Consequently unlike the simpler situation with the single mammalian NAPE-PLD, the non-specific nature of these plant PLDs and multiplicity of their corresponding genes (Wang, 2004) make it difficult to sort out the precise PLD isoform(s) in plants responsible for NAE formation in vivo. NAEs are also synthesized through NAPE-PLD-independent pathways in animals, such as through glycerophospho-NAE intermediate by $\alpha/\beta$-hydrolase 4 and glycerophosphodiesterase or through phospho-NAE intermediate by phospholipase C and a phosphatase (Ahn et al., 2008).

Biosynthesis of NAPE

Compositional similarities between NAE types and the $N$-acyl groups in NAPE in the same tissues suggest that the synthesis of NAPE may be important in determining the profile of available NAEs and their subsequent action. In animals NAPE is synthesized by $N$-acylation of phosphatidylethanolamine, in which an acyl group is transferred from the sn-1 position of a glycerophospholipid molecule to the amino group of phosphatidylethanolamine, catalyzed by
Ca\(^{2+}\)-dependent \(N\)-acyltransferase (Fig. 1.2; Okamoto et al., 2009; Schmid et al., 1996). This \(N\)-acyltransferase catalyzes the transacylation reaction from various glycerophospholipids, including phosphatidylcholine, 1-acyl-lyso-phosphatidylcholine, phosphatidylethanolamine, and cardiolipin, but not acyl-CoA, and has no direct acylation activity using free fatty acids as acyl donors (Wang and Ueda, 2009). In plants, a membrane bound enzyme, designated NAPE synthase, was purified from imbibed cottonseeds and utilizes free fatty acids for the direct acylation of phosphatidylethanolamine by a reverse serine-hydrolase type catalytic mechanism (McAndrew and Chapman, 1998). Recently, however, a candidate membrane-bound acyltransferase from Arabidopsis was cloned and characterized as an NAPE synthase \textit{in vitro} and \textit{in planta} (Faure et al., 2009). Interestingly, the NAPE synthase had acyltransferase activity \textit{in vitro} with acyl-CoAs (from \(^{14}\)C-palmitoyl-CoA and \(^{14}\)C-stearoyl-CoA), but not with free fatty acids (\(^{14}\)C-palmitic acid and \(^{14}\)C-stearic acid). Thus, this represents a new, acyl-CoA-dependent NAPE synthase distinct from either the direct acylase previously characterized in cottonseeds or from the \(N\)-acyltransferase enzyme mechanism that operates in mammalian cells.

\textbf{Fatty Acid Amide Hydrolase (FAAH)}

Fatty acid amide hydrolase (FAAH) is believed to be primarily responsible for the termination of endocannabinoid signaling in animal systems by the timely degradation of anandamide into ethanolamine and its corresponding free fatty acid. FAAH is a broad specificity amidase and esterase and hydrolyzes a wide range of NAEs and other acylamides and esters of biological relevance. Thus, FAAH modulation has become an important focus for understanding the mechanistic action of the endocannabinoid signaling system in vertebrates, and a convenient target for the development of novel therapeutics (McKinney and Cravatt, 2005).
Mammalian Fatty Acid Amide Hydrolase

A rat FAAH cDNA was isolated first by Cravatt et al. in 1996 (Cravatt et al., 1996), and functional orthologues of FAAH have been identified in other mammalian species, including human, mouse (Giang and Cravatt, 1997) and pig (Goparaju et al., 1999). These membrane-bound proteins are 579 amino acids in length and belong to an enzyme superfamily called the “amidase signature (AS)” family which is characterized by a highly conserved region (~130 amino acids) that is rich in serine, glycine and alanine residues (Chebrou et al., 1996). The AS region is common to more than 80 amidases and it corresponds to amino acids 215-257 in rat FAAH. Mammalian FAAH proteins share over 80% sequence identity, as well as similar substrate selectivities and inhibitor sensitivities (Deutsch et al., 2002). The FAAH recombinant proteins hydrolyze a broad range of alkyl/acyl amide and fatty acid ester (e.g. 2-arachidonyl glycerol and fatty acid methyl esters) substrates in vitro (Giang and Cravatt, 1997; Goparaju et al., 1999). FAAH was named in recognition of the large number of endogenous fatty acid amides that it accepts as substrates, including NAEs and fatty acid primary amides such as oleamide. Recently, the X-ray crystal structure of rat FAAH revealed several unusual features of the enzyme (Bracey et al., 2002). In contrast to the Ser-His-Asp triad typical of most serine hydrolases, the core catalytic machinery of FAAH consists of a Ser-Ser-Lys catalytic triad (S241-S217-K142), where S241 and K142 play key catalytic roles as the nucleophile and acid/base, respectively and S217 bridges between the two residues to act as a “proton shuttle” (Patricelli and Cravatt, 1999; Patricelli and Cravatt, 2000). Despite the presence of a predicted N-terminal transmembrane segment, FAAH is believed to be anchored to the membrane bilayer through a hydrophobic “foot” formed by the arrangement of three α-helices (amino acids 404-433 in the rat sequence). The enzyme also possessed channels that allow simultaneous access to
both the membrane and cytoplasmic compartments of the cell, possibly to facilitate substrate binding, product release, and catalytic turnover (Bracey et al., 2002). As expected, FAAH (-/-) knock-out mice displayed a significant elevation of anandamide and other NAEs compared with wild-type mice, increased sensitivity to exogenously applied anandamide, and a variety of neurobehavioral abnormalities consistent with altered endocannabinoid signaling, such as hypomotility, analgesia, catalepsy, and hypothermia (Cravatt et al., 2001). These findings suggest that FAAH may represent an attractive therapeutic target for the treatment of neurobehavioral disorders and have stimulated interest in the development of specific inhibitors of this enzyme.

Molecular Identification of Fatty Acid Amide Hydrolase Homologue in Arabidopsis

Based upon the occurrence of an AS domain and conservation of key catalytic residues, a candidate FAAH homologue (gene locus At5g64440) was identified in the Arabidopsis genome (Shrestha et al., 2003). A corresponding cDNA was isolated and predicted to encode a protein of 607 amino acids with a possible transmembrane domain near the N-terminus and an AS domain that shared nearly 60% identity (over a 47 amino acid stretch) with mammalian FAAH proteins. The Arabidopsis candidate FAAH cDNA was expressed as a fusion protein in Escherichia coli, and indeed encoded a polypeptide of approximately 67 kDa (including an epitope/his tag fusion; ~66 kDa without tag). The recombinant Arabidopsis FAAH (hereafter designated as AtFAAH) hydrolyzed a variety of radiolabeled NAE species. The heterologously expressed recombinant AtFAAH enzyme was associated with E. coli membranes, and was solubilized in active form by treatment of E. coli cells with 0.2 mM dodecylmaltoside (DDM). The highly purified AtFAAH had affinities for NAE substrates similar to those reported for the rat FAAH, and exhibited

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saturation kinetics with respect to all NAE substrates. The AtFAAH activity was somewhat sensitive to inhibition by the serine hydrolase inhibitor, phenylmethylsulfonyl fluoride (PMSF), but more importantly was inhibited potently by the active site-directed inhibitor of mammalian FAAH, methylarachidonyl fluorophosphonate (MAFP). Taken together, molecular and biochemical evidences indicate that the At5g64440 gene encodes a functional homologue of the mammalian FAAH, despite the low level of overall primary sequence similarity (18.5% identity over the full length proteins).

*In vivo* Roles for *Arabidopsis* Fatty Acid Amide Hydrolase

The identification of a FAAH homologue in *Arabidopsis* (Shrestha et al., 2003) paved the way for similar genetic studies in plants. Like FAAH knock-out mice, T-DNA insertional knock-outs of FAAH in *Arabidopsis* had higher levels of endogenous NAE in their seeds, and their seedlings were hypersensitive to the growth inhibitory effects of exogenous NAE (Wang et al., 2006). Conversely, AtFAAH overexpressors had lower levels of endogenous NAE in seeds, and seedlings were resistant to the growth inhibitory effects of exogenous NAE (Wang et al., 2006). Moreover, seedlings of AtFAAH overexpressors grew faster and had increased cell/organ size under normal conditions (Wang et al., 2006). These results supported the hypothesis that, like in animals, FAAH is a modulator of endogenous NAE levels in plants and that NAE depletion by the action of FAAH likely participates in the regulation of normal plant growth. Both the mammalian and *Arabidopsis* FAAH are localized to the endoplasmic reticulum (Kang et al., 2008) but lack a conventional N-terminal ER signal sequence, suggesting similar subcellular targeting mechanisms for both enzymes.
After oleamide hydrolase activity was first discovered from rat (Cravatt et al., 1996), FAAH has been intensely investigated in animal system to uncover the functions of the endocannabinoid system. However, plant FAAH homologues have been only recently studied. Therefore, our knowledge on this enzyme in plants is fragmentary and many questions remain to be addressed. Specifically, although it is clear that AtFAAH is a modulator of endogenous NAE levels in Arabidopsis, it is uncertain how conserved this enzyme is in plant species and FAAH homologues had not been identified and characterized in other plant species. Also, AtFAAH knock-out seedlings still depleted seed NAEs and lacked a dramatic phenotype compared to wild-type seedlings, indicating a possibility of existence of another NAE amidohydrolase in Arabidopsis. Furthermore, in addition to the expected phenotypes of AtFAAH overexpressors, they exhibited several unexpected phenotypes unable to be explained by NAE depletion, such as hypersensitivity to ABA (Teaster et al., 2007) and enhanced susceptibility to bacterial pathogens (Kang et al., 2008), implying a function of AtFAAH independent of its catalytic activity.
Collectively, it is clear that a better molecular and biochemical understanding of plant FAAH is needed to address these questions. Toward this goal, in this dissertation I have identified and characterized AtFAAH orthologues in diverse plant species to provide molecular and biochemical support for the existence of FAAH as a general feature of plant systems. I helped to identify an additional FAAH enzyme (AtFAAH2) in Arabidopsis. I also discovered a bifurcating function of AtFAAH(1) to influence Arabidopsis growth and/or sensitivity to ABA and initiated further studies on the domain structure and functional activity of AtFAAH(1).
Table 1.1 Biological effects of N-acylethanolamine (NAE) in plants.

<table>
<thead>
<tr>
<th>NAE species</th>
<th>Biological process affected</th>
<th>Plant species</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAE12:0</td>
<td>Inhibits PLDα activity and ABA-induced stomatal closure</td>
<td><em>Commelina communis</em>&lt;br&gt;<em>Nicotiana tabacum</em>&lt;br&gt;<em>Ricinus communis</em>&lt;br&gt;<em>Brassica oleracea</em></td>
<td>Austin-Brown and Chapman, 2002</td>
</tr>
<tr>
<td></td>
<td>Induces microtubule, actin and endomembrane reorganization</td>
<td><em>Arabidopsis thaliana</em></td>
<td>Blancaflor et al., 2003; Motes et al., 2005</td>
</tr>
<tr>
<td></td>
<td>Delays flower senescence, lipid peroxidation and ion leakage</td>
<td><em>Dianthus caryophyllus</em></td>
<td>Zhang et al., 2007</td>
</tr>
<tr>
<td></td>
<td>Enhances seedling sensitivity to ABA</td>
<td><em>Arabidopsis thaliana</em></td>
<td>Teaster et al., 2007</td>
</tr>
<tr>
<td></td>
<td>Inhibits early seedling development; inhibits root hair growth</td>
<td><em>Arabidopsis thaliana</em></td>
<td>Blancaflor et al., 2003; Teaster et al., 2007</td>
</tr>
<tr>
<td></td>
<td>Inhibits lipoxygenase activity and elevates polyunsaturated NAE levels</td>
<td><em>Arabidopsis thaliana</em></td>
<td>Keereetaweep et al., 2010</td>
</tr>
<tr>
<td>NAE14:0</td>
<td>Inhibits fungal elicitor-induced alkalization of the growth media</td>
<td><em>Nicotiana tabacum</em></td>
<td>Tripathy et al., 1999</td>
</tr>
<tr>
<td></td>
<td>Triggers expression of phenylalanine ammonia lyase (<em>PAL2</em>)</td>
<td><em>Nicotiana tabacum</em></td>
<td>Tripathy et al., 1999</td>
</tr>
<tr>
<td>NAE18:2</td>
<td>Inhibits early seedling development; inhibits root hair growth</td>
<td><em>Arabidopsis thaliana</em></td>
<td>Teaster et al., 2007</td>
</tr>
</tbody>
</table>
Figure 1.1 Molecular structure of $\Delta^9$-tetrahydrocannabinol (THC) and representative $N$-acylethanolamines (NAEs).
Figure 1.2 Formation and hydrolysis pathways for N-acylethanolamine (NAE). NAE is formed from N-acylphosphatidylethanolamine (NAPE) by NAPE-PLD (NAPE-phospholipase D) in animals and by PLD-β/γ in plants, with a concomitant release of phosphatidic acid (PA), and is hydrolyzed into the corresponding free fatty acid (FFA) and ethanolamine (EA) by fatty acid amide hydrolase (FAAH) in both animals and plants. FFA is then combined with coenzyme A (CoA) into acyl-CoA by acyl-CoA synthase. In plants, NAPE is synthesized by N-acylation of phosphatidylethanolamine (PE) via either direct acylation from FFA or transacylation from acyl-CoA, both by NAPE synthase. In animals, the acyl group is transferred from acyl-CoA to PE to produce NAPE through the sequential acylation and deacylation of phosphatidylcholine (PC) or PE by O- and N-acyltransferases. Common and animal/plant-specific pathways are color-coded.
CHAPTER 2
IDENTIFICATION AND CHARACTERIZATION OF FATTY ACID AMIDE HYDROLASE HOMOLOGUES IN DIVERSE PLANT SPECIES*

Abstract

Fatty acid amide hydrolase (FAAH) plays a central role in modulating endogenous N-acylethanolamine (NAE) levels in vertebrates, and, in part, constitutes an “endocannabinoid” signaling pathway that regulates diverse physiological and behavioral processes in animals. Recently, an Arabidopsis FAAH homologue was identified which catalyzed the hydrolysis of NAE in vitro suggesting a FAAH-mediated pathway exists in plants for the metabolism of endogenous NAE. Here, I provide evidence to support this concept by identifying candidate FAAH genes in other plant species including rice (Oryza sativa), Medicago truncatula, cotton (Gossypium hirsutum), and tomato (Solanum lycopersicum). Corresponding cDNAs were isolated and expressed as recombinant proteins in Escherichia coli. NAE amidohydrolase assays confirmed that rice and M. truncatula proteins indeed catalyzed the hydrolysis of 14C-labeled NAE in vitro. Kinetic parameters and inhibition properties of the rice FAAH were similar to those of Arabidopsis and rat FAAH, but not identical. Arabidopsis and rice FAAH, like rat FAAH, also hydrolyzed a broad range of substrate types including long-chain polyunsaturated acylamides, fatty acid primary amides, and monoacylesters. Sequence alignments revealed that five amino acid residues determined to be important for catalysis by rat FAAH were absolutely conserved within the FAAH sequences of five plant species, indicating that plant and mammalian FAAH proteins have a similar catalytic mechanism despite limited overall sequence

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identity. Also, another NAE amidohydrolase (At5g07360) was identified in Arabidopsis and expressed in E. coli which was active in vitro only when supplemented with Arabidopsis seedling homogenate. Defining the molecular properties of NAE amidohydrolase enzymes in plants will help to better understand the metabolic regulation of NAE lipid mediators.

Introduction

N-Acylethanolamines (NAEs) are fatty acid amide derivatives comprising fatty acids of various chain lengths linked to the amino group of ethanolamine. They are endogenous lipid constituents in both animal and plant systems that occur in relatively trace concentrations. Several NAE types in mammals are generally accepted as endogenous lipid mediators that regulate a wide range of physiological processes, including neurotransmission (Wilson and Nicoll, 2002), embryo development and implantation (Paria et al., 2002), immune responses (Berdyshev, 2000), and cell proliferation (De Petrocellis et al., 2000), often (but not always) through their interaction with membrane bound cannabinoid (CB) receptors (Di Marzo et al., 2002). A fatty acid amide hydrolase (FAAH) is believed to terminate the regulatory cascade triggered by NAE in these diverse processes by hydrolyzing the molecules into corresponding inactive free fatty acids and ethanolamine (McKinney and Cravatt, 2005). Thus, FAAH modulation of NAE levels has become an important focus for understanding the mechanistic action of the “endocannabinoid” signaling system in vertebrates (Cravatt et al., 2001), and a convenient target for the development of novel therapeutics (Cravatt and Lichtman, 2002).

NAEs in plants, like animals, have potent biological activities at reasonably low concentrations (reviewed in Chapman, 2004). For example, sub-micromolar to low micromolar concentrations of NAE14:0 activated defense gene expression in cell suspensions and leaves of
tobacco plants (Tripathy et al., 1999), marked by an increase in phenylalanine ammonia lyase (PAL2) transcript abundance. The activation of PAL2 expression was specific for acylethanolamides (since free fatty acids showed no effect), and was blocked by antagonists of mammalian CB receptors (Tripathy et al., 2003). In other work, Arabidopsis seedlings germinated in and maintained on micromolar concentrations of NAE12:0 showed marked developmental abnormalities in young roots, that again were selective for acylethanolamides, and in this case were restricted to the short/medium chain NAE (Blancaflor et al., 2003). While these pharmacological approaches show that the application of NAE to plants can elicit profound physiological and cellular changes, additional experimental evidence is needed to link the endogenous metabolism of these bioactive lipids to their physiological functions.

In plants, NAEs occur in a number of tissues and their levels have been reported to change in response to biotic stress and changes in development (Chapman, 2004). For example, NAEs were present at ppm levels in desiccated seeds and then dropped to barely detectable levels coincident with seed imbibition and germination (Chapman et al., 1999). The metabolism of NAE was traced in vivo in imbibed seeds, and reconstituted in vitro in microsomes of imbibed seeds, and shown to involve, in part, the hydrolysis to free fatty acids and ethanolamine, presumably by a FAAH-like enzyme activity (Shrestha et al., 2002). Bioinformatics approaches and heterologous protein expression led to the molecular identification of an amidohydrolase from Arabidopsis thaliana that hydrolyzed a wide range of acylethanolamides, including those present in desiccated seeds (Shrestha et al., 2003). Consequently, I proposed that plants, like animals, possess the capability to regulate endogenous NAE levels by a FAAH-like enzyme.

Here, I expand the knowledge-base of lipid metabolism by providing molecular and biochemical support for existence of FAAH genes and enzymes in diverse plant species.
Candidate FAAH genes were identified in rice, *Medicago truncatula*, cotton, and tomato using the FAAH gene in *Arabidopsis thaliana* (At5g64440) as a query sequence. Coding sequences from these plant species were isolated and functionally expressed in *E. coli*, and, consistent with the *Arabidopsis* and rat FAAH proteins, the recombinant rice and *M. truncatula* enzymes indeed hydrolyzed NAE. Overall, these results help to functionally define the group of enzymes that metabolize NAE in plants, information critical for understanding the regulation of endogenous NAE signaling pathways in higher plant systems.

Results

*Identification of Rice and Medicago Truncatula FAAH Candidates*

The AtFAAH gene (At5g64440) sequence was used to query other plant genome databases for candidate FAAH orthologues. FAAH orthologous genes were identified in *Medicago truncatula* and *Oryza sativa* (rice). To assess if the *M. truncatula* and rice genes encoded functional FAAH enzymes, corresponding cDNAs were first isolated and sequenced. Identification of the *M. truncatula* cDNA sequence relied on the characterization of an expressed sequence tag (EST) cDNA that was from an elicited cell suspension cDNA library (Accession AW695697). The EST sequence deposited in GenBank overlapped with the 5’ UTR and 5’ end of the ORF of the *M. truncatula* gene. The nucleotide sequence of this cDNA was determined completely on both strands and found to encode a full-length sequence of 2,336 bp. The deduced amino acid sequence was 607 amino acids in length with a predicted molecular mass of 66,207 and a pI of 5.83, properties similar to the AtFAAH (Shrestha et al., 2003).

To identify a rice cDNA, oligonucleotide primers were designed to the 5’ and 3’ ends of the predicted ORF (from the gene sequence), and a cDNA fragment was amplified from total
seedling RNA by reverse-transcriptase (RT)-PCR. The DNA fragment was cloned directly into pTrcHis2 expression plasmid (Invitrogen) for in-frame protein expression and sequenced completely on both strands for confirmation. The predicted protein product is 601 amino acids in length, has a predicted molecular mass of 65,809 and a predicted pl of 6.00, all properties that were similar to those of the *M. truncatula* and *Arabidopsis* recombinant FAAH (Shrestha et al., 2003). A coded segment identified in the cDNA was not in the ORF from some earlier gene model predictions (Accession AL606442). Together, these cDNA sequences isolated from both *M. truncatula* and rice helped to annotate correctly the corresponding genes and verified their expression.

