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# Identification of N-acylethanolamine Hydrolyzing Enzyme in *Solanum lycopersicum*

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IDENTIFICATION OF *N*-ACYLETHANOLAMINE HYDROLYZING  
ENZYME IN TOMATO (*Solanum lycopersicum*)

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Honors Thesis

By

Derek A. Stuffle

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## ABSTRACT

Identification of *N*-acylethanolamine Hydrolyzing Enzyme in Tomato (*Solanum lycopersicum*)

by

Derek A. Stuffle

*N*-acylethanolamines (NAEs) are fatty acid derivatives that occur naturally in plant and animal systems. In mammals, they regulate physiological functions, including neurotransmission, immune responses, vasodilation, embryo development and implantation, feeding behavior, and cell proliferation. NAEs are metabolized by fatty acid amide hydrolase (FAAH), which belongs to the amidase signature (AS) family. It is hypothesized that putative FAAH functions as the catalyst in the metabolism of *N*-acylethanolamine in tomato plants. To test the hypothesis, FAAH protein homologs were identified in tomato via *in silico* analysis. Among the six homologs identified, FAAH1 and FAAH2 were selected for further validation. This study is focused on 1) *in silico* analyses of *SIFAAH2*, 2) quantification of transcript levels for *SIFAAH2*, 3) determination of FAAH activity at various developmental stages of tomato, and 4) isolation of and synthesis of *SIFAAH2* cDNA for cloning. Putative *SIFAAH2* showed high homology to Arabidopsis FAAH1. Transcript levels, as measured by qPCR using RNA extracted from various developmental stages, were highest at 0 days and lowest at 4 days. Enzyme activity at certain developmental stages coincided with *SIFAAH2* transcript levels. In order to confirm that putative *SIFAAH2* encodes for an enzyme that hydrolyzes NAEs, *SIFAAH2* gene was isolated from total RNA of tomato, cDNA was synthesized by reverse transcription and the gene was amplified by PCR for further cloning in a heterologous expression system for biochemical characterization. To gain better molecular and biochemical understanding of FAAH and determine its broader functions, it is pertinent to characterize FAAH in other plant species.

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## CHAPTER 1

### INTRODUCTION

#### *Lipid Signaling Pathway*

Lipids are commonly recognized as the structural components of cells and for their role in the cellular metabolism. However, it has been recently discovered that lipids function as mediators in plant responses such as abiotic stresses, plant-microbe interactions, and they regulate plant growth and development (Chapman, 2004). Lipid-signaling enzymes, such as phospholipase D (PLD), are crucial in not only regulating lipid messengers, but also cytoskeletal rearrangement, membrane trafficking, and degradation (Wang, 2004). Lipid signaling is a vital part of cell signaling, which is now known to occur *via* activation of G protein-coupled receptors (Tang and Insel, 2004). Activators of G protein-coupled receptors include fatty acid ethanolamides, or *N*-acylethanolamines (Schmid et al, 1990; Chapman, 2000) that regulate growth, cell proliferation, defense, channel regulation, sugar sensing, and some hormonal responses (Urano et al, 2013).

#### *N-acylethanolamines*

*N*-acylethanolamines (NAEs) are fatty acid derivatives that occur naturally in plant and animal systems (Chapman, 2004). These lipid mediators were first discovered in the 1950s and were thought to have anti-inflammatory effects in vertebrates (Kuehl et al., 1957). They are expressed as NAE X:Y, in which X denotes the number of carbon atoms of the acyl chain and Y signifies the number of double bonds present in the acyl chain (Figure 1). Depending on the degree of saturation of their acyl chain, NAEs are classified as saturated or unsaturated. In eukaryotes, NAEs are synthesized from hydrolysis of *N*-acylated phospholipid precursor, *N*-acylphosphatidylethanolamine (NAPE) (Schmid 1998; Chapman 2000). The metabolic pathway

for NAEs is highly conserved in animal (Schmid, 2000) and plant systems (Shrestha et al. 2002) (Figure 2).

#### *N-acylethanolamines in Mammals*

In mammals, NAEs regulate physiological functions, including neurotransmission, immune responses, vasodilation, embryo development and implantation, feeding behavior, and cell proliferation (Chapman, 2004). In mammalian systems, the most widely studied NAE is anandamide (NAE 20:4), an endocannabinoid ligand, which upon receptor binding, triggers a series of signaling events that include pain sensation, fear, energy, anxiety, balance, appetite and memory (Alger, 2004). In addition to anandamide, other endogenous NAEs, independent of the cannabinoid receptors, exist in the mammalian systems whose therapeutic properties have been discovered (Sasso et al, 2013). These include the saturated *N*-stearoylethanolamine (NAE 18:0), and *N*-palmitoylethanolamine (NAE 16:0) and monounsaturated *N*-oleoylethanolamine (NAE 18:1) (Calignano et al, 1998; Rodríguez De Fonseca et al, 2001).

#### *N-acylethanolamines in Plants*

In plants, NAEs function as lipid mediators in defense signaling, inhibitors of PLD  $\alpha$  activity (Pappan et al, 1998), regulate seedling growth and development, and regulation of other plant-signaling pathways while only comprising of a small fraction of total lipid content. The NAE content and composition of the plants varies with the tissue type, developmental stage and pathological condition (Chapman, 2004; Kilaru et al, 2007). Like in animal systems, NAEs are formed in plants from NAPEs, and they are hydrolyzed by an amidase to yield ethanolamine and free fatty acids (FFA). Among various plant tissues, NAEs were found to be most prevalent in desiccated seeds of various plant species (Chapman et al, 1999; Chapman, 2004; Venables et al, 2005; Kilaru et al, 2007). Among seeds NAE 18:2, NAE 16:0 and NAE 18:1 are present in

higher concentration with NAE 18:2 being the most abundant. Additionally, NAE 12:0, NAE 14:0, and NAE 18:0 are found in lower concentrations and likely differ from species to species (Chapman et al, 1999; Venables et al, 2005).

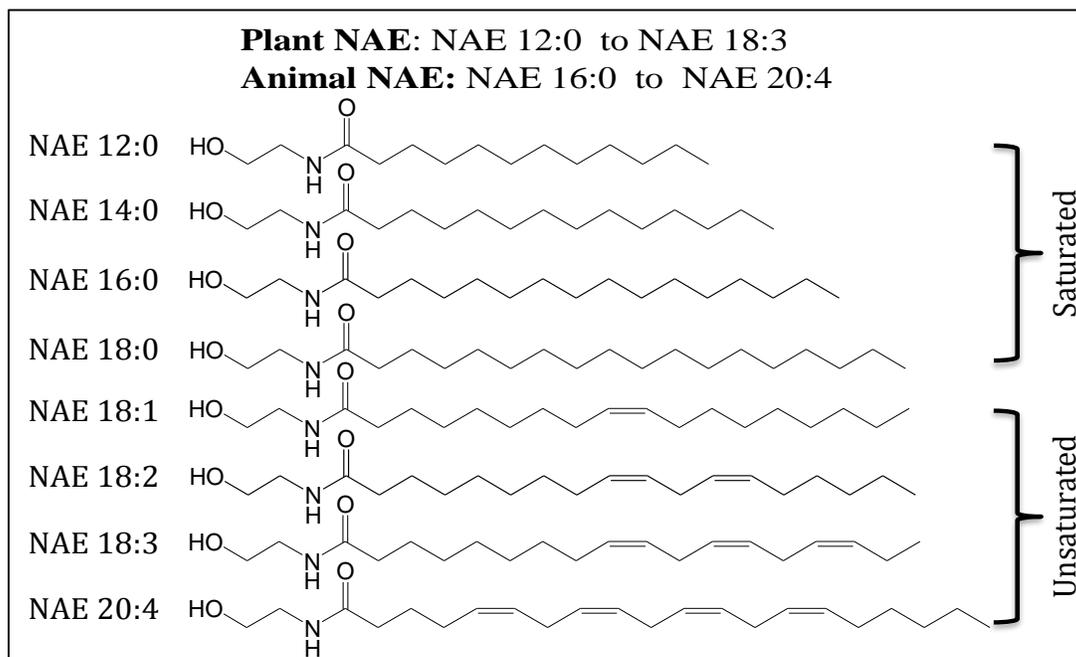
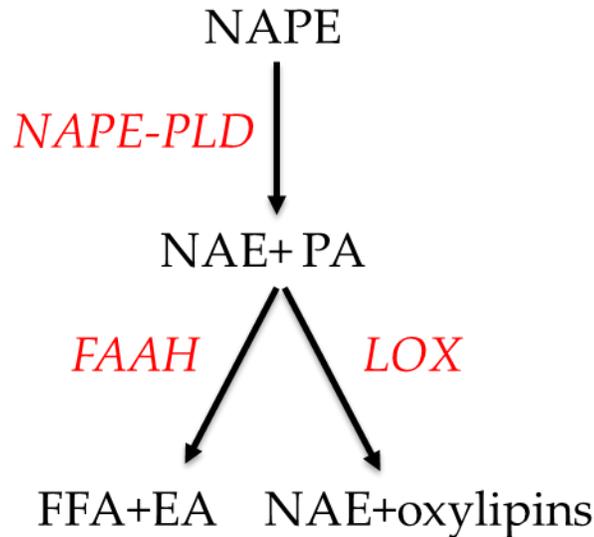


