The biosynthesis and roles of *N*-acylethanolamines in plants

Omar Arias-Gaguancela and Kent D. Chapman*
Department of Biological Sciences, BioDiscovery Institute, University of North Texas, Denton, TX, United States
*Corresponding author: e-mail address: chapman@unt.edu

Contents

Abstract

N-acylethanolamines (NAEs) are a group of lipid signaling molecules derived from the phospholipid precursor *N*-acylphosphatidylethanolamine (NAPE). NAEs can be processed by a wide range of metabolic processes including hydrolysis by fatty acid amide hydrolase (FAAH), peroxidation by lipoxygenases (LOX), and conjugation by glycosyl- and malonyl-transferases. The diversity of NAE metabolites points to participation in multiple downstream pathways for regulation and function. NAEs with acyl chains of 18C are typically the most predominant types in vascular plants. Whereas in nonvascular

Advances in Botanical Research
ISSN 0065-2296
https://doi.org/10.1016/bs.abr.2021.07.002

plants and some algae, the arachidonic acid-containing NAE, anandamide (a functional "endocannabinoid" in animal systems), was recently reported. A signaling role for anandamide and other NAEs is well established in vertebrates, while NAEs and their oxylipin metabolites are recently becoming appreciated for lipid mediator roles in vascular plants. Here, the NAE metabolism and function in plants are overviewed, with particular emphasis on processes described in vascular plants where most attention has been focused.

1. Introduction

N-acylethanolamines (NAEs) are conserved lipophilic acyl amides well-recognized for their signaling roles in animals (Alhouayek, Stafberg, Karlsson, Bergström, & Fowler, 2020; Dlugos, Childs, Stuhr, Hillard, & de Wit, 2012). Significant attention was brought to this group of acyl amides, due to their participation in a wide range of neurological processes. For example, one of the most studied NAEs in mammals, NAE20:4 (anandamide), is an endogenous ligand for cannabinoid (CB) receptors that triggers signaling cascades to regulate processes such as pain perception (Alharthi et al., 2018; Clapper et al., 2010), inflammatory responses (Lowin, Apitz, Anders, & Straub, 2015; Rettori, De Laurentiis, Zorrilla Zubilete, Rettori, & Elverdin, 2012) and anxiety behaviors (Bluett et al., 2014). This has captured the interest of many research groups, which see the therapeutic potential of NAE-like molecules as pharmacological targets (Bruijnzeel et al., 2016).

In plants, the understanding of NAE metabolism and function has gained ground using both biochemical and genetic approaches. Several lines of research have help to reveal the formation and metabolic fates of NAEs. In addition, studies have demonstrated the involvement of NAE and NAE-like metabolites in processes such as seedling establishment and development (Cotter, Teaster, Blancaflor, & Chapman, 2011; Keereetaweep, Blancaflor, Hornung, Feussner, & Chapman, 2013, 2015; Wang et al., 2006), chloroplast development (Cannon & Chapman, 2021; Keereetaweep et al., 2013; Yan et al., 2020), cellular organization (Blancaflor, Hou, & Chapman, 2003; Motes et al., 2005), flowering (Teaster et al., 2012), and biotic responses (Aziz & Chapman, 2020; Kang et al., 2008; Kim et al., 2009; Palmer, Senechal, Mukherjee, Ané, & Blackwell, 2014; Zhang et al., 2019), all of which have expanded our knowledge about these lipids. This information may be useful for future applications in crop improvement or management.

Here, a comprehensive overview of NAE metabolism and functions in plants is provided with the hope to stimulate interest towards this class of signaling molecules.

2. NAE occurence, formation and metabolic fates

2.1 NAE ocurrence in plants

N-acylethanolamines (NAEs) were initially thought to be lipophilic artifacts with minor bioactive effects. However, over the past 30 years, many studies have demonstrated the substantial biological impact associated with these lipid molecules (Blancaflor et al., 2014; Chapman, 2004). The binary designation, namely, NAE(X):(Y), is one of the most common nomenclatures to identity a particular NAE type. In this numeric based designation, X and Y represent the length of the acyl chain linked to ethanolamine and the number of double bonds in the acyl moiety, respectively. The reported NAEs (Chilufya, Devaiah, Sante, & Kilaru, 2015) in plants mostly have acyl chain lengths of 12–18C, and several degrees of unsaturation (up to 3 double bonds) (Fig. 1); however, 20C NAEs also have been identified in some nonvascular plants and algae (Gachet, Schubert, Calarco, Boccard, & Gertsch, 2017).