*The cDNAs Encode Functional NAE Amidohydrolases*

To assess if the identified FAAH cDNAs encoded functional NAE amidohydrolases, the ORFs of both *M. truncatula* and rice FAAH were cloned into the prokaryotic expression vector pTrcHis2 (Invitrogen) for in-frame recombinant protein expression in *E. coli*. Lysates of *E. coli* cells (Top10, Invitrogen), induced to express the recombinant FAAH proteins, were screened for their ability to catalyze the conversion of [¹⁴C] NAEs to [¹⁴C] free fatty acids. A similar strategy was employed successfully to demonstrate functional rat (Patricelli et al., 1998) and *Arabidopsis* (Shrestha et al., 2003) recombinant enzymes. Representative radiochromatograms of assay reaction mixture lipids are shown in Fig. 2.1. Positive controls, cells expressing rat (Rat FAAH) or *Arabidopsis* (At NAE AHase) NAE amidohydrolases, are shown in panels A and B, respectively, marking the position of both substrate (in this case [¹⁴C] NAE 18:2) and product ([¹⁴C] FFA 18:2). Cells expressing the *M. truncatula* FAAH (Mt NAE AHase) in the forward orientation with respect to the lacZ promoter (panel C) catalyzed hydrolysis of NAE, whereas
cells harboring the FAAH in the reverse orientation (panel D), did not. The same results were obtained with *E. coli* cells expressing the rice FAAH (Os NAE AHase) in the forward or reverse orientations (panels E and F, respectively). Results with the cells harboring plasmids with the prospective plant FAAH cDNAs in reverse orientation (panels D and F), indicated that *E. coli* cells had no appreciable endogenous capability to hydrolyze NAE. Hence the recovery of NAE amidohydrolase activity in cells expressing recombinant plant FAAHs (panels B, C, and E) is a clear indication that these cDNAs encode functional FAAH enzymes.

*Rice FAAH Exhibits Kinetic Properties and pH Dependence Similar to Arabidopsis FAAH*

The biochemical properties of the recombinant rice FAAH were compared to those of the AtFAAH in terms of kinetic parameters, inhibition by rat FAAH inhibitors, MAFP and URB597, and abundance of full-length proteins in *E. coli* lysates. The rice FAAH, like the *Arabidopsis* (and rat) FAAH, hydrolyzed a wide range of NAE types including NAE12:0, NAE16:0 and NAE18:2 in a concentration dependent manner (Fig. 2.2). The rice FAAH, like the AtFAAH, exhibited typical Michaelis-Menten behavior toward all NAE substrates (Fig. 2.2) and displayed a pH dependence such that enzyme behavior, judged as $V_{\text{max}}/K_{\text{m}}$, was optimal at pH 9 for essentially all NAE substrates (Fig. 2.3). Direct comparisons of apparent $V_{\text{max}}$ cannot be made here between the rice and *Arabidopsis* enzymes, since these initial velocity measurements were made with enzymes in crude lysates. Measuring enzyme activity in lysates was necessary due to poor stability of the rice enzyme to the purification procedures. This instability is readily apparent as differences in protein levels and degradation products between the different plant recombinant FAAH proteins in *E. coli* (Fig. 2.5), and helps to account for absolute differences in apparent $V_{\text{max}}$ between *Arabidopsis* and rice FAAH. Nonetheless, some inferences can be made
about substrate preferences of these enzymes by taking into account the apparent $V_{\text{max}}/K_m$ of the enzymes toward the different NAE types. The rice FAAH appeared to utilize NAE18:2 most efficiently, whereas *Arabidopsis* FAAH appeared to utilize all NAE types with similar efficiency.

**Plant FAAHs Are Inhibited by MAFP but Not by URB597**

As expected the rice FAAH activity, like the AtFAAH, was inhibited by methyl arachidonyl fluorophosphonate (MAFP) in a concentration-dependent manner (Fig. 2.4). MAFP binds to rat FAAH irreversibly and has been crystallized in the acyl domain of the active site (Bracey et al, 2002; Alexander and Cravatt, 2005). The potent inhibition of rice FAAH by MAFP and the similar kinetic properties between rice and *Arabidopsis* FAAH suggests that indeed the rice enzyme is a functional FAAH homologue. Interestingly, neither the *Arabidopsis* nor the rice FAAH was inhibited by URB597 (Fig. 2.4), another specific inhibitor of rat FAAH that covalently modifies S241 in the active site. Notably, this serine residue is conserved among rat and plant FAAH protein sequences. The URB597 inhibitor is believed to gain access to the rat FAAH catalytic site via the cytoplasmic access channel (Alexander and Cravatt, 2005), which suggests that there are some structural differences between the vertebrate and plant FAAH enzymes within this region of the FAAH protein. A structural difference is certainly supported by the low level of identity between rat and *Arabidopsis* FAAH outside the amidase signature domain (less than 20% identity; Shrestha et al, 2003). By way of comparison, the URB597 was a potent inhibitor of the rat FAAH enzyme activity in our hands with 2 nM reducing activity by 50% and 100 nM essentially eliminating enzymatic activity (data not shown). The differential inhibition of plant FAAH activity by two well-characterized inhibitors of mammalian FAAH (Alexander and Cravatt, 2005) could provide an important diagnostic tool in future detailed
comparisons between the structure/activity relationships of the mammalian and plant FAAH enzymes.

*Plant FAAHs Also Hydrolyze Fatty Acid Primary Amides and Fatty Acid Esters*

Mammalian FAAH enzymes are known to hydrolyze a broad range of alkyl amide and fatty acid ester substrates *in vitro* as well as NAE. FAAH was named in recognition of the large number of endogenous fatty acid amides that it accepts as substrates, including NAEs and fatty acid primary amides such as 9-octadecenamide (oleamide), a sleep inducer in mammals. To test the plant FAAH enzymes against a broad range of substrate types that are known to be hydrolyzed by mammalian FAAH, enzyme activity assays were also conducted with a long-chain polyunsaturated acylamide, NAE 20:4 (anandamide), a fatty acid primary amide, oleamide, and a monoacylester, 2-arachidonoylglycerol (2-AG). As shown in Fig. 2.6, these substrates were all hydrolyzed well into arachidonic acid (from anandamide and 2-AG) and oleic acid (from oleamide) by both *Arabidopsis* and rice FAAH enzymes with an efficiency similar to rat FAAH. The formation of the products was not attributable to an endogenous capability of *E. coli* cells to hydrolyze the substrates as verified by no product formation by *E. coli* cells expressing the rice FAAH in the reverse orientation (R/O in Fig. 2.6). The capability of plant FAAHs to utilize a variety of fatty acid derivatives as their substrates, like animal FAAHs, suggests that this enzyme may play a pivotal role in a number of plant physiological processes that have been evolutionally conserved in higher organisms (e.g. endocannabinoid-like signaling system).

*Identification of Cotton and Tomato FAAH Orthologues*

In addition to FAAH orthologues from rice and *M. truncatula*, cotton (*Gossypium*...
hirsutum) and tomato (Solanum lycopersicum) candidate FAAH sequences were identified. An alignment of the predicted amino acid sequences from the five plant FAAHs is shown in Fig. 2.7. Proteins were most divergent at the N-termini and highly conserved within the AS domain (about 150 aa; blue underline). Residues previously determined to be important for NAE hydrolysis by rat FAAH (Patricelli and Cravatt, 2000) were absolutely conserved in plant sequences (arrows). These conserved residues included K205, S281, S282, S305, and R307 in the Arabidopsis FAAH sequence. The cotton FAAH protein was predicted to be 611 amino acids in length, a molecular weight of 67,039, and a pI of 6.76, similar to properties of the other plant FAAHs identified up to now except for the pI value slightly higher than that of the others. The tomato FAAH protein was predicted to be 618 amino acids in length, a molecular weight of 67,814, and a pI of 6.13.

Transcript level analysis by RT-PCR performed by another group (Dr. Earl Taliercio) revealed that the cotton FAAH was significantly expressed in fiber compared to ovule and was more expressed at 10 daa (day after anthesis) than at 1 daa (unpublished data). Thus, total RNA extracted from 10 daa fiber was used to amplify the ORF of FAAH cDNA which was then cloned in pTrcHis2 expression vector and expressed in E. coli Top10 cells as with rice and M. truncatula FAAHs described above. On the other hand, tomato FAAH cDNA was cloned in pET-16b expression vector (by Dr. Adi Avni’s group) and expressed in E. coli BL21(DE3) cells.

Unlike Arabidopsis, rice, and M. truncatula FAAHs, however, no NAE amidohydrolase activity was observed with E. coli cell lysates expressing either cotton or tomato FAAH despite significant expressions of the proteins manifested by Western blot analysis (Fig. 2.8). Hydrolysis of any NAE substrates used (NAE12:0, NAE16:0, and NAE18:2) was not detected either at any reaction conditions tested (various temperature/pH conditions) or even with the enzymes affinity-purified from E. coli. This suggests that these enzymes are different from the other plant
FAAH orthologues in that they may have an additional requirement(s) to be functional, such as an eukaryotic-specific regulatory molecule that specifically binds to and activates the enzymes, a hypothesis strengthened by *Arabidopsis*-dependent activation of another *Arabidopsis* NAE amidohydrolase described below.

*Another NAE Amidohydrolase in Arabidopsis*

Depletion of seed NAE of *AtFAAH* T-DNA insertional knock-out lines and their lack of distinct phenotype compared to wild-type plants (Wang et al., 2006) all indicated that *Arabidopsis* might have alternative/additional amidases important in the regulation of NAE metabolism. *In silico* analysis indicated seven AS proteins in the *Arabidopsis* genome (Pollmann et al., 2006), of which only *AtFAAH* and amidase 1 (At-AMI1) have been characterized thus far. Among the other five putative AS proteins, the one encoded by *At5g07360* was particularly of interest due to significant sequence homology (>70%) with *Arabidopsis* FAAH and conservation of the catalytic triad (Ser-Ser-Lys) within the AS domain. Thus, its cDNA was cloned for functional expression in *E. coli* as described above for rice and *M. truncatula* FAAHs. Surprisingly, a small quantity of NAE hydrolysis was observed only when the *E. coli* lysate expressing *At5g07360* was supplemented with *Arabidopsis* seedling homogenate of 10-d old double knock-out line lacking both *AtFAAH* and *At5g07360* genes (Fig. 2.9). The double knock-out was used to remove intrinsic NAE hydrolysis activity of either *AtFAAH* or *At5g07360* present in the seedling homogenate. The barely detectable level of activity was consistent with the findings that *AtFAAH* knock-out line displayed a residual activity when measured *in vitro* with an excessive amount of seedling homogenate (~4 mg of total proteins; Fig. 2.10B), suggesting poor catalytic efficiency of *At5g07360*, at least under conditions optimized for
AtFAAH. Transcript level analysis by RT-PCR confirmed that all knock-out lines had no transcript corresponding to their gene(s) disrupted by T-DNA insertion (Fig. 2.10A). Collectively, these results suggest that At5g07360 indeed encodes a protein able to hydrolyze NAE, although not as efficient as AtFAAH, and that an Arabidopsis-specific regulator(s) absent from E. coli is required for the enzyme to be active. Additional experiments are required to characterize this new FAAH activity, and perhaps this information will be useful in establishing functional activity of tomato and cotton FAAH, but these preliminary results suggest that at least two hydrolases exist in Arabidopsis that are capable of NAE catabolism.

Discussion

The occurrence of NAE in biological systems is widespread and includes animals (Schmid and Berdyshev, 2002), plants (Chapman, 2004), and microorganisms (Merkel et al., 2005; Schmid et al., 1990). Much recent research in mammalian physiology has focused on the function of these lipids as signaling molecules since NAEs activate cannabinoid (CB) receptors (Di Marzo et al., 2002), vanilloid receptors (Di Marzo et al., 2002) and peroxisome proliferator receptor-alpha (PPARα) transcription factors (Lo Verme et al., 2005). Comparatively little information exists for NAE function in plant systems, although recent studies suggest that these lipids interact with membrane bound receptors to effect changes in ion flux and gene expression (Tripathy et al., 1999; Tripathy et al., 2003), and in other work, NAEs were shown to be potent inhibitors of phospholipase D (PLD) α (Austin-Brown and Chapman, 2002), a PLD family member that seems to be unique to higher plants (Wang, 2001). The recent discovery of NAE in Saccharomyces cerevisiae (Merkel et al., 2005) has prompted speculation that NAE function as lipid mediators in microorganisms, but direct evidence for such a role has yet to be reported. Key
to understanding the functional roles of these lipids in all organisms is to identify the
mechanism(s) that regulate their accumulation. NAE levels in animal systems appear to be
modulated by their degradation to free fatty acids and ethanolamine by fatty acid amide
hydrolase (FAAH), a member of the amidase superfamily (McKinney and Cravatt, 2005). The
recent identification of a functional FAAH homologue in *Arabidopsis* suggests that this
mechanism might also operate in plants. Here, this concept gains further support by the
identification of FAAH homologues in diverse plant species.

Several lines of evidence presented here support the identification of a FAAH family of
enzymes in higher plants. cDNAs corresponding to the genes encoding FAAH proteins from
diverse plant species, *Arabidopsis*, rice, and *M. truncatula*, were expressed in *E. coli* and these
recombinant enzymes indeed possessed FAAH-like biochemical properties (Figs. 2.1-2.4 and
2.6). All of the plant FAAH sequences including those in cotton and tomato were highly similar
with 65% identity or better over their entire lengths (Fig. 2.7). These plant sequences were
considerably diverged from mammalian FAAH sequences with generally less than 20% identity
over their entire lengths (Chapman, 2004). Nonetheless, recombinant proteins from both plants
and animal sources expressed in *E. coli* had similar, but not identical, kinetic and biochemical
properties, including similar affinities for a broad range of substrates and potent inhibition by
one active-site directed enzyme inhibitor (Bracey et al., 2002).

NAE amidohydrolase activity was previously observed in extracts of imbibed cottonseeds
and the activity was associated mostly with microsomes (Shrestha et al., 2002), suggesting that
functional FAAH homologue was indeed present in cotton being responsible for the rapid
depletion of NAE during seed imbibition and germination (Chapman et al., 1999). Nevertheless,
it remains unclear why no NAE amidohydrolase activity was found with the putative cotton
FAAH (and tomato FAAH) heterologously expressed in *E. coli* despite significant expression of the protein (Fig. 2.8). One possibility is that these enzymes, unlike other plant FAAH orthologues identified, may not be active by themselves and require an additional component(s) that is not present in *E. coli* to be functional. The requirement for an eukaryotic regulator was evidently exemplified by the *Arabidopsis At5g07360* expressed in *E. coli* that was active only in the presence of *Arabidopsis* seedling homogenate (Fig. 2.9). The *in vitro* NAE amidohydrolase assay using an excessive amount of seedling homogenates clearly supported that *At5g07360* was indeed able to hydrolyze NAE in *Arabidopsis* cells (Fig. 2.10B). Although the regulator(s) that makes *At5g07360* active remains to be identified, the *in vitro* hydrolysis of NAE by *At5g07360* (in the presence of *Arabidopsis* seedling homogenate) implies that the enzyme activity is regulated by association with an effector molecule(s) rather than by posttranslational modification or more precise protein folding that largely require *in vivo* environment to occur. The effector molecule is most likely an *Arabidopsis* protein(s) that specifically binds to and activates the enzyme because the common cofactors/coenzymes such as divalent cations, ATP, and NADH are abundant in *E. coli* cells as well. Although the molecular mechanism for regulation of *At5g07360* activity is still unclear, *Arabidopsis* apparently seems to possess an alternative route to metabolize NAE as needed depending on cellular or physiological conditions.

The presence of alternate routes for NAE metabolism in mammals is supported by pharmacological studies in FAAH knockout mice, and led to the discovery of alternate enzymes that hydrolyze NAE, such as NAE acid amidase (NAAA; Tsuboi et al., 2005), a second mammalian FAAH (FAAH-2; Wei et al., 2006) and identification of alternate NAE derivatives (Mulder and Cravatt, 2006). No close homology of mammalian NAAA and FAAH-2 with *Arabidopsis* proteins was identified. Collectively, it seems evident that NAEs are metabolized by
multiple enzymes in animal and plant kingdoms, again signifying the physiological importance
of the fine tune for endogenous NAE levels to meet cellular or physiological requirements in
both kingdoms. Taken together, these data presented in this chapter help to define a new group of
enzymes in plants that may have important regulatory functions in terms of modulating the
endogenous levels of NAE. The availability of functionally-defined molecular sequences for
FAAH enzymes should now facilitate further studies of NAE metabolism in higher plant systems.

Methods

Materials

[1-14C] Lauric acid was from Amersham Biosciences. [1-14C] Myristic acid, arachidonic
acid, oleic acid, lauric acid, linoleic acid, myristic acid, anandamide, ethanolamine,
phenylmethylsulfonyl fluoride (PMSF), and isopropyl β-D-thiogalactopyranoside (IPTG) were
from Sigma Chemical Co. (St. Louis). [1-14C] Linoleic and [1-14C] palmitic acids were
purchased from NEN (Boston, MA). Methyl arachidonyl fluorophosphonate (MAFP) was from
TOCRIS (Ellisville, MO), n-dodecyl-β-D-maltoside (DDM) was from Calbiochem (La Jolla,
CA), 9-octadecenamide, sn-2-arachidonoyl glycerol, and URB597 were from Cayman Chemical
(Ann Arbor, MI) and silica gel G (60 Å)-coated glass plates for thin-layer chromatography (10
cm×20 cm or 20 cm×20 cm, 0.25 mm thickness) were from Whatman (Clifton, NJ). Specific
types of N-[1-14C] acylethanolamines (and non-radiolabeled NAE) were synthesized from
ethanolamine and the respective [1-14C] fatty acids (and non-radiolabeled FFAs) by first
producing the fatty acid chloride (Hillard et al., 1995) and purified by TLC as described
elsewhere (Shrestha et al., 2002).
**Bioinformatics**

BLAST searches (http://blast.wustl.edu) in *Medicago truncatula* and *Oryza sativa* genome databases were done using the *Arabidopsis thaliana* FAAH gene, At5g64440, as the query sequence. Candidate orthologous FAAH cDNA sequences were identified in gene indices of different plant species curated by the Institute for Genomic Research (TIGR) and available at www.tigr.org. Exon-intron annotation of genes, nucleotide sequence analyses, and protein domain identification were performed using various Internet-based informatics tools and database comparisons including BLAST and cDART (www.ncbi.nlm.nih.gov), ProDom (Servant et al., 2002), Prosite (Sigrist et al., 2002), TMHMM transmembrane and topology predictor (Krogh et al., 2001; Sonnhammer et al., 1998), pSORT (Nakai and Kanehisa, 1992), BLOCKS (http://blocks.fhcrc.org), MEME (http://meme.sdsc.edu/meme/website/meme.html), ExPASy (http://us.expasy.org/), CLUSTALW (Thompson et al., 1994), and T-Coffee (Notredame et al., 2000). Some sequence assembly, characterization and alignments were made with DNASIS software (Hitachi).

**cDNA Isolation**

For *Medicago truncatula* FAAH cDNA isolation, an EST sequence (GenBank accession AW695697) was identified and the corresponding clone was retrieved from the Samuel Roberts Noble Foundation EST collection, and sequenced completely on both strands (2× each, commercial service). The complete nucleotide sequence was deposited in GenBank (DQ091761). Tomato FAAH cDNA cloned in pET-16b (Novagen) was courteously donated from Dr. Adi Avni and transformed into *E. coli* BL21(DE3) competent cells for protein expression. For the rice, cotton, and *Arabidopsis* (At5g07360) cDNAs, sequence-specific primers were designed to the 5’
and 3’ ends of the ORF, and were used for reverse transcriptase (RT)-PCR (for rice, 5’-ATGACGCGGTGGAGGAGGTGG-3’/5’-AGCGTTCAAGGATGTCATGAAATGC-3’, for cotton, 5’-ATGGGACTATTCAAGGCT-3’/5’-ATTCTTATGAAGTAGATTAT-3’, for *Arabidopsis*, 5’-ATGGCGAGACCATCGATCGC-3’/5’-AATGTGTAACCTACGGGGAGG-3’). Total RNAs were extracted from 2-day-old rice seedlings using Trizol reagent (Invitrogen) and from 10-daa cotton fiber and 7-day-old *Arabidopsis* seedlings using RNeasy mini kit (Qiagen), according to the manufacturer's instructions. For RT-PCR, the first-strand cDNA synthesis from total RNA was carried out at 50 °C for 30 min and incubated for 4 min at 94 °C to inactivate the reverse transcriptase. The targeted amplification of the candidate *FAAH* mRNA by Platinum Taq (RT-PCR mixture; Invitrogen) was achieved through 25 cycles of 94 °C for 1 min, 45 °C for 1 min, 72 °C for 2 min followed by a final polymerization step at 72 °C for 7 min. The RT-PCR products were gel-purified and ligated into pTrcHis2 for nucleotide sequencing and protein expression (below). Commercial DNA sequencing of both strands (complete 2× each strand) verified the identity of the cDNA as the rice, cotton, and *Arabidopsis* gene product, and the complete rice cDNA sequence was deposited in GenBank (DQ118178).