Figure 1. Structure of common saturated and unsaturated N-acylethanolamines.

### Metabolism of N-acylethanolamines

The metabolic pathway for NAEs is functionally conserved in plant (Shrestha et al, 2002) and animal systems (Schmid, 2000) (Figure 2). In animals, NAEs are generated by the hydrolysis of a minor membrane lipid constituent NAPE by NAPE-specific phospholipase D (NAPE-PLD) (Schmid, 2000). The physiology of NAEs is terminated in eukaryotes by two mechanisms: hydrolysis and oxidation. Hydrolysis catabolizes both saturated and unsaturated NAEs but alternatively, oxidation can occur on PU-NAEs to yield NAE-oxylipins. In mammals, NAEs are hydrolyzed into FFA and ethanolamine by a fatty acid amide hydrolase (FAAH), which is an amidase with broad substrate specificity (Cravatt et al, 1996; McKinney and Cravatt, 2005;

Shrestha et al, 2003; Wang et al, 2006). *In vivo* and *in vitro* experiments have indicated that plant NAEs are also hydrolyzed to FFA and ethanolamine by a membrane-associated hydrolase FAAH (Shrestha et al, 2003), functionally analogous to the enzyme in mammalian tissues (Schmid et al, 1996).



*Figure 2. Metabolic pathway of NAPE in plants. N-acyl-phosphatidylethanolamine (NAPE) is hydrolyzed by N-acyl-phosphatidylethanolamine phospholipase D (NAPE-PLD) into N-acylethanolamine (NAE). NAE is broken down into free fatty acids (FFA) and ethanolamine (EA) by fatty acid amine hydrolase (FAAH). NAE can also be broken down by Lipoxygenase (LOX) into NAEs and oxylipins.*

#### *Fatty Acid Amide Hydrolase (FAAH)*

Like other chemical transmitters, lipid messengers are also regulated by enzymes to ensure tight control over their signaling activity. For example, FAAH mediates the termination of endocannabinoid signaling in animal systems by reducing anandamide into ethanolamine and FFA (Mckinney and Cravatt, 2005).

#### *FAAH Structure*

FAAH is an integral membrane enzyme, thus making it unusual compared to other AS enzymes. Sequence comparisons identified a predicted N-terminal transmembrane domain

(amino acids 9–29) that was not present in other AS enzymes (Cravatt et al, 1996), thus indicating a rather simple mechanism for membrane binding. However, deletion of FAAH's first 30 amino acids produced a catalytically active mutant ( $\Delta$ TM-FAAH) that was still bound to membranes even in the presence of strong base, indicating that FAAH is capable of various modes for membrane integration. While the majority of other amidase signature enzymes are soluble proteins, FAAH behaves as an integral membrane enzyme, regardless of its single predicted *N*-terminal transmembrane domain (Patricelli et al, 1998). Analysis of the  $\Delta$ TM-FAAH structure showed an arrangement of three  $\alpha$  helices ( $\alpha$ 18 and  $\alpha$ 19, amino acids 410–438) that disrupts the AS fold and consists primarily of hydrophobic residues with a small number of basic amino acids interspersed throughout (Mckinney and Cravatt, 2005).

#### *FAAH in Plant Systems*

In plants, NAE levels in seeds decline dramatically during germination and continue to be low throughout seedling growth. This sudden depletion of NAEs suggests their catabolism, which is achieved by FAAH (Chapman et al, 1999), results in the mediators being broken down into FFA and ethanolamine (Mckinney and Cravatt, 2005). The levels of NAE increase in response to stress and negatively affect growth. Thus, the breakdown of NAE results in enhanced seed growth while compromising stress response (Mckinney and Cravatt, 2005). When FAAH is overexpressed, the seeds are able to break down more NAEs at a faster rate and show enhanced growth. Alternatively, when exogenous NAE is present in the absence of FAAH, the seeds grew at much lesser rate (Wang, 2006). FAAH overexpressors also showed increased susceptibility to bacterial pathogens (Kang et al, 2008), as well as unexpected phenotypes such as hypersensitivity to ABA (Teaster et al, 2007), which cannot be explained on the basis of NAE depletion by FAAH overexpressor alone. Various FAAH homologues have been recently

identified in other plant species, although most of the enzyme's characterization has been limited to *Arabidopsis*. To gain comprehensive understanding of plant FAAH, it is pertinent to characterize FAAH in additional species.

### *Significance of *Solanum lycopersicum* as Model System*

The United States ranks second as the world's largest producer of tomatoes (USDA, 2013). Tennessee ranks fourth in the country for fresh market tomatoes and has vegetable industry worth \$75 million, according to the U.S. Department of Agriculture statistics (USDA, 2013). The main threat to tomato crops is adverse weather conditions (Irazoque, 2014). Understanding the role of NAE metabolism and its ability to mediate growth and stress response are expected to develop molecular tools essential to minimize crop loss.

Tomato is a member of the *Solanaceae* family, an economically important family of flowering plants, which also includes potatoes, eggplants, and peppers. With the wide spread use of transgenics, genomic tools, and mutants, the characterization of the NAE pathway in tomato would be a vital factor in yielding highly stress tolerant cultivar. With a short generation time, a sequenced genome, and economical importance, tomato is an excellent choice for studying NAE metabolic pathway. Furthermore, a better understanding of lipid-mediated stress response in tomato will potentially result in increased productivity and sustainable agriculture.

### *Hypothesis and Objectives*

In plants, FAAH homologue has been characterized only for model plant *Arabidopsis*; under normal growth conditions, *AtFAAH* overexpressor seedlings showed enhanced growth (Wang et al, 2006). These results suggested that FAAH might act as a modulator of endogenous NAE levels and regulate growth in plants. Since NAE metabolic pathway is highly conserved

and NAEs have been reported in tomatoes (Venables et al., 2005), it is hypothesized that a putative FAAH functions as the catalyst in the metabolism of NAEs in tomato plants. To test the hypothesis, four specific objectives were developed:

- 1.) Identification of homologs of FAAH protein in tomato by *in silico* analysis using *AtFAAH1* as a template.
- 2.) Quantification of transcript levels for *S/FAAH2* at various developmental stages of tomato by quantitative-PCR (qPCR)
- 3.) Determination of FAAH activity at various developmental stages of tomato by using radiolabeled substrate.
- 4.) Isolation of and synthesis of *S/FAAH2* cDNA for cloning and heterologous expression.