In vascular plants, NAE content varies with tissue, stage of development and plant species. NAEs are present in the range of nano- to micrograms per gram of fresh weight. Concordantly, many analytical methods needed to be adapted for their detection and quantification (Chapman, Tripathy, Venables, & Desouza, 1998; Gachet et al., 2017; Keereetaweep & Chapman, 2016; Venables, Waggoner, & Chapman, 2005). In crop seeds (e.g., tomato and corn), endogenous NAE18:2, NAE18:1, NAE16:0, NAE12:0, NAE18:0, and NAE14:0 have been identified, with NAE18:2 being the most abundant species. By contrast, in legumes such as alfalfa (*Medicago sativa*) and *Medicago truncatula*, NAE18:3 represents the most abundant NAE species (Table 1). Interestingly, in vegetative tissues, NAE profiles markedly differ from that of seeds (Chapman, Venables, Markovic, Blair, & Bettinger, 1999; Venables et al., 2005). Indeed, in seeds of the model plant *Arabidopsis thaliana* (hereafter referred to as Arabidopsis), the content of 18C polyunsaturated NAEs (e.g., NAE18:2 or NAE18:3) were many times greater than in seedling tissues (Fig. 2) (Wang et al., 2006). This shows that NAE occurrence and composition are influenced by developmental

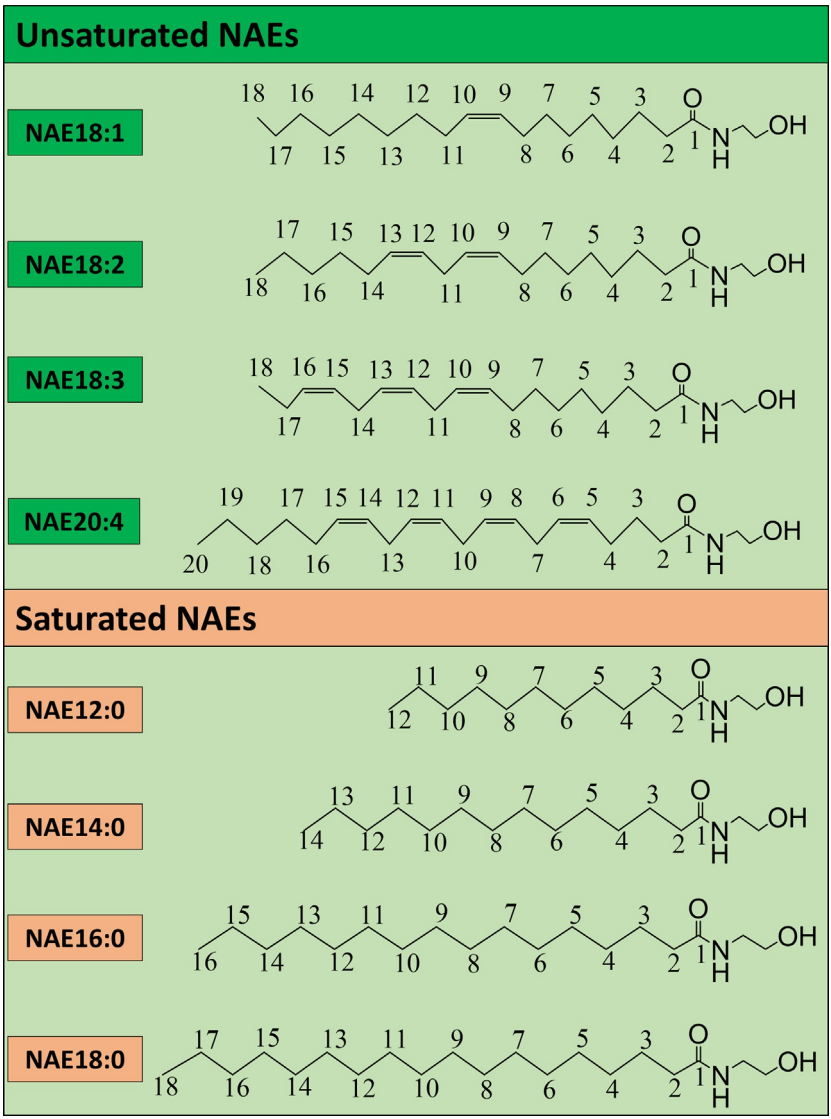

Fig. 1 Unsaturated and saturated *N*-acylethanolamines (NAEs) reported in vascular and nonvascular plants.

changes, and cell or tissue type. Further, these NAE-profiles indicate that 18C NAEs are major contributors of the total NAE pool.