**Protein Expression**

For *M. truncatula* FAAH protein expression, oligonucleotide primers (forward, 5’-ATGGGAAGAAGCGTGTAATGG-3’ and reverse, 5’-GTTAGCCCCCAGAACACATCGTAG-3’) were designed to amplify only the open reading frame (ORF) of the *M. truncatula* cDNA, and PCR conditions were as above, except that a 10-to-1 ratio of polymerases (Taq-to-Pfu; Invitrogen) was used for amplification and the template was the *M. truncatula* cDNA (GenBank accession DQ091761) in pBluescript SK-. The ORF-PCR products were gel-purified as above,
subcloned into pTrcHis2 expression vector, and the constructs introduced into chemically-
competent *E. coli* TOP10 cells as host. Transformed colonies were selected with correct in-frame
fusions and accurate nucleotide sequence for all plants were determined by sequencing plasmid DNA over the vector insert junctions and by sequencing the inserts completely on both strands. Selected transformed cell lines were grown in LB medium to an OD$_{600}$ of 0.6 to 0.7 and induced with 1 mM IPTG for 4 h. Pelleted cells were resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl and 0.2 mM DDM) at a ratio of 1-to-108 (*E. coli* cells-to-DDM molecules; 0.1 OD$_{600}$=108 cells/mL (Elbing and Brent, 2002)). After incubation on ice for 30 min resuspended cells were sonicated on ice with six 10-s bursts at high intensity with a 10-s cooling (ice bath) period between each burst. The selection of DDM as the detergent, and determination of optimal DDM concentration and content ratio was based on empirical comparisons for recovery of solubilized active *Arabidopsis* FAAH enzyme with the highest specific activity (Shrestha et al., 2003). The crude lysates were centrifuged at 105,000 $\times$g for 1 h in a Sorvall Discovery 90 model ultracentrifuge (Beckman Ti45 rotor) and the supernatants were used for NAE amidohydrolase assays.

**NAE Amidohydrolase Assays**

NAE synthesis and enzyme assays were conducted as previously described (Shrestha et al., 2002; Shrestha et al., 2003) with a few modifications. The enzyme source was solubilized in 0.2 mM dodecylmaltoside (DDM) and incubated with 100 $\mu$M [$^{14}$C] NAE (20,000 dpm) in 50 mM Bis-Tris buffer (pH 9.0) in 0.2 mM DDM for 30 min to survey for NAE amidohydrolase activity (Shrestha et al., 2002; Shrestha et al., 2003). Assays of lysates of *E. coli* cells expressing rat FAAH (WT-FAAH; (Patricelli et al., 1998) or *Arabidopsis* FAAH (Shrestha et al., 2003) served
as a comparison of NAE amidohydrolase activity, whereas non-transformed cell lysates or cell lysates with the cDNA cloned in reverse orientation with respect to the lacZ promoter served as negative controls for activity assays. Initial velocity measurements were made at increasing concentrations of $[^{14}\text{C}]$-labeled NAE species, and reactions were initiated by adding the recombinant enzyme source (2 μg E. coli lysate for Arabidopsis and 20 μg E. coli lysate for rice). Enzyme reactions were terminated by the addition of boiling isopropanol (70 °C) and lipids were extracted into chloroform. Lipid products were separated by TLC and the distribution of radioactivity was evaluated by radiometric scanning (Shrestha et al., 2002). Activity was calculated based on the radiospecific activity of $[^{14}\text{C}]$-labeled substrate. Two irreversible active-site-directed FAAH inhibitors, MAFP and URB597, were used to test the sensitivity of the plant recombinant NAE amidohydrolase activity. Inhibitors were added from a stock solution dissolved in DMSO, and activity was adjusted for minimal solvent effects where necessary based on assays in the presence of the appropriate amount of solvent alone. Protein content was determined by Coomassie blue-dye binding using bovine serum albumin as the protein standard (Bradford, 1976).

**Enzyme Assays with Various Substrates**

The enzyme source was incubated with 100 μM anandamide, 100 μM 2-arachidonoyl glycerol, or 100 μM 9-octadecenamide in 50 mM Bis-Tris buffer (pH 9.0) at 30 °C for 30 min with shaking at 120 rpm. Reactions were terminated by the addition of boiling isopropyl alcohol (70 °C) for 30 min. Total lipids were extracted into chloroform, washed twice with 1 M KCl and once with water, and separated by TLC using an organic solvent mixture of hexane, ethyl acetate, and methanol (60:40:5, v/v/v). Distribution of unreacted substrates and products formed was
evaluated by exposure of the plate to iodine vapors.

**Western Blotting**

Proteins in *E. coli* cell lysates were separated by SDS-PAGE (10% resolving gels) and were electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes (0.2 μm, Bio-Rad, Hercules, CA) in a Semidry Trans-Blot apparatus (Bio-Rad, Hercules, CA) for 40 min at constant 14 V. PVDF membranes were blocked in 5% nonfat milk (Bio-Rad), and recombinant proteins, expressed as c-Myc-epitope fusions, were localized with a 1-to-2000 dilution of mouse monoclonal anti-c-Myc antibodies (ABGENT, San Diego, CA). Immunolocalized proteins were detected by chemiluminescence following incubation with a 1-to-2500 dilution of goat anti-mouse IgG conjugated to horseradish peroxidase (Bio-Rad) according to manufacturer's instructions.
Figure 2.1 Representative radiochromatograms of lipids extracted from reaction mixtures following assays of NAE amidohydrolase (NAE AHase) activity. Enzyme activities were measured as formation of radiolabeled free fatty acid (FFA) 18:2 from radiolabeled NAE18:2 in this case and lipids were separated by Silica gel-thin layer chromatography (TLC). In all cases, *E. coli* lysate (2 μg protein for rat and *Arabidopsis*; 20 μg protein for rice and *Medicago*) was used as the enzyme source, from cells harboring the following different cDNAs in pTrcHis2 expression plasmids: A) rat FAAH cDNA, B) At FAAH cDNA, C) Mt cDNA forward
orientation, D) Mt cDNA cloned in reverse orientation so as not to direct expression of a
recombinant protein, E) Os cDNA forward orientation, F) Os cDNA cloned in reverse
orientation so as not to direct expression of a recombinant protein.
Figure 2.2 Kinetic characterization of *A. thaliana* and rice NAE amidohydrolases. Initial velocities were measured at increasing concentrations of respective [1-$^{14}$C] NAE. Reactions were initiated by the addition of *E. coli* cell lysates expressing *A. thaliana* (A, B) or rice (C, D) NAE amidohydrolases (2 μg for *A. thaliana*, 20 μg for rice) and were carried out in 50 mM Bis-Tris buffer (pH 9.0) in a final volume of 0.4 mL. Data points represent means±S.D. of triplicate assays. Plots were generated with Prism software version 3.0 (GraphPad Software, San Diego).
Figure 2.3 pH dependence of *A. thaliana* and rice FAAH enzyme activity. Initial velocities were measured at increasing concentrations of respective [1-\(^{14}\)C] NAE at each pH (buffered with 50 mM Bis-Tris buffer pH 7.0, 8.0, or 9.0 in a final volume of 0.4 mL). Reactions were initiated by the addition of *E. coli* cell lysates expressing *A. thaliana* (A) or rice (B) FAAH (2 \(\mu\)g for *A. thaliana*, 20 \(\mu\)g for rice). \(V_{\text{max}}\) and \(K_m\) values were calculated from saturation plots as in Fig. 2.2, and enzyme behavior toward each substrate was plotted vs. reaction pH.
Figure 2.4 Effects of MAFP and URB597 on the hydrolysis of $[1^{14}C]$ NAE18:2 by *A. thaliana* and rice NAE amidohydrolases. Assays were conducted with *E. coli* cell lysates expressing *Arabidopsis* (A) or rice (B) NAE amidohydrolases (2 μg for *A. thaliana*, 20 μg for rice). Reactions were incubated for 30 min at 30 °C with 100 μM $[1^{14}C]$ NAE18:2 in the absence (0 nM) or presence of increasing concentrations of MAFP or URB597. The data are means ± S.D. of triplicate assays.
Figure 2.5 Western blot analysis of recombinant, epitope-tagged *A. thaliana* and rice FAAH in *E. coli* lysates. Increasing amounts of total *E. coli* lysate (2, 5, or 10 μg protein) expressing *A. thaliana* or rice FAAH were separated by SDS-PAGE and transferred to PVDF membranes. Immobilized proteins were probed with anti-c-Myc monoclonal antibodies and visualized by indirect chemiluminescence (goat anti-mouse IgG conjugated to horseradish peroxidase). Positions of pre-stained molecular weight standards are indicated on the right, and the predicted molecular weight of the recombinant proteins is marked on the left.
Figure 2.6 TLC separation of lipids extracted from reaction mixtures following enzyme activity assays against various substrates. The substrate include anandamide (A), oleamide (B), and 2-arachidonoyl glycerol (C). In all cases, *E. coli* lysate (2 µg protein for rat and *Arabidopsis*; 20 µg protein for rice) was used as the enzyme source and lipids were visualized by exposing the TLC plates to iodine vapors. Standard lipids (Std.) were also included for comparison of product position on the plates. *E. coli* lysate expressing the rice FAAH in reverse orientation (R/O) was also included as a negative control. Black and white arrows indicate positions of products (arachidonic acid for A and C and oleic acid for B) and substrates (anandamide for A, oleamide for B, and 2-arachidonoyl glycerol for C), respectively.
Figure 2.7 Alignment of amino acid sequences of plant FAAH proteins including those identified in Arabidopsis, rice, M. truncatula, cotton, and tomato. Alignment was generated with T-Coffee algorithm. Colors denote relative similarity with red being highest and blue being lowest (not shown here). The consensus sequence (cons) below all sequences indicates residues that were identical (asterisk) or similar (colon or period) in all sequences. The amidase signature sequence is underlined in blue. Residues determined to be important for amidase catalysis by the rat FAAH (K205, S281, S282, S305, and R307 in Arabidopsis sequence) are indicated by arrows. Note that these residues are absolutely conserved in all plant sequences.
Figure 2.8 Western blot analysis of recombinant, epitope-tagged cotton (A) and tomato (B) FAAH in *E. coli* lysates. Protein expression was induced by IPTG for different times (hours) indicated above each blot. 10 μg of total proteins from each sample were separated by SDS-PAGE and transferred to PVDF membranes. Immobilized proteins were probed with anti-c-Myc monoclonal antibodies and visualized by indirect chemiluminescence (goat anti-mouse IgG conjugated to horseradish peroxidase). Positions of pre-stained molecular weight standards are indicated on the left (kDa), and the predicted molecular weight of the recombinant proteins is marked on the right (kDa).
Figure 2.9 Representative radiochromatograms for NAE amidohydrolase assay of At5g07360 expressed in E. coli. Enzyme sources were reacted with [1-\(^{14}\)C]NAE16:0. Total lipids extracted from reaction mixtures were separated by TLC and analyzed by radiometric scanning. Each radiochromatogram shows the distance (mm) on the x-axis that lipids migrated on the TLC plates and their radioactivity (counts) on the y-axis. Picks that represent NAE and FFA (free fatty acid) are indicated. A, E. coli lysate (200 \(\mu\)g of total proteins) expressing At5g07360; B, the same amount of E. coli lysate supplemented with 10-day-old Arabidopsis seedling homogenate (200 \(\mu\)g of total proteins) of double knock-out line lacking both FAAH and At5g07360; C, the same amount of seedling homogenate alone; D, no enzyme source (the same volume of buffer only).
Figure 2.10 Transcript level analysis by reverse transcriptase (RT)-PCR and NAE amidohydrolase assay of Arabidopsis T-DNA insertional knock-out lines. A, transcript level analysis by RT-PCR. For each Arabidopsis line, total RNA was extracted from 7-day old seedlings and used as template for RT-PCR reaction using gene-specific primers (FAAH1, AtFAAH; FAAH2, At5g07360). PCR-amplified 18S rRNA (18S) is shown as loading control. WT, wild-type; F1, AtFAAH T-DNA knock-out; F2, At5g07360 T-DNA knock-out; F1/2-1 and F1/2-2, two distinct lines of AtFAAH/At5g07360 T-DNA double knock-out. B, NAE amidohydrolase assay. Enzyme assay was performed for 7-day-old seedlings (black bars) and desiccated seeds (gray bars). Enzyme activity was calculated as radioactivity of the product.
formed by 1 mg of protein per minute (cpm/min/mg protein) and is shown here as relative activity to wild-type seedling (%). Error bars represent standard deviation of triplicate measurements.
CHAPTER 3

MUTATIONS IN ARABIDOPSIS FATTY ACID AMIDE HYDROLASE REVEAL THAT CATALYTIC ACTIVITY INFLUENCES GROWTH BUT NOT SENSITIVITY TO ABSCISIC ACID*

Abstract

Fatty acid amide hydrolase (FAAH) terminates the endocannabinoid signaling pathway that regulates numerous neurobehavioral processes in animals by hydrolyzing N-acylethanolamines (NAEs). Recently, an Arabidopsis FAAH homologue (AtFAAH) was identified, and several studies, especially those using AtFAAH overexpressing and knock-out lines, have suggested an in vivo role for FAAH in the catabolism of NAE in plants. It was previously reported that overexpression of AtFAAH in Arabidopsis resulted in accelerated seedling growth, and in seedlings that were insensitive to exogenous NAE but hypersensitive to abscisic acid (ABA). Here I show that whereas the enhanced growth and NAE tolerance of the AtFAAH overexpressing seedlings depend on the catalytic activity of AtFAAH, hypersensitivity to ABA is independent of its enzymatic activity. Five amino acids known to be critical for rat FAAH activity are also conserved in AtFAAH (Lys-205, Ser-281, Ser-282, Ser-305, and Arg-307). Site-directed mutation of each of these conserved residues in AtFAAH abolished its hydrolytic activity when expressed in Escherichia coli, supporting a common catalytic mechanism in animal and plant FAAH enzymes. Overexpression of these inactive AtFAAH mutants in Arabidopsis showed no growth enhancement and no NAE tolerance, but still rendered the seedlings hypersensitive to ABA to a degree similar to the overexpression of the native

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AtFAAH. Taken together, these findings suggest that the AtFAAH influences plant growth and interacts with ABA signaling through distinctly different mechanisms.

**Introduction**

Fatty acid amide hydrolase (FAAH) catalyzes the hydrolysis of acylethanolamides, such as N-acylethanolamines (NAEs) (Desarnaud et al., 1995; Deutsch and Chin, 1993; Schmid et al., 1985; Ueda et al., 1995), as well as fatty acid primary amides (Cravatt et al., 1995; Maurelli et al., 1995; Cravatt et al., 1996). FAAH is known to terminate the “endocannabinoid” signaling pathway that regulates a variety of neurobehavioral processes in animals (reviewed in Fowler, 2006; McKinney and Cravatt, 2005; Fezza et al., 2008). This membrane-bound protein is a member of an enzyme superfamily termed the “amidase signature” (AS) family (Chebrou et al., 1996; Patricelli et al., 1999). Members of the AS family (more than 80 amidases) are characterized by a highly conserved region that consists of ~130 amino acids rich in serine, glycine, and alanine residues (Chebrou et al., 1996; Patricelli et al., 1999; Cai et al., 2005; Gopalakrishna et al., 2004; Labahn et al., 2002; Neu et al., 2007). The x-ray crystal structure of rat FAAH revealed that the core catalytic machinery of FAAH, in contrast to the Ser-His-Asp triad typical of most serine hydrolases, consists of a novel Ser-Ser-Lys catalytic triad (Patricelli et al., 1999; Bracey et al., 2002; McKinney and Cravatt, 2003; Patricelli and Cravatt, 2000). *faah* (-/-) knock-out mice had higher endogenous levels of NAEs compared with wild-type mice, and exhibited a variety of physiological and behavioral abnormalities in response to endocannabinoids, such as hypomotility, analgesia, catalepsy, and hypothermia (Cravatt et al., 2001; Clement et al., 2003; Cravatt et al., 2004; Lichtman et al., 2004). These observations suggested that FAAH was a key enzyme involved in the catabolism of NAE *in vivo* and was
responsible for termination of the endocannabinoid signaling.

In plants, FAAH homologues were identified and characterized recently at the biochemical level (Shrestha et al., 2003; Shrestha et al., 2006; also see chapter 2), but much remains to be learned regarding the precise cellular function and physiological significance of this enzyme in plants. An NAE hydrolase activity was first detected in vitro in homogenates of tobacco cells (Chapman et al., 1998), and was demonstrated both in vivo and in vitro in imbibed cotton seeds through radiolabeling approaches (Shrestha et al., 2002). An Arabidopsis FAAH homologue (AtFAAH; locus At5g64440) was identified that encodes a protein of 607 amino acids with 37% identity to rat FAAH within the AS domain (Shrestha et al., 2003). Catalytic residues (Lys-205, Ser-281, and Ser-305) were absolutely conserved and a single transmembrane domain, like rat FAAH, was predicted to be present near the N terminus of the protein (Shrestha et al., 2003). Recombinant protein, expressed in Escherichia coli, was indeed active in hydrolyzing a variety of naturally occurring fatty acid amides (Shrestha et al., 2003). Functional FAAH homologues have been identified and characterized in diverse plant species (Shrestha et al., 2006). Homology modeling of the AS region of the plant FAAH revealed a highly conserved active site organization with the catalytic triad positioned in the substrate-binding site (Shrestha et al., 2006).

Several lines of evidence, especially those using AtFAAH overexpressing and T-DNA insertion mutant plants, clearly support a role for FAAH in vivo in the catabolism of NAE in plants. Exogenous NAE at low micromolar concentrations exhibited a dose-dependent reduction of Arabidopsis seedling growth (Blancaflor et al., 2003), suggesting that hydrolysis of endogenous NAE by AtFAAH might be important for normal development. Indeed, AtFAAH overexpressors displayed enhanced seedling growth and increased cell/organ size (Wang et al.,
2006). Seeds of AtFAAH overexpressors had lower endogenous NAE content, and their seedling growth was less sensitive to exogenous NAE, whereas AtFAAH knock-out seeds had elevated levels of endogenous NAE in desiccated seeds, and their seedlings were hypersensitive to exogenous NAE (Wang et al., 2006). These results suggested that FAAH is a modulator of endogenous NAE levels in plants and that NAE turnover by the action of FAAH likely participates in the regulation of plant growth.

Recently, AtFAAH overexpressors have exhibited several additional intriguing phenotypes. Overexpression of AtFAAH resulted in seedlings that were hypersensitive to the growth inhibitory effects of a plant hormone abscisic acid (ABA) (Teaster et al., 2007). AtFAAH overexpressors were also found to be hypersusceptible to several bacterial pathogens and nonhost pathogens compared with wild-type plants, and this was attributed, in part to alterations in phytohormone accumulation and signaling (Kang et al., 2008). Interestingly, however, these phenotypic effects did not seem to be directly attributed to NAE turnover by AtFAAH. Even though AtFAAH overexpressors were compromised in innate immunity compared with wild-type plants, their NAE content and compositions in mature leaves were similar to those of wild-type plants (Kang et al., 2008). Likewise, application of ABA on AtFAAH overexpressors resulted in a marked reduction in growth despite little difference in NAE content or composition between ABA-treated and untreated seedlings (Teaster et al., 2007).

Because FAAH is able to hydrolyze other types of lipid substrates in vitro (like monoacylglycerols (Patricelli and Cravatt, 1999; Ghafouri et al., 2004; Fowler et al., 2001) and fatty acid primary amides (Cravatt et al., 1995; Maurelli et al., 1995; Cravatt et al., 1996; Fowler et al., 2001), it is possible that AtFAAH-mediated hydrolysis of other endogenous substrates, yet to be identified, may explain the impact on interaction with plant defense and ABA signaling for
this enzyme, separate from its role in NAE catabolism. Here I test this possibility by ectopic overexpression of catalytically inactive, site-directed mutant forms of AtFAAH in Arabidopsis and examining the resulting effects on growth and ABA sensitivity. As expected, overexpression of the AtFAAH variants without catalytic activities led to no growth enhancement and no NAE tolerance. Interestingly the transgenic AtFAAH variant lines remained hypersensitive to ABA despite lack of enzymatic activity. Consequently, my findings suggest that AtFAAH possesses at least two co-existing activities. It influences plant growth through its amidase activity toward NAE, whereas interacting with ABA signaling through other, unknown mechanisms independent of its catalytic activity.

Results

Site-directed Mutagenesis of AtFAAH

Site-directed mutagenesis studies of rat FAAH identified five amino acids (Lys-142, Ser-217, Ser-218, Ser-241, and Arg-243), including and nearby the catalytic triad, where substitution to alanine significantly decreased catalytic activity of the protein (Patricelli and Cravatt, 2000). Amino acid sequence alignment between rat and Arabidopsis FAAH proteins over the entire AS domain showed that the residues critical for the rat FAAH activity were absolutely conserved in the AtFAAH sequence (Lys-205, Ser-281, Ser-282, Ser-305, and Arg-307; Fig. 3.1A). Therefore, the corresponding residues in AtFAAH could also be critical for its amidase activity.