## CHAPTER 2

### MATERIALS AND METHODS

#### *Identification of FAAH Protein in Tomato Via In silico Analysis*

Tomato genome has been fully sequenced and available through NCBI database. Therefore, using *Arabidopsis* FAAH (AED97893) as a template, the database was searched for putative proteins in tomato using BLASTp. Six homologs with the highest homology to *At*FAAH were converted to FASTA format and a sequence alignment was carried out using ClustalOmega. From this alignment, conserved catalytic sites were noted and relatedness of homologs to *At*FAAH was determined by generating a phylogenetic tree. Full-length putative *S*FAAH2 was translated using ExPASy translate tool and the largest reading frame with start and stop codons was determined. The 3D protein models for *S*FAAH2 and *At*FAAH were predicted with SWISS-Model tool and compared to further analyze functional homology.

#### *Plant Material*

For this research, Micro-Tom tomato plants were chosen as the model system. Micro-Tom plants share some key advantages with *Arabidopsis* in that the plants have a short life cycle, small size, and can be grown under fluorescent lights. Tomato plants were watered daily and stored in a growth chamber under fluorescent lighting at 23°C. Once the fruit was matured, seeds were extracted, washed, and stored at 4°C. To obtain plant material at various developmental stages, desiccated seeds were germinated between layers of autoclaved paper towels in a petri dish and saturated with distilled water and were harvested and weighed after 0, 2, 4, 6 and 10 days.

### *Extraction of Total RNA*

Total RNA was extracted from Micro Tom plant material harvested at 0, 2, 4, 6 and 10 days post germination (Ambion PureLink kit). About 50 mg of plant material was weighed on an analytical balance and ground to a powder in a mortar and pestle with liquid nitrogen as needed. The homogenate was incubated in 1 mL of TRIzol reagent (Ambion) at room temperature for 5 minutes, after which, 200  $\mu$ L of chloroform was added per milliliter of TRIzol and incubated for 3 minutes. The homogenate was centrifuged in microfuge tube at 12,000 g for 15 seconds. About 375  $\mu$ L of the colorless upper phase was transferred to a new centrifuge tube and equal volume of 70% ethanol was added. The liquid was transferred to a RNA-binding column (Ambion Pure Link) with a collection tube and centrifuged at 12,000 g for 15 seconds and the flow through was discarded. About 700  $\mu$ L of wash buffer I was added and centrifuged for 12,000 g for 15 seconds. The flow through was discarded and 500  $\mu$ L of wash buffer II was added to the column and centrifuged at 12,000 g for 15 seconds. This step was repeated once. The column was centrifuged for 1 minute to dry the membrane before adding 30  $\mu$ L of RNase free water directly to the membrane. The spin cartridge was placed in a recovery tube and centrifuged for 2 minutes at 12,000 g. The quality and quantity of RNA extracted was determined by Nanodrop-1000 at 280 nm (Thermo Scientific) and stored at - 80 °C until further use.

### *Total Protein Extraction*

Total protein was extracted from plant material harvested at 0, 2, 4, 6 and 10 days post germination. Samples of 50 mg were weighed out on an analytical balance and ground to powder in a mortar and pestle with liquid nitrogen. Homogenized powder was transferred into glass test tubes containing 2 mL of homogenization buffer and 4  $\mu$ L of DDM (Kim, 2013). Buffer was prepared with the following concentrations: 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM

MgCl<sub>2</sub>, 400 mM sucrose, 100 mM potassium phosphate, and an adjusted pH of 7.2. The tubes were vortexed every 5 minutes for 30 minutes before centrifugation for 20 minutes at 600 rpm and 4 °C. The supernatant was transferred to a clean tube and the concentration was determined by Nanodrop-1000 at 280 nm and stored at -80 °C until further use.

### *Quantitative PCR*

Using the RNA extracted from samples harvested at 0, 2, 4, 6 and 10 days post germination, qPCR was performed with KAPA SYBR FAST One-Step qRT-PCR kit (KAPA Biosystems). Transcript levels for *SIFAAH2* at all five developmental stages were quantified against actin, a housekeeping gene. Primers for *SIFAAH2* and *Actin* were designed using Primer3 Design software to generate an approximate product size of 150 bp and with T<sub>m</sub> around 60 °C (Table 1). The reaction mix was prepared by adding, 2 µL of forward and 2 µL of reverse primer (both 10µM), 5 µL of KAPA master mix, 3.2 µL of RNase free water and 2 µL of KAPA reverse transcriptase enzyme. A master reaction mix was prepared for 30 reactions (two technical replicates for each sample) and 9 µL of mix was pipetted into each well on a 48-well plate before adding the appropriate RNA. To each reaction, 1 µL of RNA was added to each well so that the concentration was 40 ng/µL of RNA per reaction. The reaction plate was sealed and centrifuged briefly. Using an Illumina Eco qPCR System, the thermal profile was set to 42 °C for 5 minutes for reverse transcriptase reactions and for further amplification it was set for 95 °C for 3 minutes, 95 °C for 3 seconds, and 60 °C for 20 seconds for a total of 40 cycles.

Table 1. Designed primers for *SIFAAH2* and *Actin* (control).

| <i>SIFAAH2</i> |                         | T <sub>m</sub> | GC content | Exp Product Size (bp) |
|----------------|-------------------------|----------------|------------|-----------------------|
| Forward Primer | TGCTTCTTCATTGCTATATGTCG | 59.4 °C        | 39.1 %     | 160                   |
| Reverse Primer | CCGGCAGCATTACTTGTTTT    | 60.1 °C        | 45.0 %     | 156                   |
| <i>SActin</i>  |                         |                |            |                       |
| Forward Primer | GCTGGATTTGCTGGAGATGATGA | 61.7 °C        | 43.7 %     | 145                   |
| Reverse Primer | TCCATGTCATCCCAATTGCTAAC | 61.1 °C        | 46.1 %     | 147                   |

#### *FAAH Activity Assay*

Approximately 50 µg/µl of each extracted protein sample, 4 µL of radio labeled [<sup>14</sup>C] radio labeled NAE substrate (24:0, 16:0), and 400 µL of 50 mM BTP buffer (pH 9.0) were added to individual test tubes. Bis-tris propane (BTP) buffer was prepared by dissolving 1.41 g of BTP powder in 100 mL of water and adjusting the pH to 9.0. The reaction was incubated in a water bath at 30 °C for 30 minutes while shaking at 120 rpm. Isopropanol was preheated to 70 °C and 2 mL were added to the mixture to stop the reaction (Kim, 2013). The tubes were cooled to room temperature and 1 mL of chloroform was added, vortexed thoroughly, and stored overnight at 4 °C. The following day, subsequent to bringing the tubes to room temperature, 1 mL of chloroform and 2 mL of 1 M KCl were added to the mixture and vortexed. Addition of salt solution induces phase separation subsequent to which the tubes were centrifuged at 1000 g for 5 minutes and the upper phase was discarded. The previous step was repeated twice before adding 2 mL of deionized water to wash the lipids. The remaining organic phase was transferred to 4 mL vials and the solvent was evaporated with N<sub>2</sub> gas. Once all the solvent was evaporated, the lipids were dissolved in 40 µL of chloroform and the total lipid was loaded on a silica gel-coated TLC plate. The TLC plate was developed in a chamber containing a solvent mixture of hexane, ethyl acetate, and methanol (60:40:5, v/v/v) until the solvent reached approximately 2 inches

from the top (Kim, 2013). The plate was allowed to dry and analyzed by radiometric scanning (BioScan system AR2200 imaging scanner).