In nonvascular plants, NAE occurrence has been assessed to understand evolution of lipid signaling across multiple taxa. For a long time, it was

Table 1 Endogenous concentrations of *N*-acylethanolamines (NAEs) in seeds of selected angiosperms.

Plant Species	NAE species (µg/g fresh weight)						
	NAE12:0	NAE14:0	NAE16:0	NAE18:0	NAE18:1	NAE18:2	NAE18:3
Solanum lycopersicum	0.15	0.03	0.1	0.03	0.13	0.29	0
Zea mays	0.18	0.02	0.2	0.07	0.33	0.38	0
Gossypium hirsutum	0.15	0.06	0.35	0.06	0.16	0.87	0
Abelmoschus esculentus	0.15	0.04	0.13	0.03	0.12	0.29	0
Medicago sativa	<0.02	<0.02	10.4	4.28	9.4	18.1	25.1
Medicago truncatula cv. A17	<0.02	<0.02	1.7	0.6	2.9	9.1	25.3
Glycine max cv. Dare	2.48	2.72	35.7	8.6	26.02	67.6	18.6

Cells in orange represent the most abundant NAE in a particular plant species.
Data derived from Chapman, K. D., Venables, B., Markovic, R., Blair, R. W., & Bettinger,-C. (1999). N-acylethanolamines in seeds. Quantification of molecular species and their degradation upon imbibition. Plant Physiology, 120, 1157–1164; Venables, B. J., Waggoner, C. A., & Chapman, K. D. (2005). N-acylethanolamines in seeds of selected legumes. *Phytochemistry*, 66, 1913–1918.

thought that the endogenous cannabinoid (NAE20:4) was present exclusively in nonplant systems (Gómez-Boronat et al., 2019; Pacioni et al., 2015). However, recent reports indicate that NAE20:4 alongside other NAE species (e.g., NAE18:2) are present in many nonvascular plants and even some algae. For instance, mosses (e.g., *Physcomitrella patens*), liverworts (e.g., *Conocephalum conicum*), monilophytes (e.g., *Salvinia molesta*), hornworts (e.g., *Anthoceros agrestis*), and even in the algae *Chara vulgaris* (Gachet et al., 2017). Unlike the studies conducted for vascular plants, NAE quantification

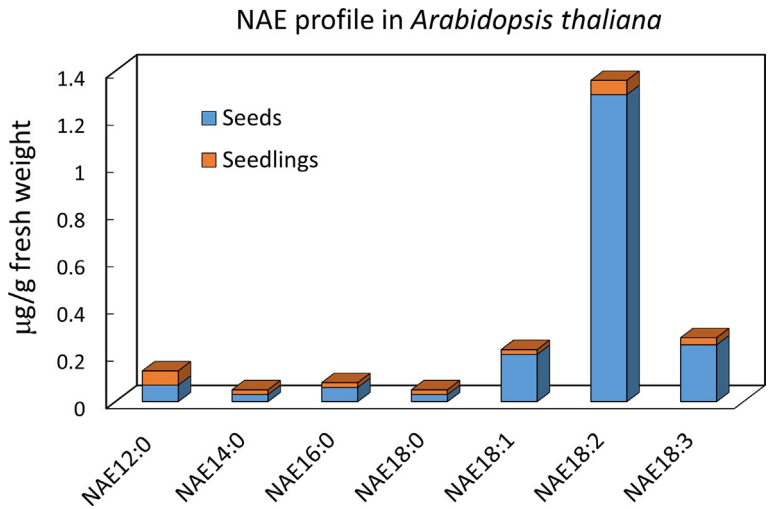

Fig. 2 NAE profile in *Arabidopsis thaliana*. Bar graphs depict the amount of NAEs found in seed and seedling tissues. *Data derived from Wang, Y. S., Shrestha, R., Kilaru, A., Wiant, W., Venables, B. J., Chapman, K. D., et al. (2006). Manipulation of Arabidopsis fatty acid amide hydrolase expression modifies plant growth and sensitivity to N-acylethanolamines.* Proceedings of the National Academy of Sciences of the United States of America*, 103, 12197–12202.*

