N-Acylethanolamines (NAEs) are endogenous constituents of plant and animal tissues, and in vertebrates their hydrolysis terminates their participation as lipid mediators in the endocannabinoid signaling system. The membrane-bound enzyme responsible for NAE hydrolysis in mammals has been identified at the molecular level (designated fatty acid amide hydrolase, FAAH), and although an analogous enzyme activity was identified in microsomes of cotton seedlings, no molecular information is available for this enzyme in plants. Here we report the identification, the heterologous expression (in Escherichia coli), and the biochemical characterization of an Arabidopsis thaliana FAAH homologue. Candidate Arabidopsis DNA sequences containing a characteristic amidase signature sequence (PS00571) were identified in plant genome data bases, and a cDNA was isolated by reverse transcriptase-PCR using Arabidopsis genome sequences to develop appropriate oligonucleotide primers. The cDNA was sequenced and predicted to encode a protein of 607 amino acids with 37% identity to rat FAAH within the amidase signature domain (18% over the entire length). Residues determined to be important for FAAH catalysis were conserved between the Arabidopsis and rat protein sequences. In addition, a single transmembrane domain near the N terminus was predicted in the Arabidopsis protein sequence, similar to that of the rat FAAH protein. The putative plant FAAH cDNA was expressed as an epitope/His-tagged fusion protein in E. coli and solubilized from cell lysates in the nonionic detergent, dodecyl maltoside. Affinity-purified recombinant protein was indeed active in hydrolyzing a variety of naturally occurring N-acylphosphatidylethanolamines, a minor membrane lipid constituent of cellular membranes (1). In animal systems, anandamide (NAE 20:4) acts as an endogenous ligand for cannabinoid receptors and has varied physiological roles, such as the modulation of neurotransmission in the central nervous system (2). Anandamide also activates vanilloid receptors, functions as an endogenous analgesic (3), and appears to be involved in neuroprotection (4, 5). In other tissues, NAEs have been implicated in immunomodulation (6), synchronization of embryo development (7), and induction of apoptosis (8). These endogenous bioactive molecules lose their signaling activity upon hydrolysis by fatty acid amide hydrolase (FAAH; see Ref. 9). In plants NAEs are present in substantial amounts in desiccated seeds (<1 μg g−1 fresh weight), and their levels decline after a few hours of imbibition (10). Individual NAEs were identified predominantly as 12C, 16C, and 18C species with N-linoleylethanolamine (NAE 18:2) generally being the most abundant. Like in animal cells, NAEs are derived from N-acylphosphatidylethanolamines (11, 12) by the action of a phospholipase D (13, 14). The occurrence of NAEs in seeds and their rapid depletion during seed imbibition to barely detectable levels in seedlings (12) suggests that these lipids may have a role in the regulation of seed germination and normal seedling development. In fact, recent experiments with Arabidopsis thaliana seedlings showed that when seeds were germinated and maintained on elevated levels (micromolar concentrations) of the naturally occurring NAE 12:0, seedling roots developed abnormally in a manner consistent with a disruption of both normal cell division and cellular expansion (15), signifying the importance of regulating NAE levels for normal plant growth and development.

The concept of NAEs acting as lipid mediators in plant systems is supported by an increasing amount of experimental evidence in vegetative tissues where normal levels of NAEs are quite low (low nanomolar concentrations). For example, NAE 14:0 appears to function as an endogenous modulator of pathogen elicitor signaling by plant cells (12, 16). NAE 14:0 levels, quantified by gas-liquid chromatography-mass spectrometry, increased 10–50-fold in leaves of tobacco plants that were treated for a few minutes with either xylanase or cryptogein elicitor proteins (17). These “elicitor-activated” levels of NAE 14:0 (submicromolar concentrations) were sufficient to induce defense gene expression (e.g. phenylalanine ammonia-lyase and PAL2 transcript abundance) in tobacco plants within hours of treatment in a manner similar to but independent of...
Arabidopsis NAE Amidohydrolase