#### *cDNA Synthesis for SIFAAH2*

*SIFAAH2* cDNA was synthesized from RNA that was extracted from four day imbibed seeds, using the method mentioned above. A High Capacity cDNA Reverse Transcriptase (RT) Kit (Thermofisher) was used to synthesize cDNA. 4  $\mu\text{L}$  of RNA template, 10.2  $\mu\text{L}$  of RNase free water, 1  $\mu\text{L}$  of RT enzyme, 0.8  $\mu\text{L}$  of dNTP mix, 2  $\mu\text{L}$  of 10x RT buffer, and 2  $\mu\text{L}$  of 10x random primer was added to a PCR tube at a total volume of 20  $\mu\text{L}$ . The thermal profile was set to 94 °C for 3 min, and 94 °C for 30 seconds, 58 °C for 1 minute, 72 °C for 1.5 minutes, and for 40 cycles, and 72 °C for 5 minutes. The quality and concentration of the extracted RNA (Figure 11) and cDNA synthesized (Figure 12) was analyzed using Nano-Drop spectrophotometer.

Forward and reverse primers for *SIFAAH2* (1  $\mu\text{L}$  each), 2  $\mu\text{L}$  of cDNA, 10  $\mu\text{L}$  of RNase free water and 12  $\mu\text{L}$  of PCR reaction mix (Emerald) was added to a PCR tube at a total volume of 26  $\mu\text{L}$ . The same thermal profile as listed above was used. The PCR product (1854 bp) was analyzed by gel electrophoresis (Figure 13).

## CHAPTER 3

### RESULTS

#### *In Silico Analysis of Fatty Acid Amide Hydrolase*

The NCBI database search revealed six homologs (Table 2) for *Solanum lycopersicum* that contained the characteristic amidase signature (AS) sequence and conserved catalytic residues. All six putative tomato FAAH shared significantly high homology to *AtFAAH*. However, among the six homologs only *SIFAAH1* (XP\_004230538.1) and *SIFAAH2* (XP\_010315156.1) expression levels were associated with seedling development (*data not shown*). Putative *SIFAAH1* and *SIFAAH2* shown highest homology (67% and 66%, respectively) with *AtFAAH* (Table 2).

Table 2. Top six results of a BLAST of *Arabidopsis* FAAH against tomato genome.

| <b>Protein</b>           | <b>Max Score</b> | <b>E value</b> | <b>% Identity</b> | <b>Accession #</b> |
|--------------------------|------------------|----------------|-------------------|--------------------|
| Putative <i>SIFAAH 1</i> | 836              | 0.0            | 67                | XP_004230538.1     |
| Putative <i>SIFAAH 2</i> | 811              | 0.0            | 66                | XP_010315156.1     |
| Putative <i>SIFAAH 3</i> | 806              | 0.0            | 64                | XP_004230539.1     |
| Putative <i>SIFAAH 4</i> | 804              | 0.0            | 65                | XP_010315155.1     |
| Putative <i>SIFAAH 5</i> | 801              | 0.0            | 65                | XP_010315154.1     |
| Putative <i>SIFAAH 6</i> | 800              | 0.0            | 59                | XP_004230544.1     |

ClustalW alignment (Figure 3) revealed conserved AS catalytic residues of Lysine (K), Serine (S) and Serine across all six putative *SIFAAH* genes. Specifically, putative *SIFAAH1* and *SIFAAH2* were found to share the highest homology with *AtFAAH*. *SIFAAH1* is being studied by another student, and thus this research focuses on identification of *SIFAAH2*. The protein encoded by *SIFAAH2* is 618 amino acids in length and contains conserved AS catalytic residues





Figure 3. Alignment of *AtFAAH* with putative *SlFAAH* homologs with arrows indicating conserved amidase signature sites.

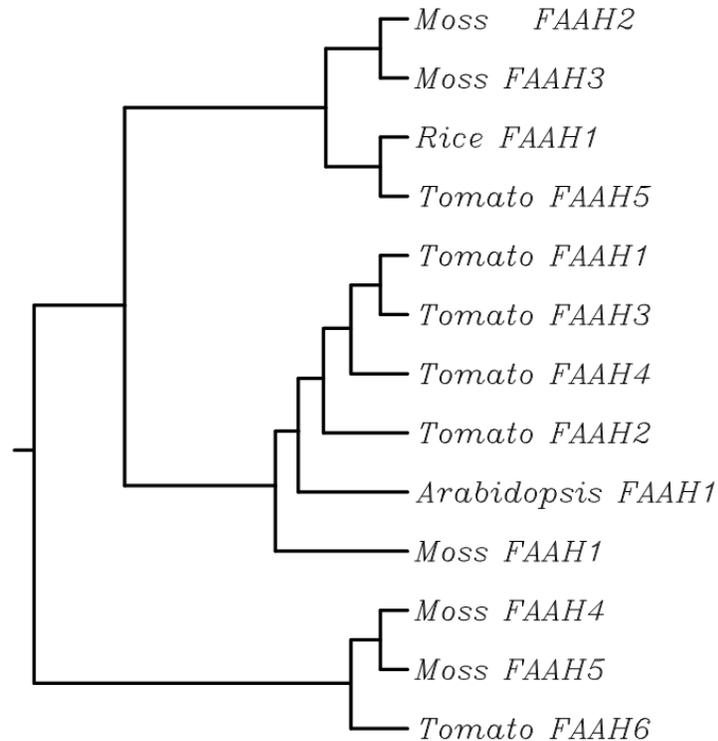


Figure 4. Phylogenetic tree between six tomato homologs and other plant FAAH, including *Arabidopsis*.

The phylogenetic tree revealed that the tomato FAAH2 is closely related to *Arabidopsis* FAAH1 (Figure 4). Tomato FAAH2 is least related to moss and rice FAAHs, except for Moss FAAH1. Of the six putative tomato FAAHs, FAAH5 and FAAH6 are least related to *Arabidopsis*, but more related to moss and rice FAAHs. Tomato FAAHs 1,2,3 and 4 are most closely related to *Arabidopsis* FAAH1, which is expected based on the NCBI database results (Table 2)