in nonvascular plants was done using whole organisms (sample tissue), and therefore, variations of NAE content associated with different tissues and developmental stages remain to be more fully investigated. Nevertheless, the detection of NAE20:4 in these taxonomic groups point to a previously unrecognized evolutionary plasticity for these lipid-signaling molecules.

2.2 NAE biosynthesis via NAPE

In mammals, multiple metabolic pathways lead to the production of NAEs (Fig. 3). One of the most common paths involves the use of membrane lipid *N*-acylphosphatidylethanolamine (NAPE) as the precursor (Hussain, Uyama, Tsuboi, & Ueda, 2017). NAPEs are found as minor membrane lipids with respect to the total phospholipid pool (Hansen, Kleberg, & Hassing, 2015). Their hydrolysis into NAEs is a conserved mechanism mediated by a membrane-associated enzyme, namely, NAPE-phospholipase D (NAPE-PLD) (Magotti et al., 2015). This phospholipase D type-enzyme cleaves NAPE phosphodiester bond, releasing NAE and phosphatidic acid (Janfelt et al., 2012). Further, NAPE-PLD catalytic activity is independent of nature of the acyl chain, which enables the formation of NAE species of various degrees of saturation and length.

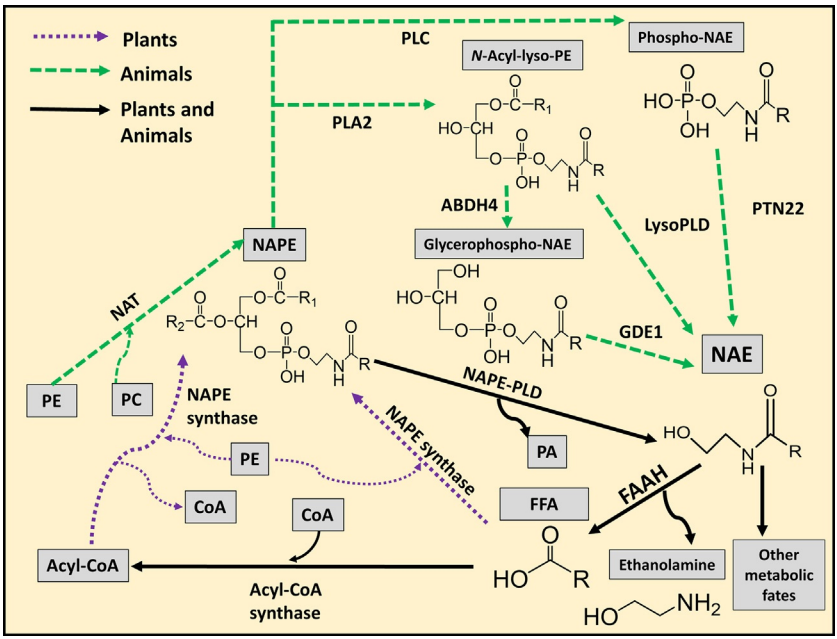

Fig. 3 NAE biosynthesis in plants and animals. Color-coded arrows represent pathways that are exclusive or conserved in plants and animals. Abbreviations: NAE, *N*-acylethanolamines; NAPE, *N*-acylphosphatidylethanolamine; NAPE-PLD, NAPE- phospholipase D; PLA2, phospholipase A2; *N*-acyl-lyso-PE, *N*-acyl-lyso-phosphatidylethanolamine; lysoPLD, lyso phospholipase D; ABDH4, α/β domain-containing hydrolase 4; GDE1, glycerophosphodiesterase 1; PLC, phospholipase C; PTN22, protein tyrosine phosphatase type 22; NAT, *N*-acyltransferase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; FAAH, fatty acid amide hydrolase; FFA, free fatty acids; R, R1, or R2 represent different fatty acid acyl chains linked to the molecule.