34991

elicitor proteins (17). Mammalian cannabinoid receptor antagonists at concentrations equimolar to NAE blocked the activation of defense gene expression induced by either NAEs or by fungal elicitor proteins (16). A membrane-associated protein was identified in leaves of tobacco (and other plant species), which specifically bound to 3H]NAE 14:0 with high affinity (Kd in the low nanomolar range). This NAE 14:0-binding protein was proposed to mediate the NAE activation of PAL2 expression in tobacco leaves, a conclusion that was based on the similarities between NAE binding properties in vitro and NAE-induced physiological responses in vivo, including experiments with cannabinoid receptor antagonists (16). Although some clear differences are evident between the emerging NAE signaling pathway in plants and the better characterized endocannabinoid signal pathway of animals, there appear to be some remarkable similarities in the formation and perception of bioactive acylethanolamines by these two diverse groups of multicellular organisms. Likewise, we propose that a FAAH-like mechanism operates in plants for NAE signal termination and overall regulation of NAE levels under various physiological conditions.

Recently, depletion of NAEs during seed imbibition/germination was determined to occur via two metabolic pathways: one lipoxygenase-mediated for the formation of NAE oxylipins from NAE 18:2, and one amidase-mediated for hydrolysis of saturated and unsaturated NAEs (18). Hydrolysis of NAEs was reconstituted and characterized in microsomes of cottonseed and appeared to be catalyzed by an enzyme similar to the FAAH of mammalian species (18). Here we report the identification of a plant orthologue of mammalian FAAH by bioinformatic approaches, isolation of its cDNA sequence, expression of the cDNA in Escherichia coli, and identification of the protein product as an NAE amidohydrolase. These results support our previous studies on the metabolism of NAEs in plant tissues and for the first time provide molecular evidence for a conserved pathway in both plants and animals for the hydrolysis of NAEs. Moreover, the results of this research now provide a means to manipulate the levels of endogenous NAEs in plants to evaluate the physiological role(s) of these bioactive lipids.

EXPERIMENTAL PROCEDURES

Materials—[1-14C]Arachidonic acid was purchased from PerkinElmer Life Sciences, and [1-14C]lauric acid was from American Biosciences. [1-14C]Myristic acid, arachidonic acid, lauric acid, linoleic acid, myristic acid, anandamide, ethanolamine, phenylmethylsulfonyl fluoride (PMSF), and isopropyl-β-D-thiogalactopyranoside (IPTG) were from Sigma. [1-14C]Linoleic, [1-14C]palmitic acids, and [1,2-14C]ethanolamine were purchased from PerkinElmer Life Sciences, and [1-14C]arachidonic acid was purchased from Calbiochem, and Silica Gel G (60 Å)-coated glass plates for thin layer chromatography (20 × 20 cm, 0.25 mm thickness) were from Whatman. Specific types of N- and 3'-UTR Sequence-specific primers were designed within the 5'-UTR and 3'-UTR of the AT5g64440 cDNA, and the complete cDNA sequence was deposited in GenBank™. Sequence alignments and some sequence analyses were made with DNAsis software (Hitachi). Protein Expression—For protein expression, oligonucleotide primers (forward, 5'-ATGGGTAAGTACGGGCATGAAACG-3' and reverse, 5'-TTTGTATTGAAGATCATAAAGGTTG-3') were designed to amplify only the open reading frame of the above At5g64440 cDNA, and PCR conditions were as above, except that a 10-to-1 ratio of polymerases (Taq-to-Plu; Invitrogen) was used for amplification, and the template was the RT-PCR product (GenBank™AY308736) in pTREHis. The open reading frame-PCR product was gel-purified as above and subcloned into expression vectors, pTREHis and pTREHis2, and the constructs were introduced into chemically competent E. coli TOP10 cells as host. Transformed colonies were selected with correct in-frame fusions and cDNA sequence by sequencing of 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 without glucose to an A600 of 0.8 to 0.7 and induced with 1 mM IPTG for 4 h. Pelleted cells were resuspended in lysis buffer (50 mM NaPO4 buffer, pH 8.0, 100 mM NaCl, and 0.2 mM DDM) at a ratio of 1-to-10 (E. coli cells-to-DDM molecules; assuming 0.1 A600 = 109 cells/ml, see Ref. 25). After incubation on ice for 30 min, resuspended cells were sonicated with ice for ten 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 enzyme with the highest specific activity. By the same criteria, DDM was determined to be better for solubilizing active enzyme than either Triton X-100 or CHAPS.