TCTTCATTGCTATATGTGCGACTTTCAAGACTCTGTGTAGCATAATTTATCTATCTATCTTGTCAATTTTT  
TTCTTTGATTGTATCTGCTGTGTTTTTGTACAGTACAAAAATTGTGACTGAAAAATGGTGTAAAAAACAAAG  
TAATGCTGCCGGCGAATGAAGTAGACCTTACTGCCGTGGAATATTTACCCGAAAAAATTGAAGCTCCACA  
TTTGACGGGGTTTTGGTTCAAGTTGTTTGTAAAGTAATCGAGGCACCTGTAATTGGTTCTTTTATTGCT  
AGTCATCTGAAGGAGAAGAATGGGATTACTGAGATTCTGAAGAATACTGTGATACCAGAGGTTCCCATGT  
TCATTCCCCAGTTCCTCTTCAAGAGCAAGAGCCTGGTGTGTTTTGCTTAGGAGAAGATGGAAAACTAA  
AGAACAGGTTGATTTAGCATTTAAGTATCTTCCACATTATGATCCTGCTAGTAGATGGAGTTCTGATTCA  
GGAGAATCATCCCAATTCGCTGCTGGAAAAATTCGTGATTATGAATATGCATACAGAAATAAGCTTACGA  
CCCCATCCATGGTTGCAGAGCACTTTATCTCAGCAATGGAGGTATTTAATAGTAAGCAGCCATCAGCACC  
ATTATTGATCTCATTTGACCCCGAGGAAGTGAGAAGGCAAGCGGCAGCTTCCACTCAAAGATTTGAGAAA  
GGAAAACAATGTCAATCTTGGAAAGGGATTTTCATTGCAGTCAAGGATGACATTGATTGCTATCCTCATC  
CTTCAAAGGTGGTTCTAAATGGTTTCATGAGGTTCCGAGGTGAAGGCAGATGGGGTTCTGTGTCAAG  
ATTACGAAACAATGGTGCAATTTTAGTAGGAAAGACAAATATGCATGAGTTTGGTATGGGCACAACAGGA  
AATAATCCAAATTATGGTACACCTAGAAATCCGCATAATCCAAAAAGGTACACAGGTGGTTCTTCCTCAG  
GTTTCAGCTGCAATTGTGCTTCTGGATTGTGCTCAGCAGCATTGGGAACAGATGCTGGAGGTTTCAGTACG  
AATTCCTGCTTCTCTTTGCGGGGTTGTGGGCTTAAAAACAACATTTGGACGGACTGACCTGACAGGGTCT  
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ATGCAGCAATCTCGGGATCCTCTCCAATGATCGAATTCAGCTGAATCCTTCCATCCCTAATTTTCCCGA  
TTTATCTTCCAGTGAGAGTTCAAATATCTTGGGATCACTTACCTTGGGAAAGTATACAAAGTGGTTTAAAC  
GATGTTAGCTCGACCGACATATCTGATAAGTGTGAAGATGTCTTAAACCAACTATTTTCGTCGGTATGGGT  
GCAAAACAACAGAGATTGTCATACCGGAGCTTCGTGAGCTGCGCACAGCTCATACTGTTACTTTTGGATC  
TGAATCACTGTCTTTTTGAATCCTGATTGCGAGGCTGGGAAAGGAGTTAGATTGACTAATGACACTCGC  
ACAAATCTGGCACTTTTCAGATCATTTGCAGCATCTGACTATATATCTGCCAGTGTCTTAGGCGAAGGA  
TAATGTACTACCATATGGAGATTTTCAAGAAAGTAGATGTCATTGTAACACCTACCACTGGAAATGACAGC  
CCCAGAAATTCAGAAAGTGTCTTGCAGTTGGAGAGACAAATTTGCAAGTTGTAGCAAGACTAATGCAA  
TTTGCTATGACAGCAAATGTTCTTGGACTTCCGGCAATTTCTGTCCCTATTGGTCATGATAAGCAAGGAC  
TTCCAATAGGCTTGCAACTTATTGGTCGTCCGTGGTGTGAAGCTTCAATCTTGCCTTTAGCTGTTGCAGT  
TGAGGAAATGTCTGCAGAAATATAGGAAGAAGCCAGTGGAGTTCTATGACATCTTGAAAGGGAACGGTTTT  
CACGACTCCACTGAGGAATAAGTTTTAAGCCTAACCTCAAAGAGAAAAACAAGATTTTTATCACAGTGTGCT  
TAGCGTTTTTGTGTTTTGTATCTTTTTAAAATAGCAGTTAAATTTCTTTTTTGGCCATGAAACTCACGC  
ACATCTTGTGTTGTATAACTTTGTTCTTAATTATCTCACTCTTACCATA

Figure 5. Determination of stop and start codons from full length coding sequence for SIFAAH2.

The full length coding sequence was determined from NCBI database, and analyzed using the ExPASy translate tool. The largest reading frame was determined and start and stop codons were identified from the full length coding sequence (Figure 5). Non-coding regions on the 5' and 3'

ends are 124 base pairs and 170 base pairs, respectively. Further analysis includes a visual comparison of the predicted structures of *AtFAAH* and *SlFAAH2* using SWISS-MODEL software (Figure 6). The spatial arrangement of the two proteins suggests the two proteins may have similar functions based on location of their catalytic residues and visual similarities.

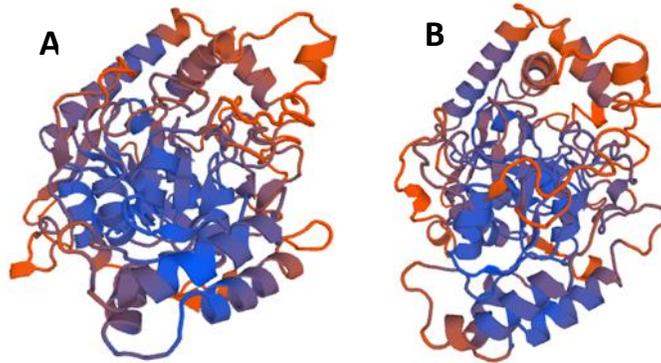


Figure 6. A) Structure of putative *SlFAAH2*. B) Structure of *AtFAAH*

#### *Quantification of Transcript Levels*

RNA was extracted in three biological replicates from imbibed seeds (10 days post-germination). The average RNA content at various developmental stages of seed development was measured using a NanoDrop<sup>™</sup> spectrophotometer (Figure 7). RNA content steadily increased from imbibed seed to 10 days post-germination. Error bars represent standard deviation between the triplicates.

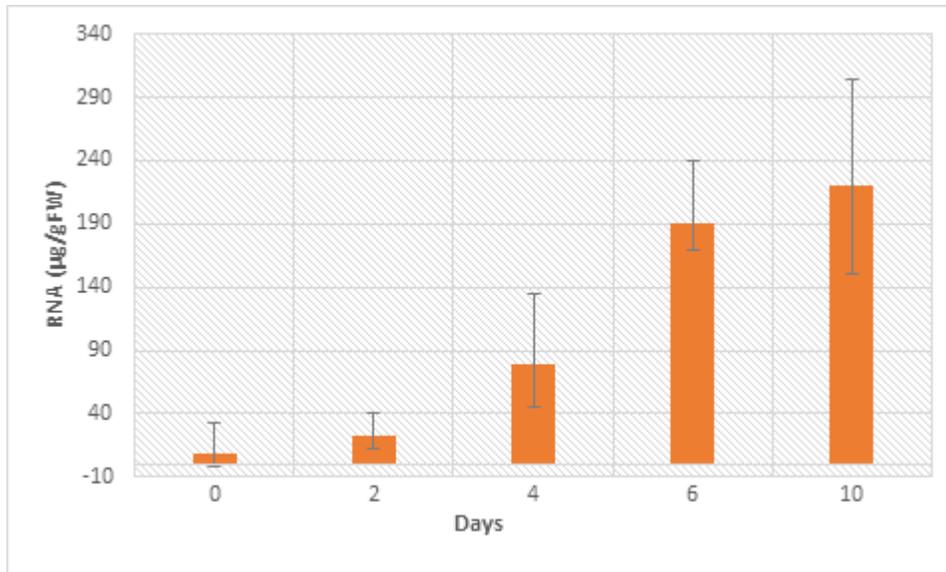


Figure 7. RNA content at various stages of tomato seed development.

Table 3. qPCR calculations for various stages of tomato seed development. Actin is used as a reference gene and the data was normalized against the control stage.

| FAAH2 | ACT   | Av ACT<br>Ct <sub>Act</sub> | FAAH2 | Av<br>FAAH2<br>Ct <sub>Faah2</sub> | ΔCt<br>(Ct <sub>Faah2</sub> -Ct <sub>Act</sub> ) | ΔΔCt | Relative<br>Expression | Fold<br>Change |
|-------|-------|-----------------------------|-------|------------------------------------|--|------|------------------------|----------------|
| 0D    | 24.32 | 24.32                       | 23.1  | 23.14                              | -1.18  | 0.00 | 1.00                   | 1.00           |
| 2D    | 24.18 | 24.18                       | 23.67 | 23.67                              | -0.51  | 0.66 | 0.62                   | -1.59          |
| 4D    | 22.86 | 22.86                       | 23.57 | 23.57                              | 0.70   | 1.88 | 0.26                   | -3.70          |
| 6D    | 23.39 | 23.39                       | 24.50 | 24.50                              | 1.10   | 2.28 | 0.20                   | -4.87          |
| 10D   | 23.35 | 23.35                       | 23.71 | 23.71                              | 0.35   | 1.54 | 0.34                   | -2.90          |

Using the following equation, the respective relative expression levels in relation to 0 day seeds were calculated (Pfaffl, 2001).

$$RE = \frac{\Delta Ct \text{ target (control - treatment)}}{\Delta Ct \text{ reference (control - treatment)}}$$

The threshold level was automatically assigned by the Eco real time qPCR program. Zero hour seeds served as the *control*, *SIFAAH2* was assigned as the *target*, and actin served as the *reference* in each calculation (Table 3). qPCR results revealed that *SIFAAH2* transcript levels were down-regulated in all stages i.e. 2, 4, 6 and 10 days after seed germination compared to the 0 day (Figure 8).