In plants, NAE formation from NAPE was first proposed in studies conducted with microsomes of cotton seedlings (Chapman, Lin, & DeSouza, 1995; Chapman & Moore, 1993). Later, many PLD isoforms, namely, α, γ, and β were cloned from Arabidopsis cDNAs (Qin, Pappan, & Wang, 1997) and transformed into *Escherichia coli* for recombinant protein production and biochemical characterization. *In vitro* assays revealed that isoforms β and γ were capable of hydrolyzing NAPE into NAE. Further, ^{14}C radiolabeling experiments with tobacco cell suspensions shown that NAE formation from NAPE was stimulated by exposure with GTPase enhancers (e.g., mastoparan) (Chapman et al., 1998; Pappan, Austin-Brown, Chapman, & Wang, 1998). Similarly, tobacco leaves treated with fungal elicitors (e.g., xylanase) induced the depletion of NAPE and increased NAE 14:0

content (≈10-fold) compared to untreated controls (Tripathy, Kleppinger-Sparace, Dixon, & Chapman, 2003; Tripathy, Venables, & Chapman, 1999). Altogether, these and other similar data support a PLD-mediated formation of NAE from NAPE in a manner that is similar to that of the bonafide pathway in animals (Fig. 3) (Kilaru et al., 2012).

2.3 Alternative pathways for NAE formation from NAPE

In animals, NAPE also is reported to be converted to *N*-acyl-lyso-PE, glycerophospho-NAE, and phospho-NAE intermediates, all of which ultimately lead to NAE formation (Maccarrone, 2017; Ueda, Tsuboi, & Uyama, 2013). Evidence in animals showed that phospholipase A2 (PLA2) converts NAPE into *N*-acyl-lyso-PE. Then, lyso phospholipase D (LysoPLD) converts *N*-acyl-lyso-PE into NAE or it can be used by α/β domain-containing hydrolase 4 (ABDH4) to generate glycerophospho-NAE (Sun et al., 2004; Simon & Cravatt, 2006), followed by the action of glycerophosphodiesterase 1 (GDE1) which removes the glycerol-3-phosphate group to finally yield NAE (Simon & Cravatt, 2008). An additional pathway uses phospholipase C (PLC) which removes 1,2-diacylglycerol from NAPE, resulting in phospho-NAE, followed by protein tyrosine phosphatase type 22 (PTN22) activity that converts this intermediate into NAE (Liu et al., 2006). These multistep routes constitute alternative pathways that lead to NAE formation, in addition to the one-step canonical PLD-mediated pathway (Fig. 3).

Attempts have been made to identify homologs of some of the mammalian enzymes for these alternative pathways in plants. For instance, the Arabidopsis gene At4g24160 (a putative ABDH4 homolog) was solubilized *in vitro* and shown to have acyltransferase, lipase and hydrolytic activities (Ghosh, Chauhan, Rajakumari, Daum, & Rajasekharan, 2009). The sequence similarity of At4g24160 to that of the mammalian enzyme, in addition to the presence of a GXSXG lipase motif in its amino acid sequence, makes this Arabidopsis gene a potential candidate for further experimental testing using NAPE as substrate (Blancaflor et al., 2014); however, other reports suggest that this gene encodes a homolog of the comparative gene identifier 58, a protein that was shown to facilitate acyl-CoA import into peroxisomes in plant leaves (James et al., 2010; Park et al., 2013), so its role in NAPE metabolism is uncertain. Further, the use of genomics and other similar technologies have made it possible to identify multiple phospholipase A and C

gene families in corn, cotton, and *Brassica napus* (Iqbal et al., 2020; Zhang, Wang, & Liu, 2018; Zhu, Zhou, Li, & Li, 2020). Data derived from these studies could be used in future work to identify A or C-type phospholipases that might act upon NAPE to generate *N*-acyl-lyso-PE or phospho-NAE, respectively.

2.4 NAPE formation

The acyltransferase, namely, *N*-acyltransferase (NAT) is responsible of NAPE formation in multiple eukaryotic species (Fig. 3). NAT transfers *O*-acyl groups of a phospholipid donor (e.g., phosphatidylcholine (PC)) to the amine head group (*N*-acyl position) of phosphatidylethanolamine (PE) in a calcium dependent manner (Dosoky, Guo, Chen, Feigley, & Davies, 2018; Ogura, Parsons, Kamat, & Cravatt, 2016). In fact, rat neuron cells incubated with PC and nanomolar concentrations of Ca_2^+ produced NAPE, whereas no evident synthesis was observed otherwise (Wellner, Diep, Janfelt, & Hansen, 2013). The same experiment also revealed that acyl moieties in glycerophospholipids in the sn-1 position, rather than free fatty acids (e.g., oleic acid), were the preferred substrates used for NAPE synthesis.