Solubilization and N2 Affinity Purification—Routinely, cultured cells (0.5 ml) were pelleted, resuspended in 8 ml of native binding buffer (50 mM NaPO4, pH 8.0, and 0.5 mM NaCl) with 8 mg of lysozyme (Sigma) and 0.2 mM DDM (final), incubated on ice for 30 min, and disrupted by sonication as above. The crude lysate was centrifuged at 105,000 × g for 1 h in a Sorvall Discovery 90 model ultracentrifuge (Beckman Ti45 rotor). The supernatant was combined with ProBond resin, precharged with Ni2+, and gently agitated for 60 min to keep the resin suspended in the lysate supernatant. The resin with adsorbed protein was settled, and the supernatant was aspirated off. The resin was washed four times to remove nonspecific proteins, and the adsorbed proteins were eluted with imidazole-containing buffer. Eluted proteins were concentrated, and imidazole was removed with 50 ml of Tris-HCl, 10 mM NaCl, and 100 mM DDM by filtration using Centricon YM-30 (Millipore, Bedford, MA) devices. Affinity-purified proteins were stored at −80 °C in 10% glycerol and were stable for more than 2 months.

Gel Electrophoresis and Western Blotting—Protein samples were diluted in 80 ml of Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.025% bromphenol blue, and 0.05% SDS and separated on 10 and 8% polyacrylamide/SDS gels (Bio-Rad) at 35 mA for 30 min and 60 mA for 60 min. For Western blot analysis, separated proteins were electrophoretically transferred to polyvinylidene fluoride (0.2 μm, Bio-Rad) membrane in a Semidry Trans-Blot apparatus (Bio-Rad) for 30 min at constant 14 V. Recombinant proteins expressed as c-Myc-epitope fusions were localized using Anti-c-Myc antibodies (mouse monoclonal, Invitrogen) and detected by chemiluminescence (Bio-Rad substrate solutions) following incubation with 1-to-2500 dilution of goat anti-mouse IgG conjugated to horseradish peroxidase (Bio-Rad). NAE Amidohydrolase Assays—NAE substrates were synthesized...
and purified, and enzyme assays were conducted as described previously (18) with a few modifications. Generally the enzyme source was incubated with 100 μM [³⁵S]NAE (20,000 dpm) in 50 mM BisTris buffer, pH 9.0, for 30 min to survey for NAE amidohydrolase activity (18). Enzyme activity was examined for time, temperature, protein, and substrate concentration dependence. For enzyme characterization, reactions were initiated with 1 μg of affinity-purified protein and incubated at 30 °C with shaking for 30 min. Assays of lysates of E. coli cells expressing rat FAAH (WT-FAAH; Ref. 26) served as a comparison of NAE amidohydrolase activity, whereas non-transformed cells lysates or cell lysates with the Arabidopsis cDNA cloned in reverse orientation with respect to the lacZ promoter served as negative controls for activity assays. Enzyme assays were terminated by the addition of boiling isopropyl alcohol (70 °C), and lipids were extracted into chloroform. Lipid products were separated by TLC, and the distribution of radioactivity was evaluated by radiometric scanning (18). Activity was calculated based on the radiospecific activity of [³⁵S]-labeled substrate. A general serine hydrolase inhibitor, phenylmethylsulfonyl fluoride (PMSF), and an irreversible active site-directed FAAH inhibitor, MAFP, were used to probe the sensitivity of recombinant Arabidopsis NAE amidohydrolase activity. Inhibitors were added from stock solutions dissolved in isopropyl alcohol for PMSF or Me₂SO for MAFP, 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 (27).

RESULTS

Tentative Identification of Arabidopsis NAE Amidohydrolase—In animal tissues, fatty acid amide hydrolase (EC 3.5.1.4), a member of the amidase signature (AS) family (28, 29), hydrolyzes NAEs to produce FFA and ethanolamine (30). A similar enzymatic activity was characterized previously in cottonseed microsomes (18). Mammalian FAAH enzymes have a conserved stretch of ~130 amino acids (31) containing a Ser/Thr-X-Glu motif within the amidase class (PS00457). Two serine residues at 217 and 241, highly conserved in the AS sequence, were found essential for enzymatic activity of recombinant rat FAAH (34). Mutation of either one of the residues into alanine caused complete loss of activity of the enzyme (34, 35). Also, mutation of serine 218 into alanine caused marked loss of activity (35). Taking these conserved residues in the AS consensus sequence into consideration, several putative plant orthologues were identified computationally. BLAST searches2 in various data bases using the AS consensus block embedded in rat FAAH (blocks.fhcrc.org) identified one A. thaliana gene (At5g64440) that was selected for further characterization (Fig. 1).