Dr. Li Kang at the Samuel Roberts Noble Foundation performed a systematic mutational analysis for each of the five conserved residues of AtFAAH by converting them to alanine to evaluate their importance for catalytic activity and biological functions in development and stress responses. Because Ser-281 and Ser-282 are located adjacent to each other on the protein
molecule, a S281A/S282A double mutant was generated to rule out the possibility that mutation of one of the two serine residues might compromise catalytic activity by structurally impacting the other residue. As a control, a S360A mutation was also generated because it is outside the amidase signature sequence and should have no impact on catalysis. Thus, the following mutants were generated for this study and expressed as recombinant proteins for functional analysis in *E. coli*: K205A, S281A/S282A, S305A, R307A, and S360A. Western blot analysis using affinity purified proteins with the mutations showed that the proteins are normally expressed in *E. coli* in roughly similar levels (Fig. 3.1B).

*Mutations in the Amidase Domain of AtFAAH Abolished Enzyme Activity*

When NAE hydrolase assays were performed with equal amounts of affinity purified, mutated proteins using NAE16:0 as a substrate, no detectable amount of the product (free fatty acid 16:0) was found in K205A, S281A/ S282A, S305A, and R307A mutants, whereas the S360A mutant exhibited fairly similar levels of product formation to wild-type (native) protein (Fig. 3.2), providing a negative control for comparison. Based on the assay results, specific activities for the mutants and their relative activities to wild-type protein were calculated and summarized in Table 3.1. Kinetic comparisons between wild-type AtFAAH and S360A mutant showed that the mutation outside of the FAAH active site had similar kinetic properties and catalytic efficiencies (*K*$_{cat}$/*K*$_{m}$) toward NAE substrates as the native FAAH (Fig. 3.3 and Table 3.2).

Enzyme activity assays were also conducted with NAE12:0, NAE18:2, a fatty acid primary amide, 9-octadecenamide (oleamide), and a monoacylglycerol, 2-arachidonylglycerol, to test the mutant enzymes against a broad range of substrate types including long- and short-chain
acylamides, long-chain polyunsaturated acylamides, primary amides, and monoacylesters (Fig. 3.4). These substrates were all hydrolyzed well by wild-type AtFAAH, but were hydrolyzed to a barely detectable degree by the site-directed mutants, except for the S360A mutant, which exhibited similar activity to wild-type protein for all the substrates tested (Fig. 3.4 and Table 3.1). Overall, all AtFAAH mutants displayed slightly better activities toward long-chain polyunsaturated NAE, and the R307A mutant hydrolyzed all the NAE substrates better than the other mutants (see Table 3.1). These results suggest that the five residues reported to be important for activity of rat FAAH (Patricelli and Cravatt, 2000) are also essential for the hydrolase activity of AtFAAH, and support a common catalytic mechanism of animal and plant FAAH enzymes.

*Overexpression of the Site-directed Mutant AtFAAH Proteins in Arabidopsis*

To test the impact of NAE turnover, or of hydrolysis of unknown lipid substrates by AtFAAH overexpressors, catalytically “dead” enzymes were overexpressed in wild-type and AtFAAH knock-out backgrounds. AtFAAH cDNAs with K205A, S281A/S282A, S305A, and R307A cloned in the pCAMBIA-1390 vector were used to transform *Arabidopsis* wild-type (Col-0) and the AtFAAH knock-out mutant using floral dip transformation (Clough and Bent, 1998). Transgenic plants were successfully obtained for S281A/S282A and R307A mutants. Western blot analysis using homogenates of 10-day-old seedlings showed that S281A/S282A and R307A mutant lines overexpressed their respective mutated proteins (Fig. 3.5A). The two backgrounds for transformation allowed for the endogenous AtFAAH to be accounted. AtFAAH protein was essentially undetectable in homogenates of wild-type and AtFAAH knock-out plants; the protein exists in very low abundance in wild-type, and it was barely detected in immunoblots.
of isolated microsomes prepared from homogenates of wild-type plants (not shown).

When NAE hydrolase assays were performed with equal amounts of total proteins extracted from 10-day-old seedlings using NAE16:0 as a substrate, both S281A/S282A and R307A mutants transformed into the wild-type background exhibited slightly reduced activities compared with wild-type plants alone, presumably due to the competition for substrate binding between the active and inactive enzymes (Fig. 3.5B; Shrestha et al., 2006). However, both of these two site-directed mutants exhibited significantly lower enzyme activity than overexpression of the native AtFAAH, clearly distinguishing the expression of site-directed mutant forms from overexpression of authentic AtFAAH. As expected, no measurable activity was found when either of the mutant forms were transformed into the AtFAAH knock-out plants (Fig. 3.5B), despite protein accumulation detected on Western blots (Fig. 3.5A).

AtFAAH Enzyme Activity Is Required for Enhanced Seedling Growth Observed in AtFAAH Overexpressing Plants

To ask whether NAE hydrolase activity of AtFAAH was required for enhanced seedling growth (Wang et al., 2006; Teaster et al., 2007), growth phenotypes of 10-day-old seedlings of the site directed mutant lines in response to NAE12:0 were determined by measuring their seedling fresh weights, primary root lengths, and cotyledon areas. Generally, both S281A/S282A and R307A mutants exhibited growth phenotypes essentially identical to those of their background lines (Col-0 and AtFAAH knock-out) when treated with either solvent (DMSO) only or NAE12:0 (Figs. 3.6 and 3.7). Overexpression of the inactive AtFAAH proteins showed no statistically significant enhancement of growth, whereas the native AtFAAH overexpressors exhibited ~30% increase in overall seedling growth when compared with wild-type plants.
Moreover, unlike the native AtFAAH overexpressors, none of these site-directed mutant lines showed any significant tolerance to exogenous NAE except that the R307A mutant transformed in wild-type background appeared to grow slightly better than either wild-type or S281A/S282A mutant in the same background.

AtFAAH Enzyme Activity Is Not Required for Hypersensitivity to ABA

It was previously shown that transcript levels of ABA-insensitive 3 (ABI3), a key transcription factor for ABA-responsive genes, are inversely associated with AtFAAH expression levels in Arabidopsis (Teaster et al., 2007). Inconsistent with this observation, overexpression of the AtFAAH protein resulted in seedlings that were hypersensitive to ABA despite lower transcript levels of ABI3 (Teaster et al., 2007). To determine whether NAE hydrolase activity of AtFAAH was required for ABA hypersensitivity observed in AtFAAH overexpressing plants (Teaster et al., 2007), the plants overexpressing the mutant forms of AtFAAH were grown for 10 days in the presence of ABA and their growth phenotypes were determined in detail as described above. Surprisingly, all seedlings expressing S281A/S282A or R307A mutants, regardless of their backgrounds, still exhibited severe hypersensitivity to ABA to a very similar degree observed for the native AtFAAH overexpressors (Figs. 3.6 and 3.7), suggesting that AtFAAH enzyme activity and NAE turnover by the enzyme are not required for the ABA hypersensitivity.

Collectively, these results indicate that the enhanced growth and the tolerance to exogenous NAE of the native AtFAAH overexpressing seedlings are attributable to elevated NAE hydrolase activity of the AtFAAH protein, whereas the hypersensitivity to ABA of the AtFAAH overexpressors is independent of the catalytic activity of the enzyme but rather
dependent on the presence of higher amounts of the protein only.

Discussion

After “oleamide hydrolase” activity was first affinity purified from rat liver membranes and the same enzyme was found to display high levels of “anandamide hydrolase” activity (Cravatt et al., 1996), FAAH has been intensely investigated in animal systems to uncover its functions in regulating the endocannabinoid signaling system and to develop new therapeutics for the treatment of human disorders (reviewed in McKinney and Cravatt, 2005; Fezza et al., 2008; Labar and Michaux, 2007). NAEs in plants are hydrolyzed by a membrane-associated hydrolase functionally analogous to the mammalian FAAH (Shrestha et al., 2003; Shrestha et al., 2006; Shrestha et al., 2002; Wang et al., 2006), and an Arabidopsis FAAH homologue was identified (Shrestha et al., 2003), suggesting that a FAAH-mediated pathway exists in plants as well for the metabolism of endogenous NAEs (reviewed in Chapman, 2004; Kilaru et al., 2007; Gertsch, 2008). However, because plant FAAH homologues have been studied only recently, our knowledge on functions of this enzyme in plants is fragmentary and many questions remain to be addressed.

Structure-function relationships for AtFAAH were predicted by homology-based modeling of the plant FAAH AS domain using the rat FAAH three-dimensional structure as a template (Shrestha et al., 2006). However no direct experimental evidence other than inhibitor studies has suggested that the plant and animal enzymes operate by a conserved mechanism. And inhibition by serine hydrolase inhibitors cannot distinguish between the Ser-Ser-Lys and Ser-His-Asp catalytic triads. Here I show that site-directed mutagenesis of five residues conserved in the AS region abolished the amidase activity of AtFAAH, supporting a conserved Ser-Ser-Lys catalytic
mechanism. All five residues were predicted to be located in the immediate vicinity of the active site pocket and the putative catalytic residues (Lys-205, Ser-281, and Ser-305) showed nearly direct overlap among all plant and rat FAAH proteins (Shrestha et al., 2006).

FAAH has an unusual catalytic feature in that, in addition to amidase activity, it possesses esterase activity at an equivalent rate (Patricelli and Cravatt, 1999). Among the five catalytically important residues of rat FAAH, the R243A mutant was reported to exhibit unaffected esterase activity despite severely compromised amidase activity (Patricelli and Cravatt, 2000). In contrast to this finding, the corresponding mutant of AtFAAH (R307A) displayed abolished esterase activity in a similar manner to other site-directed mutants tested (Fig. 3.4). This noticeable difference between rat and Arabidopsis FAAH indicates that, unlike rat FAAH, the amidase and esterase efficiencies of AtFAAH are functionally and tightly coupled. Another differential catalytic property between animal and plant FAAH enzymes has been previously shown by tolerance of plant FAAH to URB597, a specific inhibitor of animal FAAH (Shrestha et al., 2006; also see chapter 2). Collectively, these findings suggest that although the catalytic mechanism is conserved between the plant and animal enzymes, there are likely subtle differences within the AtFAAH active site that remain to be resolved at the structural level.

In addition to the expected phenotypes of plants overexpressing AtFAAH (e.g. tolerance to exogenous NAEs), these plants exhibited several unexpected phenotypes unable to be explained by NAE hydrolysis, such as hypersensitivity to ABA (Teaster et al., 2007) and enhanced susceptibility to several bacterial pathogens (Kang et al., 2008). Here I provide experimental evidence that AtFAAH influences Arabidopsis growth and responses to ABA through distinctly different molecular mechanisms.

Our previous studies suggested that hydrolysis of endogenous NAE by the amidase
activity of AtFAAH were important for normal Arabidopsis seedling growth (Blancaflor et al., 2003; Wang et al., 2006). Here I further support this hypothesis by observing growth phenotype of the plants that overexpress the inactive enzymes. Lack of both enhancement of growth and NAE tolerance by overexpressing inactive AtFAAH proteins reinforces our previous conclusions that FAAH is a modulator of endogenous NAE levels in plants and depletion of NAE by the action of FAAH is one of the key components that participate in the regulation of seedling growth.

Surprisingly, transgenic lines overexpressing S281A/S282A or R307A mutants of AtFAAH that produced inactive enzyme still exhibited ABA hypersensitivity to a degree similar to the native AtFAAH overexpressors, suggesting that AtFAAH-mediated ABA hypersensitivity is independent of catalytic activity of the enzyme toward acylamide or acylester substrates. The ABA hypersensitivity of the transgenic plants expressing mutant AtFAAH (S281A/S282A or R307A) in the AtFAAH knock-out background is almost similar to overexpressors (see Figs. 3.6 and 3.7). This can be attributed to the presence of more mutant AtFAAH protein because the expression is constitutively driven by cauliflower mosaic virus 35S-promoter. Indeed, Western analysis clearly shows more accumulation of mutant proteins in the knock-out background (see Fig. 3.5A).

I speculate that the AtFAAH protein itself might directly interact with other protein(s) involved in ABA signaling. Two conserved domains were previously identified near the C terminus of plant FAAH proteins outside of the catalytic site (Shrestha et al., 2006; Chapman, 2004), which perhaps could facilitate interactions with target proteins. A recent report indicated that mammalian FAAH can interact with a membrane protein, ERp57, in caveolin-rich membranes (Yates and Barker, 2007), but the physiological significance of this interaction
remains unclear. Future efforts will be aimed at uncovering the mechanism(s) by which AtFAAH may exert its effects on ABA sensitivity by identifying the binding partner molecule(s) of AtFAAH in *Arabidopsis* cells and identifying domains responsible for interactions.

Alternatively, differential localization of overexpressed AtFAAH protein (mutant or otherwise) might be responsible for the ABA sensitivity phenotype. However, this explanation is not entirely satisfactory because in previous experiments, when AtFAAH-green fluorescent protein was overexpressed in the *AtFAAH* knock-out background this overexpressed protein complemented the knock-out phenotype and conferred tolerance to exogenous NAE in a manner similar to overexpression of AtFAAH without green fluorescent protein (Kang et al., 2008). This suggested that the endoplasmic reticulum/plasma membrane localization of the FAAH-green fluorescent protein was at least partially reflective of the normal location of AtFAAH (to functionally restore the phenotype of knock-outs) and that this AtFAAH-green fluorescent protein overexpression was a reasonable reporter of overexpressed AtFAAH location because the NAE-tolerant growth was similar between plants overexpressing either AtFAAH protein (Kang et al., 2008). Nonetheless, an effect of AtFAAH location due to overabundance of active or inactive AtFAAH transgene product should not be entirely ruled out.

In conclusion, I have shown that the AtFAAH influences plant growth through its hydrolysis of acylethanolamides, but that interaction with ABA is independent of its hydrolytic activity. We proposed previously that NAE metabolism and its influence by FAAH resides at the balance between plant growth and the responses of plants to stress (Kilaru et al., 2007). The results presented here are consistent with this concept and offer bifurcating mechanisms that may mediate this physiological control. Although understanding the detailed mechanisms involved in these two processes will require further experimentation beyond the scope of this chapter, the
novel results presented here provide continued direction to functionally define the group of enzymes that metabolize NAE in plants. Furthermore, this work suggests the future possibility to uncouple the remarkable increase in overall plant growth seen in AtFAAH overexpressors from the concomitant increased susceptibility to stress, which could have important applications in crop biotechnology.

Methods

Chemicals and Reagents

[1-\textsuperscript{14}C] Lauric acid was purchased from Amersham Biosciences (Alameda, CA). [1-\textsuperscript{14}C] Palmitic acid and [1-\textsuperscript{14}C] linoleic acid were from PerkinElmer Life Sciences. Dimethyl sulfoxide (DMSO), isopropyl β-D-1-thiogalactopyranoside, ethanolamine, \textit{cis}-9-octadecenamide, and ABA were from Sigma. Silica Gel G (60 Å)-coated glass plates (10×20 cm or 20×20 cm, 0.25 mm thickness) were from Whatman. \textit{n}-Dodecyl β-D-maltoside was from Calbiochem (La Jolla, CA). All organic solvents (isopropyl alcohol, chloroform, hexane, ethyl acetate, and methanol) were from Fisher. Polyvinylidene fluoride membrane (0.2 μm) and goat anti-mouse (or anti-rabbit) IgG conjugated to horseradish peroxidase were from Bio-Rad. Anti-c-Myc monoclonal antibody was from Abgent (San Diego, CA). Anti-AtFAAH polyclonal antibody was generated in Biosynthesis (Lewisville, TX). \textit{N}-Lauroyl ethanolamide, \textit{N}-palmitoyl ethanolamide, \textit{N}-linoleoyl ethanolamide, and \textit{sn}-2 arachidonoyl glycerol were from Cayman Chemical (Ann Arbor, MI).

Plant Materials and Growth Measurements

\textit{AtFAAH} T-DNA insertional mutant (SALK_095108) was originally obtained from the
Arabidopsis Biological Resource Stock Center (Ohio State University, Columbus, OH) and was characterized previously (Wang et al., 2006). Transgenic Arabidopsis lines overexpressing native AtFAAH proteins under the control of the cauliflower mosaic virus 35S-promoter were previously described (Wang et al., 2006). Plants were screened for zygosity using the REDExtract-N-Amp Plant PCR kit (Sigma). Plants were propagated in soil for seed production. For growth assay, seeds were first surface-sterilized with 95% ethanol, 30% bleach containing 0.1% Tween-20 and deionized water, and stratified for 3 days at 4 °C in the dark. Seeds were grown for 10 days in nutrient media (0.5× Murashige and Skoog salts containing 1% sucrose) in a controlled environment room with a 16-h light/8-h dark cycle at 20 °C. For detailed growth measurements, seedlings grown on agar plates were tilted at an ~60° angle to facilitate reproducible measurements of root elongation. Cotyledon area and primary root length were measured from captured images of the seedlings. For fresh weight measurements, seedlings were grown in liquid media with shaking at 75 rpm, harvested by filtration, dried, and quantified in terms of seedling mass (mg) normalized to mass of seeds sown (mg). ABA or NAE, both dissolved in DMSO, were added to the appropriate final concentrations, and untreated controls contained equivalent amounts of DMSO alone (always less than 0.05% by volume). Concentrations of exogenous ABA were calculated based on the active cis-isomer.

Site-directed Mutagenesis of AtFAAH

The original construct pCAMBIA1390-AtFAAH used to generate overexpressor lines (Wang et al., 2006) was used as template in reactions of site-directed mutagenesis by using the QuikChange® II XL site-directed mutagenesis kit according to the manufacturer’s recommendations (Stratagene, La Jolla, CA). In short, 50 ng of the template DNA was used in
PCR with primers containing nucleotide corresponding to amino acid change. The PCR program includes the following steps: 95 °C for 1 min (95 °C for 50 s, 60 °C for 50 s, 68 °C for 12 min) repeat 17 more cycles, and 68 °C for 7 min. The reaction mixture was digested with DpnI at 37 °C for 2 h to remove parent plasmids. Then the DNA was precipitated and used to transform XL10-Gold competent cells. Mutations were confirmed by sequencing. The constructs were transformed into Agrobacterium strain GV3101 and used to transform Arabidopsis wild-type (Col-0) and AtFAAH knock-out plants by floral dipping (Clough and Bent, 1998). Transgenic plants resistant to hygromycin (15 mg/liter) were selected from MS medium. Putative transgenic plants were further confirmed with sequencing and reverse transcription-PCR with construct specific primers.

Recombinant Protein Expression and Purification

For proteins expressed in E. coli, AtFAAH cDNAs with the site-directed mutations were PCR-amplified, agarose gel-purified, cloned into pTrcHis2 vector (Invitrogen), and transformed into E. coli TOP10 cells. Selected transformants were grown in LB medium at 37 °C with shaking at 250 rpm to an $A_{600}$ of 0.6, and incubated with 1 mM isopropyl β-D-1-thiogalactopyranoside for 4 h. Recombinant proteins expressed in-frame with the His$_6$ tag were nickel-nitrilotriacetic acid affinity purified using the QIAexpress protein purification kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Eluted proteins were concentrated, and imidazole was removed with 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 0.2 mM dodecylmaltoside by filtration centrifugation using Centricon YM-30 (Millipore, Bedford, MA). Protein concentrations were determined by the Bradford assay using bovine serum albumin as a standard. For proteins expressed in Arabidopsis, seedlings grown in liquid media were flash
frozen and powdered in liquid nitrogen using a mortar and pestle, and suspended in homogenization buffer (100 mM potassium phosphate, pH 7.2, 400 mM sucrose, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 5 mM MgCl₂). After incubation on ice for 30 min, homogenates were centrifuged at 12,000 × g for 15 min, and the resulting supernatants were used for further experiments.

**FAAH Enzyme Activity Assays**

14C radiolabeled NAEs were synthesized from corresponding free fatty acids and ethanolamine (Shrestha et al., 2002), and combined with non-radiolabeled NAEs to achieve the desired final concentration. Enzyme activity was determined based on radiospecific activity. Protein samples were incubated with 100 μM (~12,000 cpm) NAEs (12:0, 16:0, or 18:2) or 100 μM 2-arachidonoyl glycerol in 50 mM BisTris buffer (pH 9.0) in a final volume of 0.4 ml at 30 °C for 30 min with shaking at 120 rpm. Reactions were terminated by the addition of boiling isopropyl alcohol (70 °C) for 30 min. Total lipids were extracted into chloroform, washed twice with 1 M KCl and once with water, and separated by Silica Gel-thin layer chromatography (TLC) using an organic solvent mixture of hexane, ethyl acetate, and methanol (60:40:5, v/v/v). Distribution of unreacted substrates and products formed was evaluated either by radiometric scanning (AR-2000 Imaging Scanner, Bioscan, NW Washington, DC) of the TLC plate for amidase activity assays or by exposure of the plate to iodine vapors for monoacyl esterase activity assays.