In plants, a candidate NAPE synthase gene was identified and biochemically characterized in Arabidopsis (At1g78690p) (Faure et al., 2009). Recombinant protein and membrane extracts were used to test NAPE synthase activity from ^{14}C-labeled lipid substrates. In both experiments, NAPE synthase was capable of using acyl-CoA rather than free fatty acids (Faure et al., 2009). Later work has suggested that this protein might also synthesize other acyl lipids (Bulat & Garrett, 2011), so further work is required to fully reconcile the role of this protein in NAPE formation. Alternatively, an NAPE synthase activity associated with cottonseed microsomes was capable of producing NAPE from ^{14}C-labeled free fatty acids much more readily than from ^{14}C-acyl CoA or ^{14}C-labeled PC (Chapman & Moore, 1993). Later, three distinct bioactive isozymes were separated from the original protein extract. Transient expression of YFP-tagged At1g78690p in tobacco leaves showed exclusive localization to the plasma membrane whereas NAPE synthase activity in cotton microsomes was detected in three membrane compartments: plasma membrane, Golgi apparatus and the endoplasmic reticulum (Chapman & Moore, 1993). Further, spatiotemporal analysis of At1g78690p in transgenic Arabidopsis lines, revealed preferential localization and expression of the synthase gene in roots and young tissues of

embryos (Faure et al., 2009). Similar findings also support the notion that NAPE production may differ depending on the plant species, and tissue analyzed (Kotel'nikova, 2011).

Environmental cues also influence NAPE formation. For instance, evidence has shown that chilling stress, and hydration/dehydration cycles impact NAPE formation (Chapman & Sprinkle, 1996; Sandoval, Huang, Garrett, Gage, & Chapman, 1995; Zhang & Tian, 2010). Similarly, increasing NAPE content was detected in potato cells challenged under artificial anoxic environments or treated with respiratory inhibitors (e.g., salicylhydroxamate). These abiotic factors are associated with the release of free fatty acids, and as a result, the cell counteracts the membrane destabilizing effects by promoting NAPE formation from PE and free fatty acids (Rawyler & Braendle, 2001). Overall, NAPE metabolic machineries adapt to physiological and homeostatic conditions for subsequent NAE modulation, but much remains to be learned about NAPE biosynthesis in plants.

2.5 NAE signal termination by fatty acid amide hydrolase

NAEs can be processed by fatty acid amide hydrolase (FAAH) into ethanolamine and free fatty acids (Aziz, Wang, Tripathi, Bankaitis, & Chapman, 2019; Balsevich et al., 2018; Lin, Metherel, Jones, & Bazinet, 2017). A cDNA encoding FAAH was first identified in rat, and following studies in multiple nonplant organisms have reported NAE hydrolysis by FAAH. For example, mouse, rat, and human FAAHs hydrolyzed NAE20:4 into arachidonic acid and ethanolamine (Long, LaCava, Jin, & Cravatt, 2011; Palermo et al., 2015; Strittmatter et al., 2012). Similarly, FAAH from the slime mold, *Dictyostelium discoideum*, was capable of hydrolyzing NAE20:4, along with other saturated (e.g., NAE16:0) and unsaturated NAEs (e.g., NAE18:2) (Neelamegan, Schoenhofen, Richards, & Cox, 2012). These data suggested that FAAH catalytic capabilities might be more broadly distributed in other biological systems.