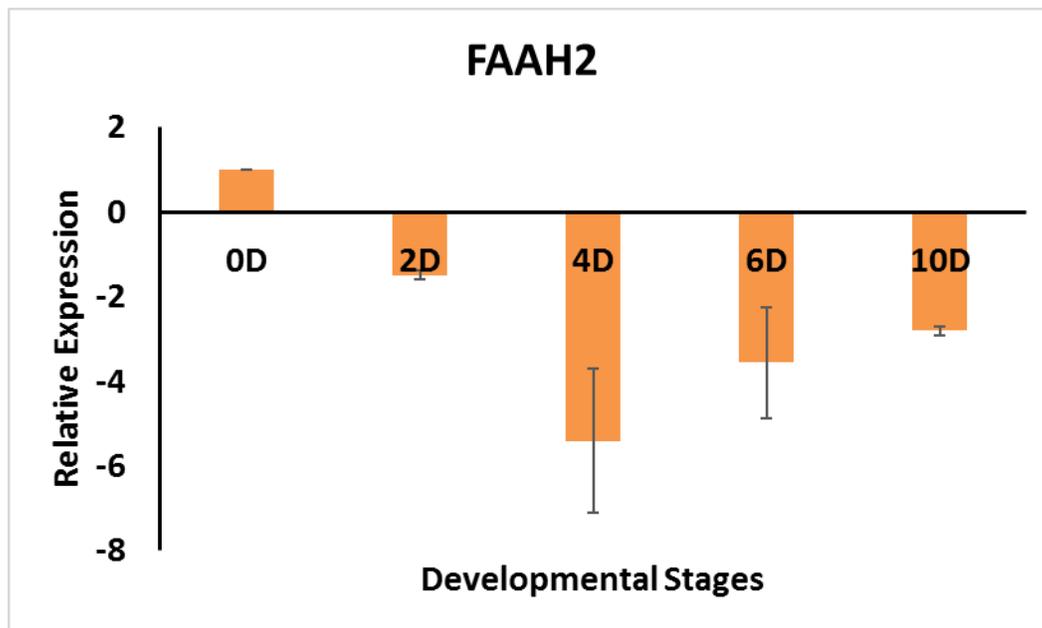


Figure 8. Transcript levels of *SIFAAH2* at various stages of seed development.

The relative expression for various stages was plotted to show the change in transcript levels of *SIFAAH2* during development (Figure 8). *SIFAAH2* protein activity for each developmental stage was determined in the next section.

#### *Protein Assay*

Protein was extracted in three biological replicates from each stage of development. The average protein content decreased after 48 hours and continued to stay relatively constant throughout development. Error bars represent standard deviation between the three replicates (Figure 9).

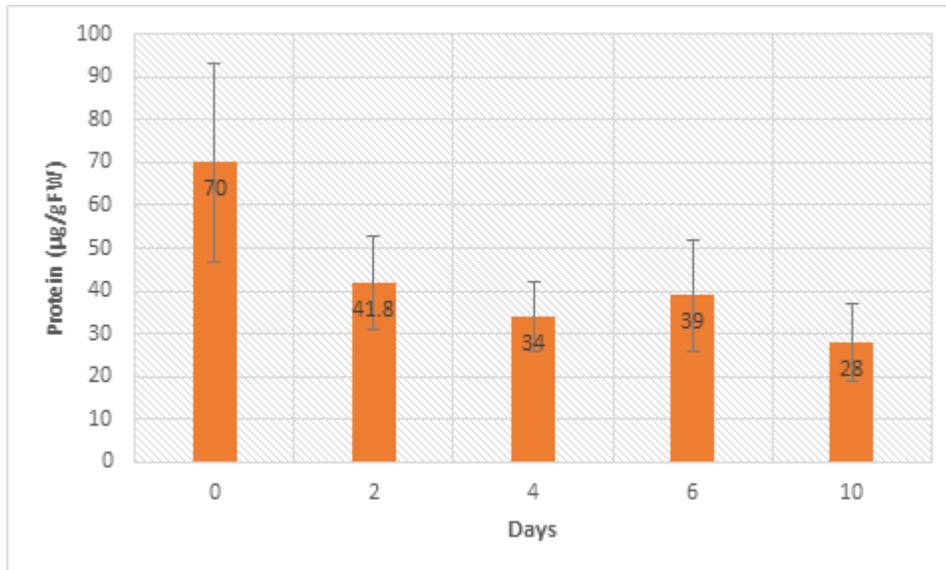


Figure 9. Protein content at various stages of seed development.

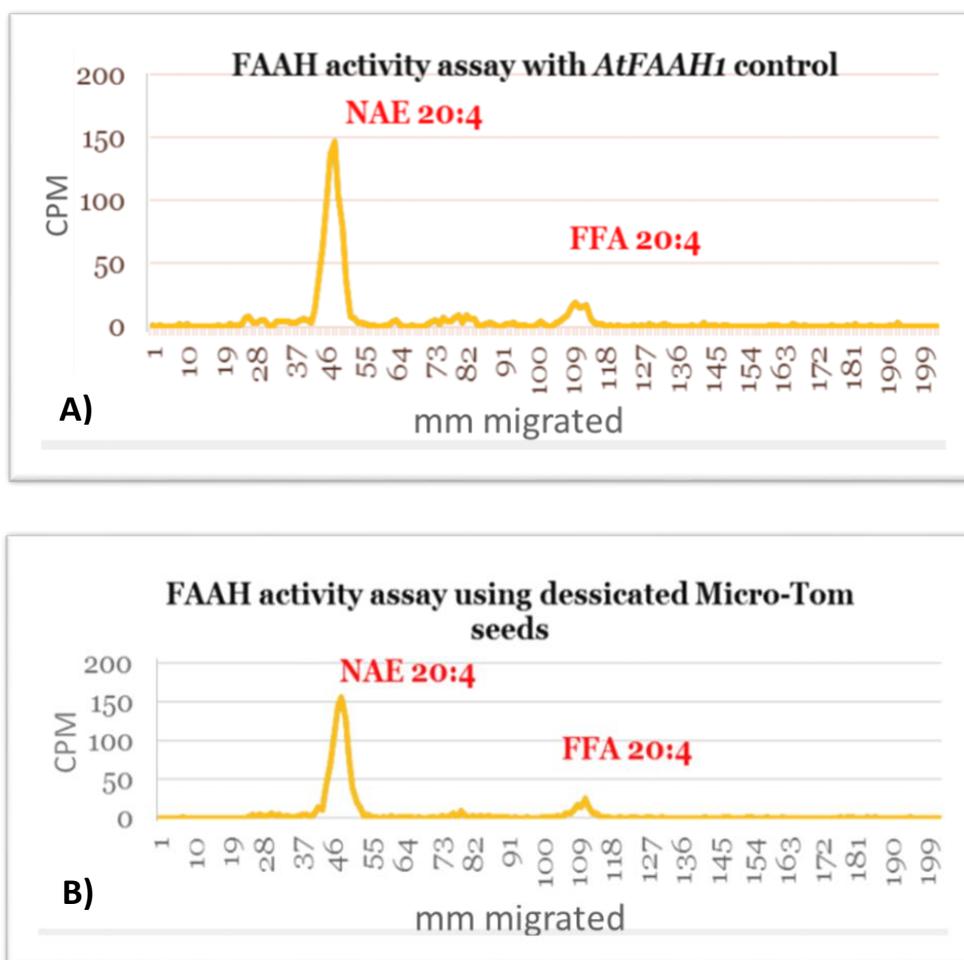


Figure 10. Radiometric scanner results for protein assay. A) *AtFAAH* control B) Tomato FAAH at 48 hours.