**Western Blot Analysis**

Protein samples were separated on 10% polyacrylamide SDS gels and electrophoretically
transferred onto polyvinylidene fluoride membranes in a Semidry Trans-Blot apparatus (Bio-Rad) for 30 min at constant 14 V. The membranes were blocked in 5% nonfat milk in Tris-buffered saline (20 mM Tris-HCl, pH 7.5, and 500 mM NaCl) containing 0.1% Tween 20. Affinity-purified proteins expressed in *E. coli* as in-frame c-Myc-epitope fusions and proteins expressed in *Arabidopsis* were localized by overnight incubation at room temperature with mouse monoclonal anti-c-Myc antibodies (Abgent, San Diego, CA) or rabbit polyclonal anti-AtFAAH antibodies, respectively. Immunolocalized proteins were detected by chemiluminescence following incubation for 1 h at room temperature with either goat anti-mouse IgG or goat anti-rabbit IgG (Bio-Rad), both conjugated to horseradish peroxidase, according to the manufacturer’s instructions.
Table 3.1 NAE hydrolysis activity of mutant proteins expressed in *E. coli*.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Specific activity</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NAE12:0</td>
<td>NAE16:0</td>
</tr>
<tr>
<td></td>
<td>µmol h(^{-1}) mg(^{-1})</td>
<td>%</td>
</tr>
<tr>
<td>WT</td>
<td>117±8</td>
<td>134±10</td>
</tr>
<tr>
<td>K205A</td>
<td>0.06±0.01</td>
<td>0.07±0.02</td>
</tr>
<tr>
<td>S281A/S282A</td>
<td>0.08±0.01</td>
<td>0.08±0.01</td>
</tr>
<tr>
<td>S305A</td>
<td>0.09±0.02</td>
<td>0.11±0.02</td>
</tr>
<tr>
<td>R307A</td>
<td>0.16±0.03</td>
<td>0.21±0.03</td>
</tr>
<tr>
<td>S360A</td>
<td>119±7</td>
<td>145±12</td>
</tr>
</tbody>
</table>

Proteins affinity purified from *E. coli* were reacted with \(^{14}\)C radiolabeled NAE12:0, NAE16:0, or NAE18:0. Specific activities were calculated based on radioactivity of the products formed. Values are shown as mean ± S.D. of triplicate measurements. For each NAE substrate, enzyme activities relative to wild-type AtFAAH without mutation (100%; WT) are indicated under “relative activity”. 
Table 3.2 Apparent kinetic parameters of wild-type AtFAAH and the S360A mutant.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$V_{\text{max}}$</th>
<th>$K_m$</th>
<th>$K_{\text{cat}}$</th>
<th>$K_{\text{cat}}/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\mu$mol h$^{-1}$ mg$^{-1}$</td>
<td>$\mu$M</td>
<td>s$^{-1}$</td>
<td>$\mu$M$^{-1}$ s$^{-1}$</td>
</tr>
<tr>
<td>WT</td>
<td>181.7</td>
<td>23.0</td>
<td>3.33</td>
<td>0.145</td>
</tr>
<tr>
<td>S360A</td>
<td>205.4</td>
<td>33.1</td>
<td>3.77</td>
<td>0.114</td>
</tr>
</tbody>
</table>

Initial velocities (for 10 min) of affinity purified wild-type (WT) and S360A proteins were measured at increasing concentrations of [1-$^{14}$C] NAE16:0. Values were estimated with Prism software version 3.0 (GraphPad Software) from triplicate measurements.
Figure 3.1 Expression of site-directed AtFAAH mutants in *E. coli*. A, conserved catalytic residues in the amidase signature (AS) sequence between rat and *Arabidopsis* FAAH proteins. Full sequences of rat and *Arabidopsis* FAAH (Ara) were aligned using T-coffee software (Swiss Institute of Bioinformatics). AS regions that consist of 125 amino acids are shown here. Five residues (Lys-142, Ser-217, Ser-218, Ser-241, and Arg-243) known to be critical for rat FAAH activity are highlighted in black boxes. These residues are also conserved in the *Arabidopsis* sequence as shown (Lys-205, Ser-281, Ser-282, Ser-305, and Arg-307). B, Western blot analysis. Wild-type AtFAAH protein without mutation (WT) and the proteins with site-directed mutation (indicated by their corresponding residues) were expressed in *E. coli*, and affinity purified proteins (1 μg) were analyzed by Western blot. All mutated AtFAAH proteins were immunolocalized at the positions expected (~69.4 kDa including epitope tags).
Figure 3.2 Representative radiochromatograms for NAE hydrolase assay of site-directed AtFAAH mutants. Equal amounts of affinity purified proteins were reacted with [1-\(^{14}\)C] NAE16:0. Total lipids extracted from reaction mixtures were separated by thin layer chromatography and analyzed by radiometric scanning. Each radiochromatogram shows the distance (mm) on the x axis that lipids migrated on the TLC plate and their radioactivity (cpm) on y axis. Picks that represent NAE and FFA (free fatty acid) are indicated. Wild-type (WT) and S360A mutant show a significant production of free fatty acid (peak at ~97 mm), whereas all mutants did not produce any detectable products. Enzyme activities for mutants compared with wild-type are summarized in Table 3.1.
Figure 3.3 Kinetic comparison of wild-type and S360A mutant. Initial velocities (for 10 min) were measured at increasing concentrations of [1-\(^{14}\)C] NAE16:0. Michaelis-Menten and Lineweaver-Burk plots are shown for comparison. Plots were generated with Prism software version 3.0 (GraphPad Software, San Diego, CA). Data points represent mean ± S.D. of triplicate assays. Kinetic parameters for wild-type and S360A mutant are summarized in Table 3.2.
Figure 3.4 Lack of enzyme activity of the mutants toward monoacylester and primary amide substrates. 1 µg of purified wild-type and S360A proteins and 10 µg of all other mutant proteins were reacted with 2-arachidonoyl glycerol (2-AG; left plate) and 9-octadecenamide (oleamide; right plate). Total lipids from the reactions were separated by TLC, and visualized by iodine vapors. Positions for substrates (2-AG and oleamide) and products (arachidonic acid and oleic acid) are indicated on the left. Wild-type and S360A mutant show a significant formation of free fatty acid products, whereas all mutants display essentially no product formation. Arachidonic acid and oleic acid standards (Std.) are also included for position comparison.
Figure 3.5 Expression of site-directed AtFAAH mutants in *Arabidopsis* and evaluation of their enzyme activities. A, Western blot analysis. *Arabidopsis* wild-type (WT) and transgenic lines (SW, RW, OE, KO, SK, and RK) were grown for 10 days as described under “Methods”. Total proteins (200 μg) from each seedling homogenates were analyzed by Western blot using anti-AtFAAH antibody. All AtFAAH proteins of overexpressors (SW, RW, OE, SK, and RK) were immunolocalized at the position expected (~66.1 kDa). SW, S281A/S282A mutant expressed in wild-type background; RW, R307A mutant expressed in wild-type; OE, native AtFAAH overexpressor; KO, *Atfaah* T-DNA insertion knock-out; SK, S281A/S282A mutant expressed in *Atfaah* knock-out background; RK, R307A mutant expressed in *Atfaah* knock-out. B, NAE hydrolysis activities. Total proteins (50 μg) from each of 10-day-old seedling homogenates were reacted with [1-14C] NAE16:0. Total activities (μmol/h) were measured based on radioactivity of
the product formed. All AtFAAH overexpressors with site-directed mutation (SW, RW, SK, and RK) exhibited significantly lower NAE hydrolase activity than the native AtFAAH overexpressor (OE). The error bars represent S.D. from triplicate measurements. Asterisks indicate a significant difference ($p < 0.0001$) compared with OE, which was determined by Student’s $t$ test.
Figure 3.6 Overall apparent growth phenotype of *Arabidopsis* seedlings expressing AtFAAH mutants in response to NAE and ABA. Seedlings were grown for 10 days in the presence of DMSO only, 30 μM NAE12:0, or 0.25 μM ABA. A, growth phenotype in liquid media.
Seedlings were grown in flasks, and then transferred onto Petri dishes to photograph. Representative images of triplicate experiments are shown here. All site-directed mutant lines (SW, RW, SK, and RK) display no growth enhancement and no NAE tolerance compared with native AtFAAH overexpressor (OE), whereas they all show ABA hypersensitivity compared with their background lines (WT and KO). B, growth phenotype in solid media. Representative images of 24 individual seedlings for each line are shown. Note that relative growths of the different lines are highly analogous to those in liquid medium (A). KO, knock-out; SW, S281A/S282A mutant expressed in wild-type background; RW, R307A mutant expressed in wild-type background; SK, S281A/S282A mutant expressed in Atfaah knock-out background; RK, R307A mutant expressed in Atfaah knock-out background.
Figure 3.7 Quantitative growth measurements of *Arabidopsis* seedlings expressing AtFAAH mutants in response to NAE and ABA. 10-Day-old seedlings were quantified in terms of seedling fresh weight (mg of seedling tissue/mg of seed sown), primary root length (cm), and cotyledon area (mm²). Values for fresh weight represent mean ± S.D. of triplicate measurements. Values for primary root length and cotyledon areas represent mean ± S.D. of 10 individual seedlings. Single and double asterisks indicate a significant difference ($p < 0.0001$) compared with wild-type (*WT*) and *Atfaah* knock-out (*KO*), respectively, which was determined by
Student’s t test. OE, overexpressor; SW, S281A/S282A mutant expressed in wild-type background; RW, R307A mutant expressed in wild-type background; SK, S281A/S282A mutant expressed in Atfaah knock-out background; RK, R307A mutant expressed in Atfaah knock-out background.
CHAPTER 4
IDENTIFICATION OF DOMAIN-SPECIFIC INTERACTING PROTEINS OF FATTY ACID AMIDE HYDROLASE IN ARABIDOPSIS: A POTENTIAL MECHANISM FOR THE MODULATION OF ABA SIGNALING

Abstract

Fatty acid amide hydrolase (FAAH) is an enzyme that hydrolyzes N-acylethanolamines, a class of lipid mediators, and is involved mainly in the endocannabinoid signaling system in animals and in various physiological processes in plants such as seedling growth, innate immunity, and hormone signaling. Previously, overexpression of active site-directed Arabidopsis FAAH (AtFAAH) mutants was shown to render Arabidopsis seedlings hypersensitive to abscisic acid (ABA) and hypersusceptible to pathogens, as observed in the native AtFAAH overexpressor, suggesting that AtFAAH might physically interact with other proteins to exert these effects independent of catalytic activity. Here I report that yeast two-hybrid screening identified HNH endonuclease domain-containing protein (HNH; At3g47490) and Arg/Ser-rich zinc knuckle-containing protein 33 (RSZ33; At2g37340) as AtFAAH-interacting proteins, which was further verified by immunoprecipitation from yeast extracts. Three consecutive arginine residues (Arg-491, Arg-492 and Arg-493) in AtFAAH were found to be necessary for the protein-protein interaction by a series of domain deletion and alanine scanning mutagenesis. Homozygous RSZ33 T-DNA insertional knock-out plants were identified from the Salk collection, and seedlings of this line, like the AtFAAH overexpressor seedlings, exhibited hypersensitivity to growth on ABA. Additional preliminary evidence for AtFAAH-protein interaction is also demonstrated here. Taken together, AtFAAH appears to interact directly and specifically with
both HNH and RSZ33 proteins. This interaction may be involved in the modulation of ABA signaling, in part, by inhibiting RSZ33 action. My data suggest a model where RSZ33 normally acts to suppress ABA signaling and its interaction with AtFAAH inhibits this activity. Future experiments with overexpression of RRR domain deletion mutants should help test this model.

Introduction

Fatty acid amide hydrolase (FAAH) catalyzes the hydrolysis of N-acylethanolamines (NAEs) and sn-2-acylglycerols and thereby terminates endocannabinoid signaling pathway in animals that regulates numerous neurobehavioral processes such as appetite, pain sensation, and memory (reviewed in Fowler, 2006; McKinney and Cravatt, 2005; Fezza et al., 2008). FAAH has been identified and characterized mostly in animal systems, and, due to its ability to regulate important physiological processes, is currently considered as a drug target for the development of novel therapeutics (reviewed in Petrocino and Di Marzo, 2010; Petrocino et al., 2009). Recently, functional homologues of FAAH were identified in several plant species including Arabidopsis thaliana, Oryza sativa (rice), and Medicago truncatula and their biochemical/enzymological properties were found to be similar, but not identical, to those of animal FAAH (Shrestha et al., 2003; Shrestha et al., 2006; chapter 2 of this dissertation). Plant FAAH was shown to function in several plant processes such as seedling growth, innate immunity, and phytohormone signaling, although the precise mechanisms for these actions remain to be resolved (Wang et al., 2006; Teaster et al., 2007; Kang et al., 2008; Kim et al., 2009).

Unlike typical enzymes that are highly specific for one, or a few, substrates, FAAH hydrolyzes the amide/ester bond of a broad range of bioactive lipid substrates in vivo including NAEs (e.g. N-arachidonoyl ethanolamine), fatty acid primary amides (e.g. 9-octadecenamide;
Maurelli et al., 1995; Cravatt et al., 1995), sn-2-acylglycerols (e.g. 2-arachidonoylgllycerol; Goparaju et al., 1998; Patricelli and Cravatt, 1999), fatty acid esters (e.g. fatty acid methyl ester Patricelli and Cravatt, 1999), and N-acyl taurines (Saghatelian et al., 2006). Among these substrates for FAAH, NAEs have been most intensively studied, and some of these NAEs are natural ligands of cannabinoid receptors (CB) that constitute the endocannabinoid signaling system in animal tissues (Devane et al., 1992). The signaling pathway involving N-arachidonoylethanolamine (NAE20:4) as a retrograde messenger in the central nervous system is well established (Kano et al., 2009). N-oleylethanolamine (NAE18:1) and N-palmitoylethanolamine (NAE16:0) also possess biological activity via CB-dependent or CB-independent pathways (Di Marzo et al., 2007; Wang and Ueda, 2009). In plants, one of the most noticeable effects of NAE (NAE12:0) is its inhibitory effect on normal seedling development. In most plant species, NAEs are present in low micromolar amounts in desiccated seeds, and their levels decline markedly after imbibition and germination to barely detectable levels (Chapman et al., 1999). Indeed, the addition of exogenous NAE12:0 (or NAE18:2) at low micromolar concentrations exhibited a dose-dependent reduction of seedling growth in Arabidopsis (Blancaflor et al., 2003; Wang et al., 2006). Together with these findings, reduction of endogenous NAE levels and enhanced seedling growth of transgenic Arabidopsis lines overexpressing FAAH suggested that FAAH is a modulator of endogenous NAE levels in plants as well, and that NAE depletion by the action of FAAH participates in the regulation of plant growth (Wang et al., 2006).

NAEs appear to regulate seedling growth in part through its interaction with plant hormone abscisic acid (ABA) signaling. Treatment of Arabidopsis seedlings with NAE elevated the transcripts for a number of ABA-responsive genes and the ABA-insensitive mutants, abi1-1,
abi2-1, abi3-1, exhibited reduced sensitivity to NAE (Teaster et al., 2007). Combined application of NAE and ABA produced a synergistic reduction in early seedling growth of Arabidopsis (Teaster et al., 2007). However, more complex level of interaction between the two metabolites appeared to operate in regulating seedling development because overexpression of Arabidopsis FAAH (AtFAAH) resulted in seedlings that had lower levels of NAE but were hypersensitive to ABA compared to wild-type seedlings (Teaster et al., 2007). The ABA hypersensitivity of AtFAAH overexpressor seedlings was unable to be explained by FAAH-catalyzed depletion of NAE because the overexpression of active site-directed mutant forms of AtFAAH lacking its catalytic activity still resulted in ABA hypersensitivity of Arabidopsis seedlings (Kim et al., 2009; also see chapter 3). The catalytic activity-independent action of AtFAAH also was exemplified by hypersusceptibility of the mutated AtFAAH overexpressor to bacterial pathogens, similar to that of the native AtFAAH overexpressor (Kang et al., 2008; Kim et al., 2009). These results suggested that AtFAAH protein interacts with ABA/defense signaling by mechanisms dependent on protein abundance but independent of catalytic activity.

One possible mechanism for the protein abundance-dependent action of AtFAAH would be by a physical interaction with other proteins involved in ABA/defense signaling, perhaps by titrating important components of the normal signaling pathway. Thus, in this chapter I attempted to identify Arabidopsis proteins that physically interact with AtFAAH using a yeast two-hybrid system, and screening an Arabidopsis seedling cDNA library. I found two AtFAAH-interacting proteins through both in vivo and in vitro approaches using yeast cells, at least one of which appeared to be involved in ABA signaling as manifested by ABA hypersensitivity of its T-DNA insertional loss-of-function mutant. These results will provide insight into the bifurcating function of AtFAAH that influences both plant growth and stress responses by distinct
mechanisms.

Results

Yeast Two-hybrid Screening Identified Two Candidate AtFAAH-interacting Proteins

An Arabidopsis cDNA library was screened for proteins (prey) interacting with full-length AtFAAH (bait; see Methods section for details). Initial yeast mating of haploids resulted in approximately $1.3 \times 10^7$ colonies on synthetic drop-out (SD) medium lacking leucine and tryptophan (LW), indicating that the mating efficiency was high enough to cover the entire Arabidopsis transcriptome. Subsequent screening of the diploid cells on SD medium lacking leucine, tryptophane, and histidine (LWH) resulted in 17 colonies, out of which 4 colonies grew well and generated a blue color on SD medium lacking leucine, tryptophane, histidine and adenine and containing X-gal (LWHAdX). To test autoactivation of the reporter genes by prey protein alone, the prey vector-cDNA constructs were isolated from the 4 colonies, and each co-transformed with empty bait vector into the yeast cells. Two of them grew and were blue on the LWHAdX medium, indicating false positives. AtFAAH alone did not activate the reporter genes.

The cDNAs that exhibited positive interaction but did not display autoactivation were sequenced by a commercial service, and turned out to encode full lengths of Arabidopsis HNH endonuclease domain-containing protein (At3g47490; hereafter designated as HNH for simplicity) and Arg/Ser-rich zinc knuckle-containing protein (At2g37340; RSZ33).

The Protein-protein Interactions Were Further Verified both In vivo and In vitro

Fig. 4.1A shows typical results obtained from the yeast two-hybrid analysis. Yeast cells grew well and produced blue color on the LWHAdX medium due to the activation of the reporter
genes only when both bait (AtFAAH) and prey (HNH/RSZ33) proteins were co-expressed, indicating \textit{in vivo} interaction in yeast cells between AtFAAH and HNH/RSZ33. Note that all clones grow well on the LW medium, suggesting that the two vectors were successfully co-transformed and expression of the two proteins does not affect normal growth of the yeast cells.

To further verify physical interaction between AtFAAH and HNH/RSZ33, I performed \textit{in vitro} immunoprecipitation assays (Fig. 4.1B). Affinity-purified AtFAAH with c-myc epitope tag was added to yeast lysate expressing HNH or RSZ33 with hemagglutinin (HA) epitope tag, and was immunoprecipitated using anti-c-myc antibody to pull down AtFAAH. The immunocomplexes were resolved and visualized by Western blot using either anti-c-myc antibody to detect AtFAAH or anti-HA antibody to detect HNH or RSZ33. HNH and RSZ33 were co-immunoprecipitated with AtFAAH but were not immunoprecipitated in the absence of AtFAAH (Fig. 4.1B), confirming interaction \textit{in vitro} between AtFAAH and HNH/RSZ33. The absence of either HNH or RSZ33 when the addition of AtFAAH was omitted, rules out the possibility of non-specific binding of the anti-c-myc antibody to HNH or RSZ33 proteins.

\textit{Identification of Domain(s) in AtFAAH Important for the Protein-protein Interactions}

Our previous study reported that AtFAAH protein had two unknown domains near its C-terminus conserved in many plant FAAH proteins (Fig. 4.2A; hereafter designated as domain 5 and domain 6 in accordance with the designation used in Shrestha et al., 2006). I speculated that either or both of the two unknown domains might be responsible for the interaction with HNH or RSZ33, leading me to a series of domain deletion analysis to probe the domains or regions necessary for the interaction. Thus, I deleted each ($\Delta$5 or $\Delta$6) and both ($\Delta$5/6) of the two domains and, in addition, first half ($\Delta$5-1 or $\Delta$6-1) and second half ($\Delta$5-2 or $\Delta$6-2) of each domain by
PCR-based deletion mutagenesis. Each of the domain-deleted *AtFAAH* cDNA constructs was co-transformed with the *HNH* cDNA into yeast cells, and tested for auxotrophic growth of the cells on the LWHAdX plate (Fig. 4.2B). Interestingly, any yeast clones with deletion of the first half of domain 5 (Δ5, Δ5/6, and Δ5-1) did not grow at all, while all other clones containing this region (Δ6, Δ5-2, Δ6-1, and Δ6-2) grew well and produced blue color (Fig. 4.2B), suggesting that the first half of domain 5 (5-1) might be essential for the interaction of AtFAAH with HNH. Since, unexpectedly, it was observed that all of these domain deletions completely abolished catalytic activity of AtFAAH toward NAE substrates (data not shown), one of the active site-directed mutants (K205A) that were previously found to have no catalytic activity (Kim et al., 2009; also see chapter 3) was included as a control to rule out the possibility that loss of catalytic activity might affect yeast growth. Also, protein expression of AtFAAH and HNH in the yeast cells that did not grow (Δ5, Δ5/6, and Δ5-1) was confirmed by Western blotting (Fig. 4.2C). Survival of the cells with K205A mutant on LWHAdX plate (Fig. 4.2B) and normal expression of the AtFAAH and HNH proteins on Western blot (Fig. 4.2C) all suggest that no growth of the cells harboring Δ5, Δ5/6, and Δ5-1 mutants was attributed solely to the breakdown of protein-protein interaction resulted from the missing domain.