In plants, the molecular basis for NAE hydrolysis by FAAH was initially revealed following identification of the Arabidopsis homolog to rat FAAH (37% amino acid similarity within the amidase signature domain) (Kim et al., 2009; Shrestha, Dixon, & Chapman, 2003). Experiments with recombinant Arabidopsis FAAH (AtFAAH) have shown its capacity to hydrolyze a wide range of NAEs, including NAE18:2, NAE16:0, NAE14:0, and NAE12:0. Similar work in other plant systems showed that recombinant rice and *Medicago truncatula* FAAHs, namely, OsFAAH and MtFAAH, also metabolized NAEs in a manner that resembles that of the Arabidopsis FAAH

(Shrestha, Kim, Dyer, Dixon, & Chapman, 2006). Enzyme kinetics studies also have shown that AtFAAH is capable of hydrolyzing NAE18:2-derived oxylipin, 9-hydroxy octadecadienoyl ethanolamide (9-NAE-HOD) with a similar catalytic efficiency to that of the unsubstituted NAE18:2 parent molecule (Aziz et al., 2019), which was not the case for rat FAAH. Further, AtFAAH has also been shown to irreversibly cleave the amide bond of *N*-acyl-L-homoserine lactones (e.g., 3-oxo-C12-HSL), bacteria-derived lipids known to participate in quorum signaling (Palmer et al., 2014).

Recent studies in the moss, *Physcomitrella patens,* identified nine FAAHs (PpFAAHs). PpFAAH1–4 were closely related to AtFAAH whereas PpFAAH5–9 were more similar to rat FAAH (Haq & Kilaru, 2020). Biochemical characterization of one of these enzymes (PpFAAH1) revealed amidohydrolase activity toward endogenous NAE20:4 and other NAEs (e.g., NAE16:0). Collectively, this information shows an expanded role for FAAHs to hydrolyze a wide range of NAE and NAE-like structures, and further supports a conserved catalytic mechanism across multiple species.

2.6 FAAH structural features accommodate NAEs

FAAH is a widely conserved amidase in eukaryotes which is characterized by a Ser-Ser-Lys catalytic triad of amino acid residues (Haq & Kilaru, 2020; McKinney & Cravatt, 2003, 2005). FAAH is a homodimer protein with each monomer consisting of a core of twisted beta sheets surrounded by alpha helices (Dainese & Oddi, 2020). The resolved crystal structures of AtFAAH (Aziz et al., 2019) and rat FAAH (Bracey, Hanson, Masuda, Stevens, & Cravatt, 2002) have made it possible to gain additional insights about their structural conformations and enzymatic behavior. Analysis of their structures has helped to recognize some key structural similarities and differences between plant and mammalian FAAHs.

Unlike rat FAAH, AtFAAH has an N-terminal region that is postulated to anchor the protein to *endo*- and plasma membranes and facilitate its interaction with lipophilic substrates (Fig. 4). Additional structural differences were revealed in the acyl binding and membrane access channels, namely, ABC and MAC, respectively (Aziz & Chapman, 2020; Aziz et al., 2019). Rat FAAH has two cavities for acyl binding and substrate access, separated by a region known as the "dynamic paddle." In contrast, FAAH from Arabidopsis and the FAAH modeled from *P. patens*, have a more open cavity for substrate access and binding (Aziz et al., 2019; Haq & Kilaru, 2020). The absence of a "dynamic paddle" seems to be a key conserved evolutionary