The structure and organization of the At5g64440 gene is relatively complex with 21 exons including 5'-UTR (untranslated region) and 3'-UTR (Fig. 1A). The predicted gene is 4689 nucleotides in length and encodes a predicted protein of 607 amino acids with a molecular mass of 66.1 kDa. Based on the presence of the conserved residues characteristic of the canonical AS sequence, this gene seemed likely to encode an Arabidopsis NAE amidohydrolase. To assess if this gene was expressed and to isolate a full-length cDNA for functional studies, oligonucleotide primers were designed within the 5'- and 3'-UTR, and a cDNA fragment was amplified by RT-PCR from Arabidopsis leaf RNA (Fig. 1B). The RT-PCR product was sequenced and found to be 99.9% identical with the corresponding TC139316 annotated at TIGR. The protein domain prediction tools, ProDom (20), identified six domain families in the Arabidopsis protein, five of which were also found in rat FAAH (Fig. 1C). A single putative transmembrane segment was identified near the N terminus (TMHMM; Refs. 22 and 23) similar to the predicted topological organization in rat FAAH.

Alignment of the deduced amino acid sequences from the Arabidopsis NAE amidohydrolase cDNA and the rat FAAH (28) showed only 18.5% identity over the entire length. Alignment within the AS sequence of 125 amino acids (Fig. 2A, underlined residues) showed 37% identity with five residues determined to be important for catalysis to be absolutely conserved (Ref. 31; Lys-142, Ser-217, Ser-218, Ser-241, and Arg-243, denoted by arrows, Fig. 2A). Comparison of a 47-amino acid motif within the AS showed the Arabidopsis protein had close to 60% identity with FAAHs from several mammalian species (Fig. 2B). Organization of predicted secondary structure within this Arabidopsis and rat FAAH AS motif was similar (Fig. 2C), and the structure of the rat enzyme has been confirmed by x-ray crystallography (36). In addition, this putative A. thaliana NAE amidohydrolase and rat FAAH have similar predicted molecular weights (~66 kDa), similar predicted topologies (single transmembrane segment near the N terminus with the C terminus facing the cytosol, via TMHMM transmembrane and topology predictor; Refs. 22 and 23), and similar predicted subcellular locations (secretory pathway, pSORT; Ref. 24). Although the Arabidopsis NAE amidohydrolase shared several domains with glutamylin-rNA amidotransferases (Fig. 1C), these amino acid-modifying enzymes are localized to chloroplasts in higher plants, and there is no indication of stromal targetting sequences at the N terminus of the At5g64440 gene product.

Functional Identification of Arabidopsis NAE Amidohydrolase—The Arabidopsis putative NAE amidohydrolase was cloned into pTrecHis and pTrecHis2 for expression in E. coli of N-terminal and C-terminal epitope- and polyhistidine-tagged fusion proteins, respectively. E. coli lysates were surveyed for expression of enzyme activity using [³⁵S]NAE 18:2 (radiolabeled on the carbonyl carbon) as substrate. Representative chromatograms shown in Fig. 3 indicate that, like the recombinant rat FAAH (expressed in the same vector), the recombinant Arabidopsis protein effectively hydrolyzed [¹⁴C]NAE 18:2 to [¹°C]FFA 18:2. As a control, E. coli expressing the Arabidopsis cDNA in reverse orientation showed no hydrolytic activity (Fig. 3). In these preliminary experiments with crude E. coli lysates, the Arabidopsis NAE amidohydrolase activity was linear up to 60 min at protein amounts up to 50 μg. The enzyme was optimally active at alkaline pH (e.g. pH 8–9) and was inactivated by treatment at 100 °C for 10 min. The Arabidopsis NAE amidohydrolase did not hydrolyze ceramide, nor did ceramide influence NAE hydrolysis (not shown). The Arabidopsis NAE amidohydrolase did not catalyze the reverse reaction of NAE hydrolysis (formation of NAE) under any conditions tested (not shown). Higher activity was reproducibly recovered in cells expressing C-terminal fusions, compared with cells expressing N-terminal fusions. Similar to reports for the rat protein (26), the recombinant Arabidopsis NAE amidohydrolase was mostly associated with E. coli membranes.