**Three Arginine Residues in AtFAAH Are Necessary for the Protein-protein Interactions**

Previous computational studies revealed seven amino acid residues in domain 5-1 of AtFAAH that were absolutely conserved in plant FAAH sequences (Shrestha et al., 2006). These residues include Q488, R491, R492, R493, M495, Y497, and F502 in *Arabidopsis* sequence (Fig. 4.3A). To identify the amino acid residue(s) necessary for the interaction of AtFAAH with HNH/RSZ33, the seven amino acids were individually substituted to alanine by site-directed
mutagenesis and analyzed by yeast two-hybrid system in the same way described above. This so-called alanine scanning analysis revealed that individual amino acid substitution did not alter the protein-protein interaction, and thereby the growth of yeast cells on LWHAdX medium. But interestingly, when the three consecutive arginine residues (R491, R492, and R493) were simultaneously mutated to alanine, no interaction of AtFAAH with either HNH or RSZ33 was observed in yeast cells (Fig. 4.3B). This result suggests that the three arginines are necessary for the interaction of AtFAAH with both HNH and RZS33 and that the two proteins may compete for the binding site on AtFAAH. NAE amidohydrolase assays showed hydrolysis of NAE to free fatty acid by the yeast cells expressing AtFAAH with the triple arginine mutation (Fig. 4.3C), again suggesting that non-growth of the cells was attributable solely to the disruption of protein-protein interaction resulted from the mutation of the three arginines.

RSZ33 Knock-out Plants Display Hypersensitivity to ABA

If the ABA hypersensitivity observed in AtFAAH overexpressor (Teaster et al., 2007; Kim et al., 2009; also see chapter 3) was due to alteration of ABA signaling by direct interaction of AtFAAH with HNH or RSZ33 in Arabidopsis cells, it is possible that Arabidopsis HNH or RSZ33 knock-out lines would exhibit growth phenotype different from wild-type plant in response to ABA, either hypersensitive or insensitive to ABA. Thus, I obtained homozygous T-DNA insertional knock-out lines of HNH (SALK_006389C) and RSZ33 (SALK_083782C), and examined their growth phenotypes in response to ABA in comparison with wild-type and AtFAAH overexpressor (Fig. 4.4). Interestingly, RSZ33 knock-out line exhibited marked ABA hypersensitivity to a degree very similar to the AtFAAH overexpressor, when compared to wild-type plant, in terms of all phenotypic characteristics tested—seedling fresh weight, primary root
length, and cotyledon area. The *HNH* knock-out line, however, appeared to be somewhat more sensitive to ABA than wild-type but it was neither statistically significant nor as dramatic as *RSZ33* knock-out line. All lines tested grew well in the presence of solvent (DMSO) only. Taken together, the observations that both AtFAAH overexpression and *RSZ33* loss-of-function rendered *Arabidopsis* seedlings hypersensitive to ABA and that these two proteins interacted with each other suggested a model that *RSZ33* may normally act to suppress ABA signaling and that AtFAAH might inhibit *RSZ33* action and this inhibition enhances ABA responses in *Arabidopsis* plants (See Fig. 4.5).

**Additional Preliminary Evidences for AtFAAH-protein Interaction**

Seedlings with *Atfaah* disruptions do exhibit some ABA tolerance (Kim et al., 2009; also see Figs. 3.6 and 3.7) relative to wild-type seedlings, suggesting that AtFAAH-*RSZ33* interaction may be relevant *in vivo* for modulation of ABA signaling. This concept also helps reconcile previous experimental results where overexpression of AtFAAH with green fluorescent protein (GFP) fused to the C-terminus renders seedlings less sensitive to ABA, whereas overexpression of AtFAAH with GFP fused to the N-terminus displays ABA hypersensitivity despite similar FAAH specific activity of both GFP-fused seedlings relative wild-type or AtFAAH (without GFP) overexpressor. So I had hypothesized that something other than catalytic activity was responsible for the modulation of ABA sensitivity and perhaps it is the interaction with *RSZ33*, an interaction that might be blocked by GFP tag at the C-terminus, but not at the N-terminus. A direct interaction, however remains to be tested.

To identify proteins that physically interact with AtFAAH *in planta*, AtFAAH was immunoprecipitated from *Arabidopsis* seedling homogenate overexpressing AtFAAH fused at
the N-terminus with GFP using anti-GFP antibody. *Arabidopsis* homogenate overexpressing AtFAAH without GFP was included as a negative control for comparison. The resulting immunoprecipitants were then subjected to SDS-PAGE to visualize proteins that had been co-immunoprecipitated with AtFAAH. Several protein bands were detected over the gel which were not present in the control (Fig. 4.6A). These proteins were extracted out of the gel, digested by trypsin, and subjected to electrospray ionization tandem mass spectrometry (ESI-MS/MS) for sequence determination and protein identification by BLAST comparisons to *Arabidopsis* protein databases. As expected, one of the proteins was identified with a high sequence coverage as AtFAAH (*N*-acylethanolamine amidohydrolase; At5g64440) with GFP tag (Fig. 4.6A and B), suggesting a good reliability of this technique for protein identification. For unknown reasons, neither HNH nor RSZ33 was detected by this approach. Among other proteins detected on the gel and identified with high sequence coverage, polyadenylate-binding protein 8 (PABP8; Fig. 4.6A and B), which possessed a RNA recognition motif (RRM) responsible for RNA binding, was of interest because RNA metabolism had been reportedly implicated in ABA signaling.

The observations that PABP8 was detected by co-immunoprecipitation but not by yeast two-hybrid analysis suggest that the interaction between PABP8 and AtFAAH might be mediated by another protein that directly interacts with AtFAAH. Thus, I performed yeast two-hybrid analysis to see if PABP8 interacts with either HNH or RSZ33. PABP8 was found to indeed interact with HNH (Fig. 4.6C), but not with RSZ33. Although *in planta* occurrence and physiological significance in *Arabidopsis* of this protein-protein interaction remain to be studied, it was surprising that HNH identified by yeast two-hybrid approach (Fig. 4.1) mediates interaction between AtFAAH and PABP8 identified by co-immunoprecipitation (Fig. 4.6A and B). Yeast three-hybrid approach may be useful to see the simultaneous interaction of the three
proteins. Interestingly, this interaction between AtFAAH and PABP8 was found to occur only in the absence of ABA by co-immunoprecipitation followed by SDS-PAGE and ESI-MS/MS (Fig. 4.7), possibly accounting for the competition of HNH and RSZ33 for the binding site on AtFAAH; HNH (and PABP8) may normally stay bound to AtFAAH and in the presence of ABA, RSZ33 replaces HNH resulting in enhancement of ABA signaling.

Discussion

The marked reduction of NAE levels during seed germination of plants (Chapman et al., 1999) and the inhibition of Arabidopsis seedling development by exogenously applied NAE (Blancaflor et al., 2003; Wang et al., 2006) suggest a physiological significance of NAE depletion in normal seedling growth in plants. Evidence revealed that NAE might regulate this seed-to-seedling transition in part through its interaction with ABA signaling. ABA, like NAE, was rapidly depleted during Arabidopsis seed germination and the combined application of both NAE and ABA resulted in a synergistic reduction in germination and seedling growth of Arabidopsis (Teaster et al., 2007). Moreover, NAE-treated seedlings had elevated transcripts for a number of ABA-responsive genes and ABA-insensitive mutants, abi1-1, abi2-1, abi3-1, exhibited reduced sensitivity to NAE (Teaster et al., 2007). These findings imply that normal seedling establishment requires the coordinated depletion of both NAE and ABA that function together in modulating the growth of very young seedlings when conditions are not favorable for establishment.

Although it is clear that NAE metabolism modulates seedling growth by interacting with ABA signaling, this interaction appears to be complex because AtFAAH overexpressors, which had lower endogenous levels of NAE, were hypersensitive to ABA (Teaster et al., 2007).
Insights into the complexities of AtFAAH action have been revealed by a recent finding that AtFAAH has a bifurcating function to influence seedling growth. AtFAAH modulates growth via its hydrolytic activity that controls NAE levels; however in the absence of this catalytic activity, AtFAAH still interacts with ABA signaling and plant defense signaling independent of its catalytic activity (Kim et al., 2009). These findings suggest that in addition to regulating NAE levels, the AtFAAH protein itself might interact with other proteins to modulate ABA/defense signaling. Thus, in this chapter I attempted to identify Arabidopsis proteins that physically interact with AtFAAH and are involved in ABA signaling to understand molecular mechanism for the catalytic activity-independent action of AtFAAH, and found two proteins (HNH and RSZ33) directly interacting with AtFAAH, one of which appeared to function in ABA signaling, by using both in vivo and in vitro techniques.

Yeast two-hybrid approaches relying on nuclear localization of the recombinant proteins might not have been suitable for the membrane-bound AtFAAH. Mammalian FAAH is a family of membrane proteins predominantly localized in the endoplasmic reticulum (Patricelli et al., 1998; Arreaza and Deutsch, 1999). Likewise, AtFAAH was also found to associate with the endoplasmic reticulum in plant cells (Kang et al., 2008; Kim et al., 2010) indicating shared cellular targeting mechanisms with mammalian FAAH. However, unlike typical membrane-bound proteins utilizing their transmembrane segments to traverse the hydrophobic environment of the lipid bilayer, crystal structure of rat FAAH showed a hydrophobic foot formed by the arrangement of three α-helices used to anchor the protein to the membrane despite the presence of a predicted N-terminal transmembrane segment (Bracey et al., 2002; Patricelli et al., 1998). This unusual membrane-interaction mechanism of FAAH may enable nuclear localization of the protein by strong nuclear localization signal provided by the yeast two-hybrid system. Although
it is still unclear whether plant FAAH proteins have the same mechanism for membrane association, my subsequent in vitro pull-down assay (Fig. 4.1B) clearly supported that the initial screening by yeast two-hybrid approach was reliable for the identification of AtFAAH-interacting proteins.

A series of domain-deletion mutagenesis and site-directed alanine scanning analysis revealed that the three consecutive arginine residues (R491, R492, and R493) were necessary for the interaction of AtFAAH with both HNH and RSZ33 (Figs. 4.2 and 4.3), pointing to a highly specific region-selective interaction. The two AtFAAH-binding partners possibly compete for the binding site of AtFAAH by means of electrostatic attraction to the highly basic arginine side chains. The positive charge of a single arginine residue may not be sufficient to form the electrostatic interaction between the proteins, explaining why individual mutation of the arginines still retained the ability to mediate the protein-protein interaction.

The physiological significance of the competition of the proteins for AtFAAH will remain elusive until functional characterization of HNH facilitates investigation of molecular nature of its interaction with AtFAAH. Knowledge about Arabidopsis HNH endonuclease domain-containing proteins is poor. HNH (His-Asn-His) endonuclease domain is found in several bacterial toxins (e.g. E. coli colicin) that non-specifically digest cellular DNA or RNA in target cells, leading to cell death (Smarda et al., 1990). Although sequence analysis identified a few Arabidopsis proteins containing a domain conserved with the bacterial HNH endonuclease domain, neither endonuclease activity nor cellular function of the proteins has been reported as of now.

While little is known about HNH, RSZ33 (also known as AtRSZ33) has been functionally characterized as a nuclear protein that is located in nuclear speckles as part of a spliceosome.
complex by interacting with other splicing factors. It appears to participate in constitutive and/or alternative RNA splicing for regulation of gene expression (Lopato et al., 2002). This plant-specific Arg/Ser-rich protein has a unique domain structure consisting of RNA recognition motif (RRM), two zinc knuckles, and an acidic C-terminal domain (Lopato et al., 2002). RSZ33 is reported to be expressed during embryogenesis and early stages of seedling formation (Kalyna et al., 2003). Ectopic overexpression of RSZ33 resulted in various Arabidopsis developmental changes including increased cell expansion, changed polarization of cell elongation and division, and changes in auxin-responsive promoter activity (Kalyna et al., 2003). Although there is no direct evidence for implication of this protein in ABA signaling, recent growing evidence suggests that RNA metabolism or turnover is associated with ABA signaling (reviewed in Kuhn and Schroeder, 2003; Razem et al., 2006; Kuhn et al., 2008). RSZ33 may down-regulate expression of genes whose proteins are involved in ABA signaling (e.g. ABI genes) by turning over their RNA transcripts to control plant response to ABA. In this regard, transcript levels of ABI genes will need to be evaluated in RSZ33 knock-out plants and/or its overexpressor. It would also be interesting to see how Arabidopsis seedlings overexpressing RSZ33 respond to ABA treatment; if the down-regulation of ABA-responsive genes by RSZ33 is the case, the overexpressor is expected to be insensitive, or at least less sensitive than wild-type plant, to ABA unless other factors compensate. This speculation is supported simply by the observation that RSZ33 knock-out plant was hypersensitive to ABA (Fig. 4.4). The fact that HNH and PABP8, as well as RSZ33, all possess RRM supports a hypothesis that AtFAAH, when overexpressed, affects RNA metabolism through one or more of these effector molecules to alter ABA signaling. Indeed AtFAAH overexpression is associated with changes in ABI3 transcript abundance and seedling growth arrest by ABA and/or NAE was shown to be inversely associated with ABI3
transcript levels (Teaster et al., 2007). While much work remains to be done to fully understand the mechanism(s) of NAE metabolism and ABA signaling, AtFAAH and its interaction with cellular machinery may be part of this process that is outside the direct catalytic hydrolysis of NAE lipid mediators.

The impact on RSZ33 action may only be dramatically evident once an appropriate stoichiometry is met by overexpression of AtFAAH and large number of AtFAAH protein molecules can interact with RSZ33 molecules and prevent them from entering nucleus by sequestering them in cytosol, thereby enhancing ABA signaling of AtFAAH overexpressor. *In vivo* microscopic analysis and/or *in vitro* cell fractionation followed by Western blot analysis should help uncover subcellular (or topological) mechanism for the effect of AtFAAH overexpression on the suppression of RSZ33 function in *Arabidopsis* cells. In addition, although the yeast system used in this study provides an eukaryotic environment similar to plant system, future efforts will be needed to visualize the interaction *in planta* to see if the interaction of the proteins indeed occurs in their native environment and, if so, where it occurs by using fluorescence microscopic techniques such as fluorescence resonance energy transfer (FRET) or biomolecular fluorescence complementation (BiFC). In any case, the ABA hypersensitivity of *RSZ33* knock-out line (Fig. 4.4) indirectly supports the hypothesis that FAAH overexpression is mediating its ABA hypersensitive action through its interaction with RSZ33.

In contrast to the impact of AtFAAH on its binding partners, the NAE-hydrolyzing activity of AtFAAH may be down-regulated by RSZ33 via direct interaction through its C-terminal domain; AtFAAH may mimic structural features of many other signaling molecules consisting of an internal catalytic domain and a C-terminal regulatory domain. Enhanced seedling growth observed in *RSZ33* knock-out line in solvent control, even more enhanced than in AtFAAH
overexpressor (Fig. 4.4), supports this possibility that AtFAAH catalytic activity is down-regulated by RSZ33 and, in the absence of RSZ33, decreased levels of NAE by increased FAAH catalytic activity enhance seedling growth of RSZ33 knock-out Arabidopsis. This however, is speculation at this point and requires further studies and direct experimental evidence.

Together with previous findings of NAE hydrolysis by AtFAAH and seedling growth of AtFAAH overexpressors and knock-outs, the data shown in this chapter propose a molecular model for bifurcating function of AtFAAH (Fig. 4.5). NAE hydrolysis activity and/or protein expression might be regulated by a combinatorial input of both growth and stress cues. This influences either NAE levels/composition via catalytic activity of AtFAAH (through its catalytic site) to enhance growth or ABA signaling via direct interaction with RSZ33 (through the three arginine residues) to suppress growth. The fact that both AtFAAH overexpression and RSZ33 knock-out caused ABA hypersensitivity (Fig. 4.4) suggests that AtFAAH might inhibit RSZ33 action that normally negatively regulates ABA signaling. Our understanding of molecular mechanism for the bifurcating function of AtFAAH is still fragmentary. Also, further biochemical and molecular studies are required to understand physiological functions of FAAH and NAE and their signaling mechanisms in plants. Precise molecular dissection of plant FAAH action will facilitate development of an attractive strategy to improve plant productivity and stress resistance, just as mammalian FAAH currently being investigated as a novel drug target for treatment of human disorders.

Methods

Yeast Two-hybrid Screening

Yeast two-hybrid screening was performed using Matchmaker™ GAL4 Two-Hybrid
System 3 (Clontech) which supplied pGBK7T and pGADT7 vectors carrying GAL4 DNA binding domain and GAL4 activation domain, respectively and yeast strain AH109 harboring three distinct reporter genes, HIS3, ADE2 and MEL1 encoding enzymes to synthesize histidine, adenine and α-galactosidase, respectively. The pGBK7T and pGADT7 also carried auxotrophic markers for transformant selection, TRP1 and LEU2 encoding enzymes to synthesize tryptophane and leucine, respectively. Yeast strain Y187 pre-transformed with cDNA library from 11 source tissues of Arabidopsis (Col-0) cloned in pGADT7 (prey strain; Clontech) was mated with the AH109 cells transformed with AtFAAH cDNA cloned in pGBK7T (bait strain). In short, the bait strain was incubated at 30 °C in 50 ml of synthetic drop-out (SD)/-Trp liquid medium until the OD₆₀₀ reaches 0.6. The cells were pelleted, resuspended to a cell density of >1×10⁸ cells/ml in SD/-Trp medium, combined with 1 ml of the prey strain in a sterile 2 liter flask, and incubated at 30 °C for ~20 hr with a gentle shaking (~40 rpm). The mated culture was pelleted and resuspended in 10 ml of 0.5x YPDA (yeast extract, peptone, dextrose, and adenine hemisulfate) liquid medium containing 50 μg/ml kanamycin. 100 μl of 1/10,000 dilution of the culture was spread on LW agar plate and incubated at 30 °C for 4 days. The number of diploid cells was calculated by the number of colonies × 10 ml/100 μl × 10,000 (dilution factor). The remaining culture was incubated on LWH agar plates at 30 °C for 4 days. Large colonies (>2 mm in diameter) were transferred onto LWHAdX plates and incubated at 30 °C for 3 days. Plasmids were extracted from large blue colonies using ChangeSwitch® Plasmid Yeast Mini Kit (Invitrogen), transformed into E. coli TOP10 cells (Invitrogen), and incubated on Luria-Bertani (LB) plate containing 100 μg/ml ampicillin to select transformants with pGADT7 vector. Plasmids were extracted, sequenced, and sequences were blasted against Arabidopsis genome database (www.ncbi.nlm.nih.gov) for identification. For confirmation, the DNA constructs
carrying cDNAs (or empty vectors) were co-transformed into AH109 cells and incubated on LW plates. Transformant cells were suspended in sterile water. 5 μl of the cell suspensions were spotted on LW and LWHAdX plates and incubated for 2 days for imaging.

**In vitro Pull-down Assays**

Yeast cells expressing HNH endonuclease domain-containing protein or RSZ33 with hemagglutinin (HA) tag were grown in 50 ml of YPDA (yeast extract-peptone-dextrose-adenine) medium at 30 °C with shaking until mid-log phase (OD600=0.6). Cells were washed with cold water and resuspended in 1 ml of cold yeast breaking buffer (50 mM sodium phosphate pH7.4, 1 mM EDTA, and 5 % glycerol). Cells were then lysed with ~300 μl of acid-washed glass beads (0.5 mm in diameter) by vortex for 3 min (6 × 30-sec vortex/30-sec cooling on ice) and cleared by centrifugation at 13,000 rpm for 5 min at 4°C. The supernatant was combined with 50 μg of affinity-purified AtFAAH with c-myc tag from *E. coli* and 20 μg of agarose-conjugated anti-c-myc antibody (50 % slurry in PBS; MBL international) and incubated overnight at 4 °C with gentle shaking. The affinity-purified AtFAAH was omitted for controls. The immunocomplex was washed four times with the yeast breaking buffer by centrifugation at 2,500 xg for 10 sec, mixed with 20 μl of 2x Laemmli sample buffer, and boiled for 5 min. Agarose was removed by centrifugation at 2,500 xg for 10 sec and the resulting supernatant was analyzed by Western blot.