Protein assay results for 48 hour imbibed tomato seeds revealed catalytic activity. Using *At*FAAH as a control, the lipid product from the protein assay was extracted and separated on a TLC plate. The plate was analyzed by a radiometric scanner and a positive result for 2 day imbibed seeds was obtained. The results for *At*FAAH control (Figure 10.A) and for the 48 hour imbibed seeds (Figure 10.B) both had two peaks present in the graph, the highest peak is radiolabeled NAEs that were not hydrolyzed, and the smaller peak is FFA that were a product of the reaction.

### *SIFAAH2 Gene Amplification*

Concentration of extracted RNA from 4 day (post germination) was determined to be 260 ng/ $\mu$ L using a NanoDrop<sup>™</sup> spectrophotometer (Figure 11). Using the same method, the concentration of synthesized cDNA was determined to be 4598.3 ng/ $\mu$ L (Figure 12).

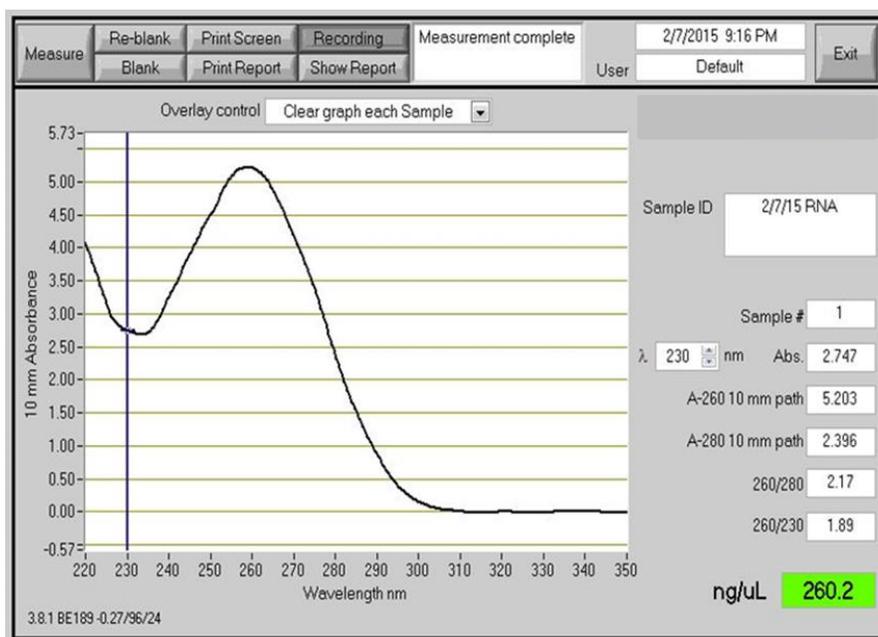


Figure 11. Concentration of RNA

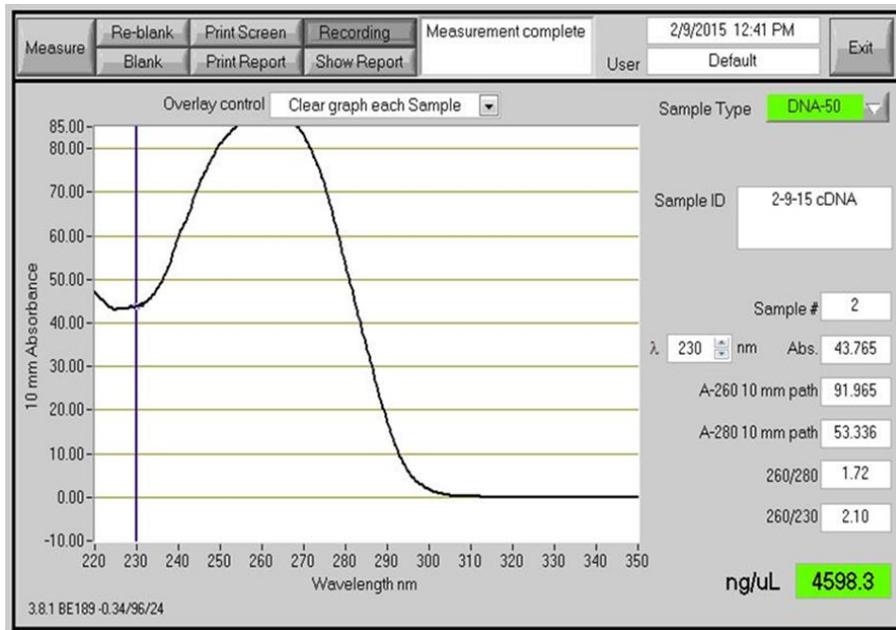


Figure 12. Concentration of synthesized cDNA

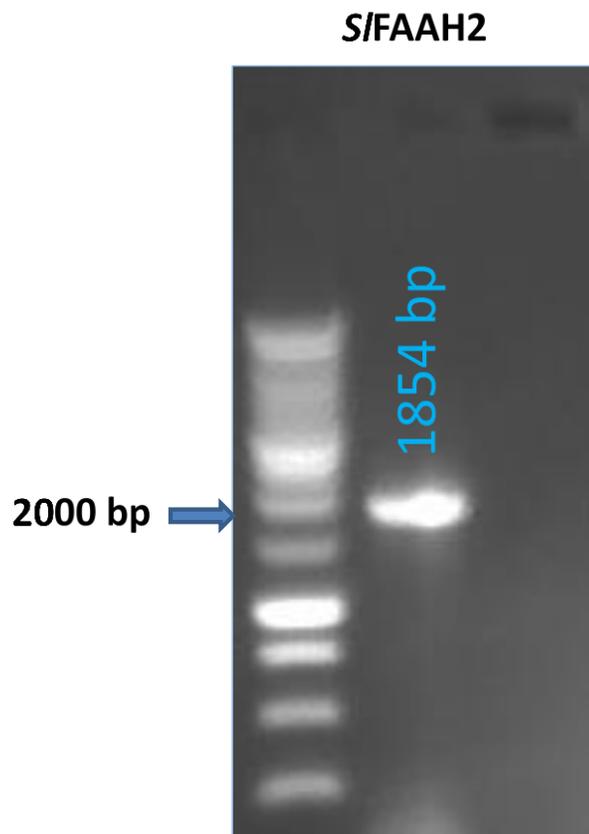


Figure 13. PCR product was analyzed by gel electrophoresis.

The amplified *S/FAAH2* gene was ran on an 1% agarose gel containing ethyl bromide. The resulting band migrated just under the 2000 bp reference band. Prior *in silico* analysis of *S/FAAH2* revealed the gene was 1854 bp in length (Figure 13).

## CHAPTER 4

### DISCUSSION

Similar to the catabolism of endocannabinoids by FAAH in mammals, *Arabidopsis* FAAH terminates NAE activity by hydrolysis (Teaster et al. 2007). *In silico* studies with putative *S/FAAH2* revealed a high homology with *Arabidopsis* FAAH1 (At5g64440). The results concluded a 66% homology to *Arabidopsis* FAAH1 and an E value of zero (table 1). The E value describes the number of results one can expect by chance alone. Therefore, an E value of zero indicates a significant match to *Arabidopsis* FAAH1. An alignment of putative tomato FAAH homologs with *Arabidopsis* FAAH confirm the conserved amidase signature catalytic sites as well as the overall homology between the genes (Figure 5). Further analysis of the predicated structures of *AtFAAH1* and *S/FAAH2* show that the two proteins may have similar functions based on their spatial arrangement (Figure 6). This illustrates the highly conserved FAAH-mediated breakdown of NAE across different species.