Affinity Purification of Recombinant Enzyme—The Arabidopsis NAE amidohydrolase, expressed as a C-terminal fusion protein, was solubilized in DDM and subjected to native Ni²⁺-affinity purification, SDS-PAGE, Western blot analyses, and enzyme activity assays (Fig. 4). A protein of ~70 kDa was enriched under native conditions by Ni²⁺-affinity purification and was detected by the c-Myc antibody (Fig. 4, A and B, arrows, Rec. protein lanes). Likewise, NAE amidohydrolase activity was enriched in this native affinity-purified protein fraction (Fig. 4C) by ~375-fold, relative to the DDM-solubilized supernatant (SupS) fraction. More stringent denaturing condi-

---

2 W. Gish, blast.wustl.edu.
tions led to purification of the recombinant protein to homogeneity (single 70-kDa band on gel), but also inactivated the enzyme irreversibly (not shown). Hence we proceeded with the biochemical characterization of the active enzyme preparation, affinity-purified to near-homogeneity.

Biochemical Characterization—Recombinant NAE amidohydrolase activity was evaluated by incubating affinity-purified NAE amidohydrolase with [1-14C]NAE 20:4, [1-14C]NAE 18:2, [1-14C]NAE 16:0, [1-14C]NAE 14:0, or [1-14C]NAE 12:0 and measuring the rate of conversion to their respective [1-14C]FFA products. NAE amidohydrolase exhibited saturation kinetics with respect to all NAE substrates tested including those identified in plant tissues and those not found in plant tissues. The enzyme exhibited typical Michaelis-Menten kinetics when initial velocity measurements were made at increasing substrate concentrations (Fig. 5), and parameters calculated from these plots are summarized in Table I. The relative apparent \( K_m \) values of the Arabidopsis enzyme varied by a factor of about 4.
depending upon NAE type. Surprisingly, the Arabidopsis enzyme had a higher affinity toward the non-plant NAE 20:4 than toward the more abundant endogenous plant NAEs 16:0 and 18:2. The highest maximum rate of NAE hydrolysis also was estimated for NAE 20:4 compared with the endogenous plant NAEs, although the range of the difference was not as great. The specificity constant ($k_{\text{cat}}/K_m$) was calculated for the Arabidopsis enzyme toward all NAE substrates and supported the conclusion that NAE 20:4 appeared to be the best substrate for the plant enzyme in vitro. Similar published data for the rat FAAH indicated this enzyme showed a 10-fold preference for NAE 20:4 over NAE 16:0 (37). With respect to NAE 20:4, the best substrate for both the plant and animal FAAH, the catalytic efficiency of the Arabidopsis NAE amidohydrolase reported here (2.4 \times 10^3 \text{ M}^{-1} \text{s}^{-1}) is about 10 times less than that reported for the rat FAAH (2.2 \times 10^5 \text{ M}^{-1} \text{s}^{-1}). This may be due to different assay conditions, different detergents, or a difference in relative enzyme purity. In any case the same trend was

![FIG. 2.](https://example.com/figure2.png)

**FIG. 2.** Comparative alignment of Arabidopsis NAE amidohydrolase amino acid sequence (GenBank™ accession number AT308736) with mammalian FAAH. **A**, full-length alignment of Arabidopsis amino acid sequence (GenBank™ accession number AT308736) with rat FAAH (GenBank™ accession number U72497) (28). These proteins are members of the AS sequence-containing superfamily that includes amidase or amidohydrolase (EC 3.5) enzymes involved in the reduction of organic nitrogen compounds and ammonia production (31, 33). The AS region is underlined and consists of about 125 amino acids. There is 18.5% identity between the Arabidopsis protein and rat FAAH when compared over the entire length of the proteins, whereas there is 37% identity within the AS. Conserved residues essential for rat FAAH activity (Lys-142, Ser-217, Ser-218, Ser-241, and Arg-243) are indicated with arrows. **B**, alignment of more conserved AS sequence motif (30) for the enzymes that hydrolyze NAEs; mouse (GenBank™ accession number U82536) (53), porcine (GenBank™ accession number AB027132) (60), rat (GenBank™ accession number U72497) (28), and human (GenBank™ accession number U82535) (53). Identical residues within this motif between the plant and animal FAAHs are highlighted in black boxes. Within this motif there is 55–60% identity between the Arabidopsis and mammalian FAAH enzymes. **C**, secondary structure prediction (PSIPRED, 61, 62) of the AS (C, coil; H, helix; E, strand) is depicted above the rat and Arabidopsis (At) AS sequence motifs. Residues with similar secondary structure are highlighted in black boxes, illustrating the high degree of similarity within the active site (or AS sequence in NAE amidohydrolase) see Ref. 30. This structural organization has been confirmed for rat FAAH by x-ray crystallography (36) and suggests a functional link between these rat and Arabidopsis motif sequences despite limited primary amino acid sequence identity.