**Site-directed and Deletion Mutagenesis**

The construct pGBKT7-AtFAAH used for yeast two-hybrid screening was used as template in PCR reactions for mutagenesis. Site-directed mutagenesis was performed using QuikChange® II XL site-directed mutagenesis kit according to the manufacturer’s instructions (Stratagene, La
Jolla, CA). 50 ng of the template DNA was used in PCR with primers containing nucleotide corresponding to amino acid change. The PCR program includes the following steps: 95 °C for 1 min (95 °C for 50 s, 60 °C for 50 s, 68 °C for 10 min) repeat 17 more cycles, and 68 °C for 7 min. The reaction mixture was digested with DpnI at 37 °C for 2 h to remove parent plasmids. Then the DNA was precipitated and used to transform XL10-Gold competent cells (Stratagene). Mutations were confirmed by sequencing. Domain deletion mutagenesis was performed by PCR with 5’-phosphated forward primers hybridizing 3’-sequence flanking the target domain to be deleted and 5’-phosphated reverse primers hybridizing 5’-sequence flanking the target domain. PCR reactions were done with *Pfu* Turbo DNA polymerase (Stratagene) at 95 °C for 2 min, 30 cycles of 95 °C for 30 sec, 62 °C for 30 sec and 72 °C for 10 min, and at 72 °C for 10 min. The PCR products were isolated by gel-extraction, self-ligated by T4 DNA ligase (Promega) at room temperature (~23 °C) overnight, and used to transform *E. coli* TOP10 competent cells (Invitrogen). Mutations were confirmed by sequencing. The resulting pGBK7-AtFAAH with mutations was extracted from the bacterial cells and transformed into yeast AH109 cells.

**FAAH Enzyme Activity Assays**

Protein samples were incubated with 100 μM (~12,000 cpm) NAEs (12:0, 16:0, or 18:2) or 100 μM 2-arachidonoyl glycerol in 50 mM BisTris buffer (pH 9.0) in a final volume of 0.4 ml at 30 °C for 30 min with shaking at 120 rpm. Reactions were terminated by the addition of boiling isopropyl alcohol (70 °C) for 30 min. Total lipids were extracted into chloroform, washed twice with 1 M KCl and once with water, and separated by Silica Gel-thin layer chromatography (TLC) using an organic solvent mixture of hexane, ethyl acetate, and methanol (60:40:5, v/v/v). Distribution of unreacted substrates and products formed was evaluated either by radiometric
scanning (AR-2000 Imaging Scanner, Bioscan, NW Washington, DC) of the TLC plate for amidase activity assays or by exposure of the plate to iodine vapors for monoacyl esterase activity assays.

*Ionization Tandem Mass Spectrometry (ESI-MS/MS)*

Protein bands were excised from the gel and vortexed with 100 μl of 25 mM NH₄HCO₃/50 % acetonitrile for 10 min. This step was repeated at least twice before drying the gels completely by using a Speed Vac (Savant Instruments). The dried gel pieces were treated with 0.25 μg trypsin in 25 mM NH₄HCO₃ and the mixtures were incubated for 6 h at 37 °C. The supernatant from the trypsin-digested mixtures were collected in separate tubes, and peptides were extracted by treating the gel pieces with 30 μl of 25 mM NH₄HCO₃. 8 μl of the extracted peptides were analyzed by ESI-MS/MS. For liquid chromatography, an Agilent 1100 binary pump (Agilent technologies) was used, together with a reversed-phase capillary column, 6 mm × 150 mm. A 60-min gradient of 0~100 % CAN in 0.1 % formic acid was used for the separation of the peptides. The peptides eluted were then analyzed by ESI-MS/MS. The spectrophotometer was operated in data-dependent mode, automatically switching to MS/MS mode. For each scan, the most intense, doubly or triply charged, ions were sequencially fragmented in the linear trap by collision-induced dissociation using collision gas, and results MS/MS peaks. The most intense MS/MS peaks, in positive ion mode, were automatically selected after defining an intensity threshold. Monoisotopic masses from the tryptic digests were used to identify the corresponding proteins to search in *Arabidopsis thaliana* databases in SWISS-PROT and NCBI using MASCOT search algorithm (http://www.matrixscience.com). The database search was done with peptide mass fingerprint data with a mass accuracy of 0.1 Da. Search options were allowed only
specific tryptic cleavage and included one missed cleavage site.

**Western Blot Analysis**

Protein samples were separated on 10% polyacrylamide SDS gels and electrophoretically transferred onto polyvinylidene fluoride membranes in a Semidry Trans-Blot apparatus (Bio-Rad) for 30 min at constant 14 V. The membranes were blocked in 5% nonfat milk in Tris-buffered saline (20 mM Tris-HCl, pH 7.5, and 500 mM NaCl) containing 0.1% Tween 20. AtFAAH proteins cloned in pGBK7 (or in pTrcHis2 for purification) and proteins cloned in pGADT7 were localized by overnight incubation at room temperature with mouse monoclonal anti-c-Myc antibodies (Abgent, San Diego, CA) or anti-HA antibodies (Abgent), respectively. Immunolocalized proteins were detected by chemiluminescence following incubation for 1 h at room temperature with goat anti-mouse IgG (Bio-Rad) conjugated to horseradish peroxidase, according to the manufacturer’s instructions.

**Plant Materials and Growth Measurements**

Transgenic *Arabidopsis* lines overexpressing AtFAAH proteins under the control of the cauliflower mosaic virus 35S-promoter were previously described (Wang et al., 2006). HNH and RSZ33 T-DNA insertional mutants (SALK_006389C and SALK_083782C) were obtained from the Arabidopsis Biological Resource Stock Center (Ohio State University, Columbus, OH). Plants were screened for zygosity using the REDExtract-N-Amp Plant PCR kit (Sigma). Plants were propagated in soil for seed production. For growth assay, seeds were first surface-sterilized with 95% ethanol, 30% bleach containing 0.1% Tween-20 and deionized water, and stratified for 3 days at 4 °C in the dark. Seeds were grown for 10 days in nutrient media (0.5× Murashige and
Skoog salts containing 1% sucrose) in a controlled environment room with a 16-h light/8-h dark cycle at 20 °C. For detailed growth measurements, seedlings grown on agar plates were tilted at an ~60° angle to facilitate reproducible measurements of root elongation. Cotyledon area and primary root length were measured from captured images of the seedlings. For fresh weight measurements, seedlings were grown in liquid media with shaking at 75 rpm, harvested by filtration, dried, and quantified in terms of seedling mass (mg) normalized to mass of seeds sown (mg). ABA dissolved in DMSO was added to the appropriate final concentrations, and untreated controls contained equivalent amounts of DMSO alone (always less than 0.05% by volume). Concentrations of exogenous ABA were calculated based on the active cis-isomer.
Figure 4.1 Yeast two-hybrid and in vitro pull-down assays for identification of Arabidopsis proteins interacting with AtFAAH. A, interaction between AtFAAH and HNH/RSZ33 in yeast. The DNA constructs carrying cDNAs (or empty vectors indicated by minus (-) symbol) were co-transformed into AH109 cells and incubated on LW medium. 5 µl of transformant cell suspensions were spotted on LW and LWHAdX media and incubated for 2 days. Yeast cells grew well in blue on the LWHAdX medium only when both bait and prey proteins were co-expressed. LW, medium lacking Leu/Trp; LWHAdX, medium lacking Leu/Trp/His/adenine and containing X-gal; pGBK7, bait vector with GAL4 DNA-binding domain; pGADT7, prey vector with GAL4 activation domain; p53/T-antigen, positive control. B, in vitro pull-down assay. Affinity-purified AtFAAH with c-myc epitope tag was added (+) or not added (-) to yeast lysate expressing HNH or RSZ33 with hemagglutinin (HA) epitope tag (indicated under “lysate”), immunoprecipitated using anti-c-myc antibody, and visualized by Western blot using anti-c-myc or anti-HA antibodies (indicated under “WB”). HNH and RSZ33 were co-immunoprecipitated with AtFAAH but were not immunoprecipitated in the absence of AtFAAH.
Figure 4.2 Domain deletion mutagenesis to identify the domain responsible for the protein-protein interaction. A, two unknown domains conserved in plant FAAH sequences. C-terminus of AtFAAH (amino acid 481~607) is shown here. The two domains are color-coded with red indicating domain 5 and blue indicating domain 6. First (5-1 and 6-1) and second (5-2 and 6-2) halves of each domain are solid- and dotted-underlined, respectively. B, interaction between AtFAAH with domain deletions and HNH in yeast. The domains deleted are denoted by symbol. Yeast cells did not grow at all on the LWHAdX medium when domain 5-1 was missing in AtFAAH. FAAH (K205A), FAAH with active site-directed mutation (K205A) as a control. C, Western blot analysis of the domain deletion mutants. Yeast lysates expressing both AtFAAH
with domain 5 (Δ5), 5-1 (Δ5-1), or 5 and 6 (Δ5/6) deleted and HNH were probed with anti-c-myc antibody for AtFAAH and anti-HA antibody for HNH. Note that both AtFAAH and HNH were normally expressed in all domain deletion mutants tested.
Figure 4.3 Site-directed mutagenesis to identify the domain responsible for the protein-protein interaction. A, seven amino acid residues conserved in plant FAAH sequences. Sequences corresponding to domain 5-1 of AtFAAH are shown here with red indicating the residues conserved in all sequences. B, interaction between AtFAAH with mutation of three arginines (R491, R492, and R493) to alanine and HNH/RSZ33 in yeast. Yeast cells did not grow at all on the LWHAdX medium when all the three arginines were simultaneously mutated. C, NAE amidohydrolase assay of yeast cells expressing AtFAAH (R491/492/493A) and HNH or AtFAAH (R491/492/493A) and RSZ33. [1-14C]NAE16:0 was used as substrate (NAE). AtFAAH (R491/492/493A) displays significant NAE-hydrolyzing activity, verified by the formation of free fatty acid product (FFA).
Figure 4.4 Growth phenotype of *Arabidopsis HNH* and *RSZ33* T-DNA insertional knock-out seedlings in response to ABA. Seedling were grown for 10 days in the presence of solvent (DMSO) only or 0.25 μM ABA. A, overall apparent growth phenotype in solid media. WT, wild-type (Col-0); FAAH-OE, AtFAAH overexpressor; HNH-KO, *HNH* T-DNA insertional knock-out (SALK_006389C); RSZ33-KO, *RSZ33* T-DNA insertional knock-out (SALK_083787C). *RSZ33* knock-outs exhibit ABA hypersensitivity to a degree similar to AtFAAH overexpressors. B, quantitative measurements of growth phenotype. 10-Day-old seedlings were quantified in terms of seedling fresh weight (mg of seedling tissue/mg of seed sown), primary root length (cm), and cotyledon area (mm²). Black and white bars indicate solvent (DMSO) control and ABA-treated, respectively. Values for primary root length and
cotyledon area represent mean ± S.D. of 10 individual seedlings. Asterisks indicate a significant difference ($p<0.0001$) compared with ABA-treated wild-type, which was determined by Student’s $t$ test.
Figure 4.5 Proposed molecular model for bifurcating function of AtFAAH. FAAH activity and/or protein expression might be regulated by a combinatorial input of both growth and stress cues. This influences either NAE levels/composition via catalytic activity of FAAH to enhance growth or ABA signaling possibly via direct interaction with RSZ33 to suppress growth. FAAH overexpression and RSZ33 knock-out caused ABA hypersensitivity (Fig. 4.4), suggesting that FAAH might inhibit RSZ33 action that negatively regulates ABA signaling. AtFAAH also interacts with HNH (with PABP8 not shown here) but its significance is unknown.
Figure 4.6 Identification of AtFAAH-interacting proteins by co-immunoprecipitation from Arabidopsis seedling homogenates. A, SDS-PAGE analysis of co-immunoprecipitants. 10-day-old Arabidopsis seedlings homogenates expressing AtFAAH (F) or GFP-tagged AtFAAH (GF) were immunoprecipitated with anti-GFP antibody, resolved in SDS-PAGE and stained with Coomassie blue. AtFAAH and PABP8 appeared in GF, but not in F, are indicated on the right of the gel and sizes of protein standards are indicated on the left. M, markers for protein size. B, identification of AtFAAH (with GFP) and PABP8 by ESI-MS/MS. Full sequences of AtFAAH...
(without GFP; top) and PABP8 (bottom) are shown here, where regions matched with trypsin-digested peptides are shown in red. Sequence coverage of AtFAAH and PABP8 are 22\% and 32\%, respectively, both of which are well above the minimum value considered reliable (~15\%). C, interaction between HNH and PABP8 by yeast two-hybrid analysis.
Figure 4.7 Effect of ABA on the interaction between AtFAAH and PABP8. 10-day-old Arabidopsis seedlings homogenates expressing AtFAAH (F) or GFP-tagged AtFAAH (GF) treated with solvent (DMSO) only or 0.25 μM ABA were immunoprecipitated with anti-GFP antibody, resolved in SDS-PAGE and stained with Coomassie blue. Positions of AtFAAH and PABP8 expected to appear on the gel are indicated on the right of the gel and sizes of protein standards are indicated on the left. M, markers for protein size. Proteins expected to be AtFAAH and PABP8 based on their positions were extracted, sequenced by ESI-MS/MS as shown in Fig. 4.7, and identified as AtFAAH and PABP8, respectively. Note that the PABP8 band appeared in the solvent control but not in the ABA treatment.
CHAPTER 5
SUMMARY AND SIGNIFICANCE

*N*-acylethanolamines (NAEs) comprise fatty acids of various chain lengths and numbers of double bonds linked to the amino group of ethanolamine via an amide bond. Several NAE types in mammals (e.g. NAE16:0, NAE18:1, and NAE20:4) are generally accepted as endogenous lipid mediators that regulate a wide range of physiological processes. Especially, NAE20:4 (known as anandamide) is considered to be an endocannabinoid that bind to cannabinoid receptors in brain tissues and regulate numerous neurobehavioral processes. In plants, NAEs are present in micromolar amounts in desiccated seeds and are depleted after imbibition and germination to barely detectable levels (Chapman et al., 1999). NAE12:0 and NAE18:2 were shown to have potent growth inhibiting properties when supplied exogenously to *Arabidopsis thaliana* seedlings (Blancaflor et al., 2003; Wang et al., 2006). These results suggest that NAE may have a role in the negative regulation of seed germination and normal seedling development. This effect of NAE was found to be, in part, by association of NAE metabolism with abscisic acid (ABA) signaling (Teaster et al., 2007).

The enzymatic machinery for metabolic pathway of NAEs appears to be functionally conserved in both animal and plant systems. NAEs are formed from *N*-acylphosphatidylethanolamine (NAPE) precursor by the action of NAPE-specific phospholipase D (PLD). The hydrolysis of NAEs to free fatty acids and ethanolamine is accomplished by the action of an amidase with broad substrate specificity, the fatty acid amide hydrolase (FAAH) (Cravatt and Lichtman, 2002). FAAH is believed to terminate the regulatory cascade triggered by NAEs in a wide range of physiological processes by degrading them (Cravatt and Lichtman,
Thus, FAAH-modulation of NAE levels has become an important focus for understanding the mechanistic action of the endocannabinoid signaling system in vertebrates, and a convenient target for the development of novel therapeutics (McKinney and Cravatt, 2005). This membrane-bound protein belongs to an enzyme superfamily called the “amidase signature (AS)” family. The FAAH recombinant proteins hydrolyze a broad range of alkyl amide and fatty acid ester (2-arachidonyl glycerol and fatty acid methyl esters) substrates in vitro (Giang and Cravatt 1997; Goparaju et al., 1999). Recent crystal structure analysis of rat FAAH revealed its core catalytic machinery consisting of a Ser-Ser-Lys catalytic triad and an unusual mechanism for membrane association of this protein, which was through a hydrophobic foot formed by three \( \alpha \)-helices (Patricelli and Cravatt, 1999; Patricelli and Cravatt, 2000). Based upon the occurrence of an AS domain and conservation of key catalytic residues, a candidate FAAH homologue gene (At5g64440) was identified in the \textit{Arabidopsis} genome (Shrestha et al., 2003). Molecular and biochemical evidences indicated that the At5g64440 gene encodes a functional homologue of the mammalian FAAH.

As expected, seeds of \textit{Arabidopsis} FAAH (AtFAAH) overexpressors had significantly lower endogenous NAE content than wildtype, and their seedling growth was less sensitive to exogenously applied NAE, whereas AtFAAH knockout seeds had elevated levels of endogenous NAEs, and their seedlings were hypersensitive to exogenous NAE (Wang et al., 2006). Moreover, AtFAAH overexpressors displayed enhanced seedling growth and increased cell and organ size. These results supported the hypothesis that FAAH is a modulator of endogenous NAE levels in plants and, again, that NAE depletion (by the action of FAAH) likely participates in the regulation of normal plant growth. AtFAAH seemed to be involved, in part, in ABA signaling in \textit{Arabidopsis} seedlings (Teaster et al., 2007). The levels of \textit{ABI3}, a key regulator of a number of
ABA-responsive genes, were inversely associated with expression level of AtFAAH. Paradoxically, AtFAAH overexpressors exhibited hypersensitivity to ABA, suggesting AtFAAH might be involved in ABA signaling independent of its catalytic ability to influence NAE flux (Teaster et al., 2007). Recently, AtFAAH overexpressors were found to be more susceptible to several bacterial and non-host pathogens (Kang et al., 2008).

As noted, FAAH has been intensely investigated in animal system in an attempt to uncover the functions of the endocannabinoid signaling system. Now evidence reveals that NAEs in plants are hydrolyzed by a membrane-associated hydrolase functionally analogous to the mammalian FAAH (Wang et al., 2006; Shrestha et al., 2002). However, since plant FAAH homologues have been studied relatively later, and less intensely than animal FAAHs, our knowledge on this enzyme in plants is fragmentary and many questions remain to be addressed. In this dissertation, I have identified and characterized AtFAAH orthologs in diverse plant species to provide molecular and biochemical support for existence of FAAH genes and enzymes in plant kingdom. I also examined a bifurcating function of AtFAAH to influence Arabidopsis growth (i.e. catalytic activity-independent action in addition to NAE-hydrolyzing activity of AtFAAH) and its molecular mechanism in context of the relationships between domain structure and functional activity of AtFAAH.

Recent discovery of an Arabidopsis FAAH homologue which catalyzed the hydrolysis of NAE in vitro suggested a FAAH-mediated pathway exists in plants for the metabolism of endogenous NAE (Shrestha et al., 2003). In chapter 2, I provided evidence to support this concept by identifying candidate FAAH genes in other plant species including rice, Medicago truncatula, cotton, and tomato. Corresponding cDNAs were isolated and expressed as recombinant proteins in E. coli. NAE amidohydrolase assays confirmed that rice and M.
truncatula proteins indeed catalyzed the hydrolysis of NAE in vitro. Kinetic parameters and inhibition properties of the rice FAAH were similar to those of Arabidopsis and rat FAAH, but not identical. Arabidopsis and rice FAAH, like rat FAAH, also hydrolyzed a broad range of substrate types including acylamides and acylesters. Five amino acid residues important for catalysis by rat FAAH were absolutely conserved within the five plant FAAH sequences, indicating that plant and mammalian FAAH proteins have similar structure/activity relationships despite limited overall sequence identity. Also, another NAE amidohydrolase (At5g07360) was identified in Arabidopsis and expressed in E. coli which was active in vitro only when supplemented with Arabidopsis seedling homogenate. These findings will help define the molecular properties of NAE amidohydrolase enzymes in plants, a key step to better understand the metabolic regulation of NAE lipid mediators.

Next, following up our previous report that overexpression of AtFAAH in Arabidopsis resulted in enhanced seedling growth, and in seedlings that were insensitive to exogenous NAEs but hypersensitive to ABA, I demonstrated in chapter 3 that whereas the enhanced growth and NAE tolerance of the AtFAAH overexpressing seedlings depend on the catalytic activity of the enzyme, hypersensitivity to ABA is independent of its enzymatic activity. Five amino acids known to be critical for rat FAAH activity and conserved in plant FAAH include Lys-205, Ser-281, Ser-282, Ser-305, and Arg-307 (in the Arabidopsis sequence). Site-directed mutation of each of these conserved residues in AtFAAH abolished its hydrolytic activity when expressed in E. coli, supporting a common catalytic mechanism in animal and plant FAAH enzymes. Overexpression of these inactive AtFAAH mutants in Arabidopsis showed no growth enhancement and no NAE tolerance, but still rendered the seedlings hypersensitive to ABA to a degree similar to the overexpression of the native AtFAAH. These findings suggest that the
AtFAAH influences plant growth and interacts with ABA signaling through distinctly different mechanisms. AtFAAH might physically interact with other proteins to exert these effects independent of catalytic activity. Thus, in an attempt to indentify proteins that interact with AtFAAH and are involved in ABA signaling, various molecular techniques were employed in the next chapter.