Transcript levels for *S/FAAH2* were highest in 0 and 2 day seeds and lowest for 4 day seeds. This agrees with the results from the protein assay in which FAAH activity was observed at 48 hours of seed development (Figure 10). At this time, it is unclear whether *S/FAAH1* or *S/FAAH2* is responsible for the hydrolysis of NAEs. In order to determine which enzyme, or enzymes, are responsible for this catalytic activity, cloning of both genes into an expression system must be carried out. The extracted RNA and cDNA from four day seeds were both of good quality as well as quantity (Figure 11, 12). *S/FAAH2* was amplified by PCR and separated by gel electrophoresis to confirm the correct gene had been transcribed (Figure 13).

## CHAPTER 5

### CONCLUSIONS AND FUTURE WORK

This research identified FAAH2 in tomato plants, and most importantly, in a crop plant. *In silico* analysis revealed that *S/FAAH2* shares high homology with *AtFAAH1*. Sequence alignment revealed conservation of catalytically important amino acid residues found in other systems. Comparison of predicted structures for *AtFAAH1* and *S/FAAH2* further illustrated that *S/FAAH2* serves a similar function in tomato. Further study included extraction RNA of at various developmental stages and quantification of transcript levels by qPCR. Activity of *S/FAAH2* at various stages was analyzed via enzyme assay followed by cDNA synthesis and isolation of FAAH by PCR amplification. This study will lead to characterization of NAE pathway in a crop plant, which will ultimately lead to develop molecular and biochemical tools necessary to improve tolerance to abiotic stress and increase crop productivity.

To further characterize this enzyme in tomato, cloning of *S/FAAH2* into a heterologous expression system will be carried out. The synthesis of cDNA from extracted RNA has been carried out and primers have been designed for cloning. *S/FAAH2* cDNA will be inserted into plasmid by a ligation reaction and then cloned in-frame into bacteria vector by heat shock technique before it is then transformed into *E.coli*. Positive transformed colonies will be selected and the expressed protein can be extracted and purified by column chromatography to yield pure *S/FAAH2*.

## REFERENCES

- Alger, B.E. (2004) Endocannabinoids: getting the message across. *Proc. Natl Acad. Sci. USA*, 23, 8512–8513.
- Chapman, Kent D. (2004). Occurrence, Metabolism, and Prospective Functions of N-acylethanolamines in Plants.
- Chapman KD, Venables B, Markovic R, Blair Jr RW, Bettinger C *Plant Physiol.* (1999); 120(4):1157-64.
- Cravatt BF, Demarest K, Patricelli MP, Bracey MH, Giang DK, Martin BR, et al. (2001). Supersensitivity to anandamide and enhanced endogenous cannabinoid signaling in mice lacking fatty acid amide hydrolase. 98:9371–9376.
- Cravatt BF, Giang DK, Mayfield SP, Boger DL, Lerner RA, Gilula NB. (1996). *Nature* 384:83–87.
- Irazoque, Andrea C. (2014). Agriculture under Threat.
- Kang L, Wang YS, Uppalapati SR, Wang K, Tang Y, Vadapalli V, Venables BJ, Chapman KD, Blancaflor EB, Mysore KS. (2008). Overexpression of a fatty acid amide hydrolase compromises innate immunity in Arabidopsis. 56(2):336-49.
- Kilaru A, Blancaflor EB, Venables BJ, Tripathy S, Mysore KS, Chapman KD. (2007). The Nacylethanolamine-mediated regulatory pathway in plants. *ChemBiodivers.*4(8):1933- 55.
- Kim, Sang-Chul et al. (2013). Analysis of Fatty Acid Hydrolase Activity in Plants. *Methods in molecular biology.* 1009:115-27.
- Kuehl FF, Jacob TA, Ganley OH, Ormond RE, Meisinger MAP (1957). *J Am Chem Soc* 1957;79:5577–8.
- McKinney, Michele K., and Benjamin F. Cravatt. (2005). STRUCTURE AND FUNCTION OF FATTY ACID AMIDE HYDROLASE. - *Annual Review of Biochemistry*, 74(1):411.
- Pappan K, Austin-Brown S, Chapman KD, Wang X. (1998). Substrate selectivities and lipid modulation of plant phospholipase D alpha, -beta, and -gamma. *Arch Biochem Biophys* 353(1):131-40.
- Patricelli MP, Lashuel HA, Giang DK, Kelly JW, Cravatt BF. (1998). *Biochemistry* 37:15177–87.
- Pfaffl., M. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research*, 29 (9).
- Rodriguez de Fonseca, F., Navarro, M., Gomez, R., Escuredo, L., Nava, F., Fu, J., Murillo-Rodriguez, E., Giuffrida, A., LoVerme, J., Gaetani, S., et al. 2001. An anorexic lipid mediator regulated by feeding. *Nature* 414: 209–212.

- Sasso O, Moreno-Sanz G, Martucci C, Realini N, Dionisi M, Mengatto L, Duranti A, Tarozzo G, Tarzia G, Mor M et al. (2013) papp. Antinociceptive effects of the N-acylethanolamine acid amidase inhibitor ARN077 in rodent pain models. *Pain* 154(3):350-60.
- Schmid HH, Schmid PC, Natarajan V. (1996). The N-acylation-phosphodiesterase pathway and cell signalling. *Chem Phys Lipids* 80(1-2):133-42.
- Shrestha R, Kim SC, Dyer JM, Dixon RA, Chapman KD. (2006). Plant fatty acid (ethanol) amide hydrolases. *1761(3):324-34*.
- Shrestha R, Dixon RA, Chapman KD. (2003). Molecular identification of a functional homologue of the mammalian fatty acid amide hydrolase in *Arabidopsis thaliana*. *12;278(37):34990-7*.
- Shrestha R, Noordermeer MA, van der Stelt M, Veldink GA, Chapman KD. (2002). N-acylethanolamines are metabolized by lipoxygenase and amidohydrolase in competing pathways during cottonseed imbibition. *Plant Physiol* 130(1):391-401.
- Schmid HH. (2000); Pathways and mechanisms of N-acylethanolamine biosynthesis: can anandamide be generated selectively? *Chem Phys Lipids*, 108(1-2):71-87.
- Schmid HH, Schmid PC, Natarajan V. (1990). N-acylated glycerophospholipids and their derivatives. *Prog Lipid Res* 29(1):1-43.
- Tang C-M, Insel PA. (2004). GPCR Expression in the Heart: “New” Receptors in Myocytes and Fibroblasts. *Trends in Cardiovascular Medicine* 14(3):94-99.
- Teaster, N. D., Motes, C. M., Tang, Y., Wiant, W. C., Cotter, M. Q., Wang, Y. S., Kilaru, A., Venables, B. J., Hasenstein, K. H., Gonzalez, G., Blancaflor, E. B., and Chapman, K. D. (2007) N-acylethanolamine metabolism interacts with abscisic acid signaling in *Arabidopsis thaliana* seedlings. *The Plant cell* 19, 2454-2469
- Tomato Genome. (2012). The tomato genome sequence provides insights into fleshy fruit evolution. *Nature* 485, 635-641
- Wang, X. (2004). Lipid signaling. *Current Opinion in Plant Biology*, 7(3), 329-336.
- Wang YS, Shrestha R, Kilaru A, Wiant W, Venables BJ, Chapman KD, Blancaflor EB. (2006); Manipulation of *Arabidopsis* fatty acid amide hydrolase expression modifies plant growth and sensitivity to N-acylethanolamines. *103(32):12197-202*.
- Urano D, Chen JG, Botella JR, Jones AM. (2013). Heterotrimeric G protein signalling in the plant kingdom. *Open Biol* 3(3):120186.
- USDA. (2013). U.S. Department of Agriculture *Economic Research Service*
- Venables BJ, Waggoner CA, Chapman KD. (2005). N-acylethanolamines in seeds of selected legumes. *Phytochemistry* 66(16):1913-8.