![FIG. 3.](https://example.com/figure3.png)

**FIG. 3.** Representative radiochromatograms of NAE amidohydrolase activity assays surveyed in E. coli harboring expression plasmids. Lysates from cells expressing recombinant rat FAAH-A (A) (26) were compared with lysates of cells designed to express the Arabidopsis NAE amidohydrolase cDNA in forward (B) or reverse orientation (C) with respect to the lacZ promoter. In all cases CDNs were in pTrcHis2 expression plasmids, and recombinant protein expression was induced by 4 h of incubation with 1 mM IPTG. For assays, 100 mM $[1^{14}C]\text{NAE 18:2 (~20,000 dpm)}$ in 50 mM BisTris buffer, pH 9.0, was used. The reactions included 50 \text{ng} of protein of the respective cell lysate and were incubated for 30 min at 30 °C with shaking. Lipids were extracted and separated by TLC. The positions of $[1^{14}C]\text{NAE 18:2}$ substrate and $[1^{14}C]\text{FFA}$ product are indicated.
A 70-kDa immunoreactive protein was evident in total protein extracts, purified under native conditions. A small but detectable amount of Rec. protein in DDM.

The results presented here predict and functionally confirm that the Arabidopsis gene AT5g64440 (Fig. 1) encodes a homologue of the mammalian FAAH. Although there was limited primary amino acid sequence identity over the length of the Arabidopsis protein compared with the rat protein (18%), there was substantially higher similarity within the amidase catalytic domain both at the primary (37–60% depending on the length compared) and secondary structural levels (Fig. 2). Expression of the Arabidopsis cDNA in E. coli indicated that the Arabidopsis protein product was capable of hydrolyzing a wide range of NAE substrates to free fatty acids (Figs. 3–5 and Table I), a feature also of the mammalian enzyme (30, 39). Kinetic parameters summarized in Table I were derived from these plots.

DISCUSSION

The results presented here predict and functionally confirm that the Arabidopsis gene AT5g64440 (Fig. 1) encodes a homologue of the mammalian FAAH. Although there was limited primary amino acid sequence identity over the length of the Arabidopsis protein compared with the rat protein (18%), there was substantially higher similarity within the amidase catalytic domain both at the primary (37–60% depending on the length compared) and secondary structural levels (Fig. 2). Expression of the Arabidopsis cDNA in E. coli indicated that the Arabidopsis protein product was capable of hydrolyzing a wide range of NAE substrates to free fatty acids (Figs. 3–5 and Table I), a feature also of the mammalian enzyme (30, 39). Kinetic parameters summarized in Table I indicate that the plant enzyme has similar affinities for NAE substrates as the FAAH from several mammalian species (28, 39–43). Moreover, the inhibition of the Arabidopsis NAE amidohydrolase by
MAFP (Table II), the active site-directed irreversible inhibitor of rat FAAH (36, 44), strongly suggests a conserved enzyme mechanism between the plant and animal NAE amidasases supporting the predictions from sequence/domain comparisons.