In chapter 4, a yeast two-hybrid screening approach and in vitro pull-down assays identified HNH endonuclease domain-containing protein (HNH) and Arg/Ser-rich zinc knuckle-containing protein 33 (RSZ33) as AtFAAH-interacting proteins. A series of domain deletion and alanine scanning mutagenesis identified three consecutive arginine residues (Arg-491, Arg-492, and Arg-493) in the AtFAAH sequence to be necessary for the protein-protein interaction. RSZ33 T-DNA insertional knock-out plant, like the AtFAAH overexpressor, exhibited hypersensitivity to ABA. Thus, AtFAAH is believed to directly interact with both HNH endonuclease domain-containing protein and RSZ33, and to modulate ABA signaling in part by inhibiting RSZ33 action that possibly suppresses ABA signaling in Arabidopsis. Collectively with the findings in chapter 3, AtFAAH appears to have a bifurcating function to control Arabidopsis growth possibly depending on environmental conditions; a combinatorial input of both growth and stress cues influences either NAE levels/composition via its catalytic activity to enhance growth or ABA signaling via direct interaction with RSZ33 to suppress growth.

In summary, I found in this dissertation that FAAH-catalyzed NAE degradation and thereby physiological changes and multiplicity of enzymatic routes for NAE hydrolysis are a conserved cellular mechanism between mammals and higher plants. I also found that plant FAAH may have evolutionarily diverged in such a way that it has adapted to have additional regulatory functions in plants that may be unnecessary for animals, such as responses to
environmental stresses. Historically, plant NAE/FAAH researchers have often followed on advances made in the mammalian NAE metabolism field to guide corresponding studies of this pathway(s) in plants. Perhaps with the discovery of bifurcating properties of plant FAAH, mammalian researchers will be prompted to look to plants for new insights into the regulation of the endocannabinoid signaling pathway. My studies also have provided both broad and detailed pictures of similarities (e.g. occurrence, enzymatic properties, etc.) and differences (e.g. URB597 inhibition, catalysis-independent activity, etc.) of the FAAH enzyme between animal and plant systems, which was largely ambiguous in the past due to lack of information on plant FAAH sufficient to compare with the animal counterpart. FAAH-regulated signaling of fatty acid amides/esters in higher organisms now provides new ideas about lipid mediators and their enzymes in regulation of cellular physiology, in addition to the canonical signaling pathways involving membrane phospholipids and their intracellular enzymes.

Regulation of a broad range of cellular, physiological and behavioral processes in vertebrates through NAE catabolism by FAAH suggests that FAAH may represent an attractive therapeutic target for the treatment of human and animal disorders, and has stimulated interest in the development of specific inhibitors of this enzyme (reviewed in McKinney and Cravatt, 2005; Fowler, 2006). Plants also have the machinery for the catabolism of NAEs, suggesting similar applications for plant biology will come from a more detailed understanding of the roles of FAAH and NAEs in plant development. In these regards, my results from this dissertation should help to functionally define the group of enzymes that metabolize NAEs in plants, information critical for understanding the regulation of endogenous NAE signaling pathways in higher plant systems. Further, my research will expand the knowledge-base of lipid metabolism, and thereby lead to new insights into lipid signaling in plants and may uncover new mechanisms for
manipulation of various physiological processes important to plant growth, diseases and productivity.
APPENDIX A

cDNA AND PROTEIN SEQUENCES OF PLANT FATTY ACID AMIDE HYDROLASES
Arabidopsis thaliana

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<td>541</td>
<td>FVLAANLGAP PAISVPVGYD KELPGFLQIQ MGRPWAETAV LGLAAAIVEEL APVTPKbauF</td>
</tr>
<tr>
<td>601</td>
<td>YDILNTN</td>
</tr>
</tbody>
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Medicago truncatula

361 TACGGGCGAA CAAGCATGGA GGGGTCCTTG TGTGATTCTG GGACAGTGGA AGTTATTGGA 961 TACGGGCGAA CAAGCATGGA GGGGTCCTTG TGTGATTCTG GGACAGTGGA AGTTATTGGA
241 GKAEMFVDGL GYTNMNPYG TARNPSHDR YTGGSSSPGA ALVSSGLSCA AIGDGGGSV 181 RFQQGNPISI LGDFIAPDIRM TDDCPCPSK SVDCTDKVSA LRLKCVLPI
301 RIPSICGIJ GLKTYGRTDR MTLADCMCTC VEVASPLAS VEDALVLSA IAGSPMDK1
361 TLRPSDLCVP NLVSPDNNNI LGSVKIGKTY EWFHDVSDRD ISNCTEDALN LCCSFSQCI 421 EEITLPELEEE MRTAHVVSIG TESISDLPNHP YRAGKRTFET LTDRTSLALF GSFSTSDKYVA
481 SCRIRRRMYY YHNEAKKVV TDMAPTTGIT APEIQSSLKL LGEESYVSM YLMRFTVAGN
541 LGFLPAITVP VGDHKGQLPLQ GLQILGRPWG EASLLRVASA IEEILQKRKR RPSAFHDILN 601 A
Gossypium hirsutum (cotton)

1 ATGGGACTAT TCAAGGCTGC TGGCGTTGTT TACAAGCCGG TCGAGGAGAT TGATCTTGGT
61 CCTGACAGCA ATGGGACTAT TCAAGGCTGC TGGCGTTGTT TACAAGCCGG TCGAGGAGAT
121 CTGGTCAAAA TATTGCTGTG GTTCTTGGAG TCAAGGATCA TTGGAACGTT ATTGTTGTAT
181 ATATGGGATG AAGACGAGTT CAACGGGCAA TGAATTGTCT TCCTTTGACC
241 TTAGAGAAGT CAGTAGATGA TTCAAATTCC AGCAGCTTCC GACGCTGGAC AATAGCGGAT
301 TATTCAAGAG CCTATAGTTC TGGAGAAATA ACTCCCCGGA AGGTTGCGGA GCAATTTATA
361 AATGCCGTGC ATGAATCTTC CCGTGCTGCT TTGCCAATGT CCTTCTTCAT TAACTATGAT
421 GCTGAAGATA TCCTGAAACA AGCTACAGAA TCAACTCTTC GGTACGAAAG AGGGGATCCG
481 ATATCAGCTC TAGACGGAGT CCCAATAGCT ATCAAGGATG AAATAGATTG TTCTCCATAT
541 GCTGAAGATA TCCTGAAACA AGCTACAGAA TCAACTCTTC GGTACGAAAG AGGGGATCCG
601 ATATGGGATG AAGACGAGTT CAACGGGCAA TGAATTGTCT TCCTTTGACC
661 CCAACAACAG AAGGTGACAA GTGCGGCTAC AAGGTGACAA GTGCGGCTAC
721 TGTGTTTGGTGT GGTATGGACT TTCGCTGTGCT TTGCCAATGT CCTTCTTCAT TAACTATGAT
781 TTAGAGAAGT CAGTAGATGA TTCAAATTCC AGCAGCTTCC GACGCTGGAC AATAGCGGAT
841 CCAACAACAG AAGGTGACAA GTGCGGCTAC AAGGTGACAA GTGCGGCTAC
901 TGGTGCTGCT CTCTGGTGGT GGATGAGGGA GGATGAGGGA GGATGAGGGA
961 GTGTGTTTATAG CATCATCACC AACTCTTGGG GGATGAGGGA GGATGAGGGA
1021 AACTCGGACAG TGGAGGATG TGGAGGATG TGGAGGATG TGGAGGATG
1081 TAGTGCGCCA TTAGTGCCCA ACTCCTACAT CATAGGCCAA CAATTTTACC TCCAAGTAT
1141 CTTTCCCCAA TGGCAGCTGC TGGCAGCTGC TGGCAGCTGC TGGCAGCTGC
1201 GATGTGGTCC ACGATGGAG TGGAGGATG TGGAGGATG TGGAGGATG
1261 TTTTGGTGGTAT AAGACTGTGTA AAGACTGTGTA AAGACTGTGTA AAGACTGTGTA
1321 CAGCTGCGCC CAAACTTGGC CAAACTTGGC CAAACTTGGC CAAACTTGGC
1381 AAAAAGCTGG ATTTTGCA
1441 CTTTCCAAACA CAAACTTGGC CAAACTTGGC CAAACTTGGC CAAACTTGGC
1501 CATAAGACAG TTTTGGTGCT TGGAGGATG TGGAGGATG TGGAGGATG
1561 TACATCATTAT TTAGTGCCCA CCTAGGGAAA CCTAGGGAAA CCTAGGGAAA
1621 CTTTGGTGGTAT AAGACTGTGTA AAGACTGTGTA AAGACTGTGTA AAGACTGTGTA

1 MTGKRVMVPA KDVLDSIKY EEPIVQAPHL TGFWFRFFVR LIEAPLIGPF LLTMLKKENK
61 IDQLLRNTVF PEEPMFKPEY PPQEKEHSVV ELDEDGRPEG RVESNCLPLP HYDVKCLWEN
121 SSATFRYWKI RIMYAYAYQSR KVCTPSVAES IISIMEENGI DKFEPYPLLFS FDAEVRQQA
181 AASTQRFESG NPLSILODFG IAIEKIDIDCH PHFSTDGSTW MHEVRDVEAD AVCVSRILSC
241 GVIIFGTNKNM HEGFMTGTTGN NSNYGTARP HFADRTGGS SSQAAVAS GLCSALGTD
301 GSSVRIPPSS LHEVVFVMPH YGLSTIEVHF YLQSTGVEGV VDGPSSPGAAS GLCSALGTD
361 PANRISMKPS TPCLPLSSDL DDTDALRSLR IGIYTPWNNN VHSTEVSDK EDALNLSKA
418 DCVPEVEVMVV PEIEMRMTAH LVSLGKDEVSL LPNSIDEGK VQKLSYDTRT SLALEFQSTA
421 ADYVAQGCHR RRIKRTYHEMI ERIKRTYHEMI ERIKRTYHEMI ERIKRTYHEMI ERIKRTYHEMI
481 HGCEVVEVVI PEIEMRMTAH LVSLGKDEVSL LPNSIDEGK VQKLSYDTRT SLALEFQSTA
541 VVPANLGLLP AISPVYGLQI EQLPTEVQPLT RAVAAEVEKL GESKRRPVTY
601 YDVLGAN
Solanum lycopersicum (tomato)

1 ATGGTTGAAAA AACAAGTAAT GTGCAGCCCGC AATGAAGTAG ACCTTACTGG GGTGAATTAT
61 TTACCCGGAAA AATAAGGAAC TCCACTATTG AGCGGGTTTT GTTCAAGATT GTTGGTTAAA
121 GTAATGAGGC ACTTGTCTTTT ATTTGGTATG ACTGTAACGG TACAGGAAAC AcTTAAGGAA
181 ATTACTGAGA TTCTGAAGAA TACTGTGATA CCAGAGGTTC CCATGTTCAT TCCCCAGTTC
241 CCTCTTCAAG AGCAAGAGCC TGGTGTTGTT TGCTTAGGAG AAGATGGAAA ACCTAAAGAA
301 CAGGTTGATT TAGCATTTAA GTATCTTCCA CA CTATGATC CTGCTTGTAG ATGGAGTTCT
361 GATTCAGGAG AATCATCCCA ATTCCGCTGC TGGAAAATTC GTGATTATGA ATATGCATAC
421 AGAAATAAGC TTACGACCCC ATCCATGGTT GCAGAGCACT TTATCTCAGC AATGGAGGTA
481 TTTAATGAGTA AGCAGCATTG ACACCATTA TTGATCTCAT TTGACCCCGA GGAAGTGAGA
541 AGGGTATTTCA TTGATCAGTA GGAATGACAT GTATTGATCT CATCATCCTTC AAAAAAGGTT
601 TTCAATAGGTT TTATCATGAGTT AGCAGACCAT TAAGGCAGGA GAGGGCAGGA AAGATCAGAA
661 CCAAGAAACTT GCGCAATTTT AGTGAAGAAG ACAATATGCC ATGAGTTTGG TATGGGCAACA
721 ACAGGAAATA ATCCAAATTG TGCTTACACT ATCTGAAATT ATGTCACTTT AGCTGCAATA
781 AGGTGTTCTCT CCTCGGAGTG ACGGCTGAGG AATGGTATTC TCGCTTGTGT AGCTGCAATA
841 GATGAGATTT TCAAGAAAGT AGATGTCATT GTAACACCTA CCACTGGAAT GACAGCCCCA
901 GAATATCCAG AAGCTGTTCA GCCATTTTGA ATGGTCATT GTGGGCAAGT TCTACTTCTT
961 AACACCATGC TGGACTTGGC TATGGGTGCA TGGAGGAGAC ATATGCTGG ACTGTCCTCA
1021 ATTATGGGAC CCACTAGGAG TACTGTGAGG GTAGCCATAC TGGCGATGC AGCAATCCTG
1081 GATATCCCTC CACACTTACC AATCTTGGGA TCACTTATCC TCCATATTT TCCGATTTA
1141 TCTTCACAGTG AGAGTCTCAA TATCTTGGGG ATCATTCTACT TGGGAAGTGA TACAGTTG
1201 TTCAATAGGTT TTATCATGAGTT AGCAGACCAT TAAGGCAGGA GAGGGCAGGA AAGATCAGAA
1261 TTTGCTCCGGCG AGGGATGTAA AAGCAAGAAG ATGGTCTTGG TCGATCGCTCG TGGAGGGCA
1321 AGCAGCTATG TTCTTGGGAC TGAGGCGATG AAGAGGAGAA AGAGAGGACA AGAGAGGACA
1381 GCTGAGGAGA GATGAGTATT GACTAAGATG ACTGCAAGGA ATGGAGAGCA ATGGAGAGCA
1441 TTGGCGAGAT TTCTTGGGAC TGAGGCGATG AAGAGGAGAA AGAGAGGACA AGAGAGGACA
1501 ATGGAGAGAT TTCTTGGGAC TGAGGCGATG AAGAGGAGAA AGAGAGGACA AGAGAGGACA
1561 GAATATCCAG AAGCTGTTCA GCCATTTTGA ATGGTCATT GTGGGCAAGT TCTACTTCTT
1621 ATGGGACTTT TTGTTATGC ATGAGTCTAT CACCTTGATG GTATTGGTGA TTGGGGCTTA
1681 CATGGATGC AATGGGAGAT TTCTTGGGAC TGAGGCGATG AAGAGGAGAA AGAGAGGACA
1741 TATGTTGGCG TTTGTTTGGG ATGGAGAGCA ATGGAGAGCA ATGGAGAGCA ATGGAGAGCA
1801 TTGGGACTTT TTGTTATGC ATGAGTCTAT CACCTTGATG GTATTGGTGA TTGGGGCTTA

1 MVKQGVMLPA LMVKEPIAPHL TGFWFKLFLV VIEAVPVGSF IASHLKEKNG
61 ITEILKNTVII PEVMFPIDIV PLOQEEPGVV CLGEDGKPE EVDLAFKLYL HYDAPRCWSS
121 DSGEQQPFRCW KIRYDEYAY RNKLTTPSYS AEHFISAMEV FNSKQGPSAPL LIFSPDPEEVR
181 RQAQAATQRFK EKQKLQSLIE HIFVAKVDDI DCYHPHSGGG SWKHFEVRQV KAGDPVSRSL
241 RNNAGLQVVK TMNHEFGMGT CTTGNPYNTP RNPHNPKRTY GGSSSAAAG VASGLCASAAL
301 GTDAGGVSRI PASLGCYVGL KTTFGRDTLT GLSLWEAGTVT IIGPITATVE DAILVYAAIS
361  GSSPTDRIQL NPSIPFPDL SSSESSNILG SLTLGKYTKW FNDVSSTDISSDKCEDVNLQ
421  FRRYGCKTTE IVPELRELRTAHTVTFGSE SLSFLNPDCE AGKGVRLTND TRTNLALFRS
481  FAASYISAQ CLRRRIMYYH MEIFKKVDVI VTPTTGATPEIFESALAVGETNLQVVARL
541  MQFAMTANVL GLPAISVPILG HDKQGLPIGL QLIGRPWCEA SILRLAVAVE EMSAERYK
601  VEYDILKGN GFHDSTE
APPENDIX B

cDNA AND PROTEIN SEQUENCES OF MUTATED ARABIDOPSIS FATTY ACID AMIDE HYDROLASE
Only the region of sequences that has the mutation is shown here. Junction site of deletion mutation and site for substitution mutation are underlined.

Domain 5 deletion
1441 GCTTCAGACT ATATCGCTGC _TCTCCTGAT GCTCTCAAAA ATGGAGAAAC CAATATTCAA
481 ASDYIAAPPD ALKNGETNIQ VTTDLRFVL AANLLGPFAI SVPGYDKEG LPIGLQIMGR

Domain 6 deletion
1561 CCTCCTGATG CTCTCAAAAA TGGAGAAACC AATATTCAAG TGACAACGTGA _TGGTTTAGCT
481 ASDYIAAQCL RRRLMEYHLN IFKDVDTVVT PTTGMPAVPI PPDALKNGET NIQVTGDGLA

Domain 5/6 deletion
1441 GCTTCAGACT ATATCGCTGC _TGGTTTAGCT GCCGCAGTGC AGGAACGTGC TCCAGTACC
481 ASDYIAAGLA AAVEELAPVT KKPAIFYDIL NTN

Domain 5-1 deletion
1441 GCTTCAGACT ATATCGCTGC _AAAGACGT GATGTCATTG TGACCCCTAC AACTGGAATG
481 ASDYIAAKDV DVIVTPPTGM TAPVIPDADL KNGETNIQVT TDLRFVLAAN LLLGFAISV

Domain 5-2 deletion
1501 ATCTTCTTCT CTGATGCTCT CAAAATGGA GAAACCAATA TTCAAGTGAC AACTGATTTA
481 ASDYIAAQCL RRRLMEYHLN IFPPDALKNG ETNIQVTDDL MRFLAANLL GFPAISVPVG

Domain 6-1 deletion
1561 CCTCCTGATG CTCTCAAAAA TGGAGAAACC AATATTCAAG TGACAACGTGA _TGGTTATGAT
481 ASDYIAAQCL RRRLMEYHLN IFKDVDTVVT PTTGMPAVPI PPDALKNGET NIQVTGDYD

Domain 6-2 deletion
1621 TTGTGTCTAG CTGCAATCTC CCTCGGCTTC CCTGCCATAT CAGTCCCGGT _TGGTTTAGCT
541 FVLAANLLGF PAISVPGLA AAVEELAPVT KKPAIFYDIL NTN

Q488A
1441 GCTTCAGACT ATATCGCTGC _TGGATCTT AGGCAGAAGAT TGATGGAGTA TCACCTGAAT
481  ASDYIAAACL RRRLMEYHLN IFKDVDVIVT PTTGMTAPVI PPDALKNGET NIQVTTDLMR
R491A
1441 GCTTCAGACT ATATCGCTGC TCAATGTCTT _GGCGAAGAT TGATGGAGTA TCACTTGAAT
481  ASDYIAAQCL RRRLMEYHLN IFKDVDVIVT PTTGMTAPVI PPDALKNGET NIQVTTDLMR
R492A
1441 GCTTCAGACT ATATCGCTGC TCAATGTCTT AGGGCAAGAT TGATGGAGTA TCACTTGAAT
481  ASDYIAAQCL RRRLMEYHLN IFKDVDVIVT PTTGMTAPVI PPDALKNGET NIQVTTDLMR
R493A
1441 GCTTCAGACT ATATCGCTGC TCAATGTCTT AGGCGAGCAT TGATGGAGTA TCACTTGAAT
481  ASDYIAAQCL RRRLMEYHLN IFKDVDVIVT PTTGMTAPVI PPDALKNGET NIQVTTDLMR
M495A
1441 GCTTCAGACT ATATCGCTGC TCAATGTCTT AGGCGAGAT TGQCGGAGTA TCACTTGAAT
481  ASDYIAAQCL RRRLAEYHLN IFKDVDVIVT PTTGMTAPVI PPDALKNGET NIQVTTDLMR
Y497A
1441 GCTTCAGACT ATATCGCTGC TCAATGTCTT AGGCGAGAT TGATGGAGGC TCACTTGAAT
481  ASDYIAAQCL RRRLMEYHLN IFKDVDVIVT PTTGMTAPVI PPDALKNGET NIQVTTDLMR
F502A
1501 ATCGCAGAAG ACGTTGATGT CATTGTGACC CCTACACTG GAATGACAGC TCCAGTGATA
481  ASDYIAAQCL RRRLMEYHLN IAKDVDVIVT PTTGMTAPVI PPDALKNGET NIQVTTDLMR
R491/492/493A
1441 GCTTCAGACT ATATCGCTGC TCAATGTCTT _GCCGACAGAT TGATGGAGTA TCACTTGAAT
481  ASDYIAAQCL _AAALMEYHLN IFKDVDVIVT PTTGMTAPVI PPDALKNGET NIQVTTDLMR


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hydrolysing phospholipase D exhibit significantly decreased levels of N-acylphosphatidylethanolamines. Biochem. J. 389, 241-247.


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