We suggest the annotation of “glutamyl-tRNA amidotransferase similarity” now accompanying this At5g64440 gene be modified to include the new functional information herein regarding NAE hydrolysis. Most amidasases, including the mammalian FAAHs, carry the glutamyl-tRNA amidotransferase similarity annotation due to the presence of sequence similarity within the amidase signature domain, often as the only identifiable domain within this family of proteins. Based on our functional studies, it is unlikely that this At5g64440 gene product functions as a glutamyl-tRNA amidotransferase, and this activity has not been attributed to membrane-bound mammalian FAAH enzymes. Additionally, in plants these glutamyl-tRNA amidotransferases are localized in the stroma of chloroplasts as soluble, oligomeric complexes of multiple subunits (45, 46). In fact, nuclear-encoded, chloroplast-localized orthologues of the glutamyl-tRNA amidotransferase subunits have been cloned from Arabidopsis and expressed/characterized by the Soll group (GenBank™ accession numbers, AF21841, AF240465, AF239836, and AF224745), and these proteins share less than 24% amino acid sequence identity with the At5g64440 NAE amidohydrolase. Thus there is a need to clarify the descriptive annotation within these amidase protein subfamilies.

The signal-mediated activation of NAE metabolism constitutes a major regulatory feature of the endocannabinoid signaling system in animal tissues through the rapid generation and timely degradation of bioactive acylethanolamines (4, 9, 28, 47, 48). Although a principal role for NAE 20:4 as an endogenous ligand for cannabinoid receptors has emerged as a paradigm for endocannabinoid signaling (2, 38), other types of NAEs as well as other fatty acid derivatives likely interact with this pathway directly or indirectly to modulate a variety of physiological functions in vertebrates (49–52). An increasingly detailed understanding of the degradation of these bioactive NAEs by FAAH has pointed to this metabolic step as a key regulator of NAE levels and hence NAE function in vivo (9, 29, 30). Recent major advances in the understanding of FAAH function in mammals at the structural level (36), mechanistic level (32), and the physiological level (9) have been made possible only through the cloning, expression, and manipulation of the cDNA/gene encoding FAAH (53). We anticipate the identification of this plant cDNA will facilitate a similar increased appreciation for this lipid pathway in plant physiology.

Research in the last decade has made it apparent that NAE metabolism occurs in plants by pathways analogous to those in vertebrates and invertebrates (12, 18), pointing to the possibility that these lipids may be part of an evolutionarily conserved mechanism for the regulation of physiology in multicellular organisms. Two physiological situations in plant systems have been identified in which the endogenous levels of NAEs are transiently modulated. The first is in the perception of fungal elicitors by plant cells, wherein the levels of endogenous NAE 14:0 were elevated 10–50-fold in leaves of tobacco plants following elicitation (17). In other work, NAEs (mostly C12, C16, and C18 types) were quantified in desiccated seeds of higher plants but were metabolized rapidly during the first few hours of seed imbibition/germination (10), in part by an amidohydrolase-mediated pathway (18), suggesting that the transient changes in NAE content may play a role in seed germination. In fact, Arabidopsis seedlings germinated and grown in the presence of exogenous NAE exhibited dramatically altered developmental organization of root tissues (15).

With evidence of conserved enzymatic machinery in plants for the formation and degradation of NAEs, and the potent biological effects caused by altered exogenous NAE levels, it is now important to begin to address NAE function in plants by forward and reverse genetics approaches. The identification of an Arabidopsis cDNA clone encoding a functional NAE amidohydrolase re-enforces the similarity between plants and animals in terms of NAE metabolism, but more importantly provides a tool for the future manipulation of NAE levels in plants as a means to understand the physiological role(s) of these bioactive lipids in the plant kingdom.

Acknowledgments—We thank Drs. John Ohlrogge and Fred Beisson, Michigan State University, for sharing their catalogue of Arabidopsis lipid genes in advance of its publication. Dr. Cecilia Hillard, Medical College of Wisconsin, with permission of Dr. Benjamin Cravatt, Scripps Research Institute, kindly provided the cloned rat WT-FAAH and ΔTM-FAAH in pTrcHis2 for our studies.

REFERENCES
1. Schmid, H. H. O., Schmid, P. C., and Nataranjan, V. (1996) Chem. Phys. Lipids 80, 133–142
2. Wilson, R. I., and Nicoll, R. A. (2002) Science 296, 678–682
3. Pertwee, R. G. (2003) Prog. Neurobiol. 63, 569–611
4. Hansen, H. S., Moesgaard, B., Hansen, H. H., and Petersen, G. (2000) Chem. Phys. Lipids. 108, 135–150