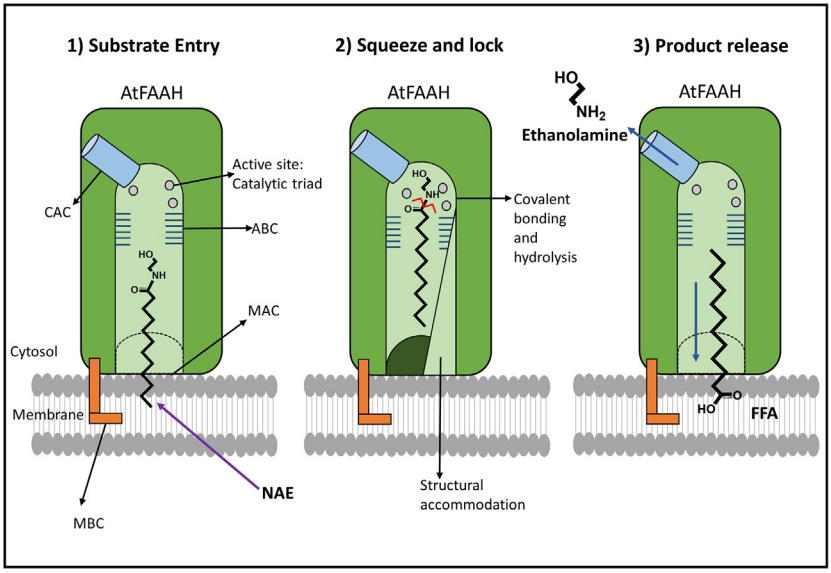

Fig. 4 Arabidopsis fatty acid amide hydrolase (AtFAAH) uses a "squeeze and lock" mechanism to accommodate and hydrolyze NAEs. Abbreviations: CAC, cytosolic access channel; ABC, acyl-binding channel; MAC, membrane access channel; MBC, membrane binding cap; FFA, free fatty acids. *Model derived from Aziz, M., Wang, X., Tripathi, A., Bankaitis, V. A., & Chapman, K. D. (2019). Structural analysis of a plant fatty acid amide hydrolase provides insights into the evolutionary diversity of bioactive acylethanolamides.* The Journal of Biological Chemistry, *294, 7419–7432.*

trait in vascular and nonvascular plants, as observed in sequence homology and studies of their 3D structures. Such differences alter the means by which plant FAAHs can accommodate NAEs and other lipid substrates into their active site. Based on X-Ray crystallographic data, AtFAAH is proposed to undergo a "squeeze and lock" mechanism upon substrate binding. When the ligand has reached the active site of the ABC, there is a conformational change of the helix region conformed by amino acids 531 to 537—"squeeze" the substrate in the binding site. In parallel, residues 25–28 in the MAC undergo movement along with Leu55 to secure the substrate-ligand complex—"lock." Upon reaction completion, the water-soluble product ethanolamine is released into the cytosol via the cytoplasmic access channel, while the fatty acid product is released back to the hydrophobic environment of the membrane (Fig. 4). In contrast, rat FAAH-substrate accommodation relies on a "dynamic paddle" formed by Phe432 and Trp531, to partially close its short MAC, and to further secure the ligand complex into

its wider ABC (Aziz & Chapman, 2020; Aziz et al., 2019). This suggests that in animals, NAE-processing mediated by FAAH may have evolved substrate preferences that are more restrictive than FAAHs found in plants.

2.7 A second group of FAAH enzymes in plants

Recently, an analysis of FAAH amino acid sequences with special reference to amino acid residues in the substrate binding pocket have made it possible to categorize FAAH amino acid sequences from vascular plants into two major phylogenetic clusters, namely, group I and II FAAH (Aziz & Chapman, 2020). Angiosperm plants such as Arabidopsis and *Ricinus communis* have only group I FAAH whereas most angiosperms have genes that encode for multiple group I and II FAAHs, as predicted for cotton plants (*Gossypium arboretum*, *G. raimondii*, and *G. hirsutum*), Solanaceous plants (e.g., *S. lycopersicum*, *S. tuberosum*) and legumes (e.g., *Glycine* max, *Medicago truncatula*). This in silico analysis highlights the possibility that through evolution plants may have adapted to utilize one or both FAAH groups, perhaps providing for an expanded substrate repertoire beyond processing NAEs.

Homology modeling of group I and II FAAH proteins from soybean (*G. max*) based on the AtFAAH three-dimensional crystal structure (PDB: 6DHV), revealed some similarities and differences that were conserved among members of each FAAH group (Aziz & Chapman, 2020). Like group I FAAH, the group II FAAH protein from soybean maintained a substrate binding pocket with a MAC, an ABC, and a cytosolic access channel. Also, they both have the characteristic N-terminal region for membrane anchoring, and a conserved catalytic triad. In contrast, the main differences between group I and II FAAHs reside in the substitution of key conserved amino acids surrounding the substrate binding pocket. For example; the cytosolic access channel of group I FAAH was highly hydrophilic and had a more polar substrate binding pocket, whereas group II FAAH had a more hydrophobic cytosolic access channel as well as a more hydrophobic substrate binding pocket, characterized by the presence of more aromatic amino acids than group I FAAHs. Based on these differences it is reasonable to think that that group II FAAHs might process substrates with less polarity compared to group I FAAHs. In fact, computational docking shown that GmFAAH II can accommodate alkamides (molecules structurally similar to NAEs), and *N*-acyl L-homoserine lactones (AHLs) in a more efficient manner compared to GmFAAH I (Aziz & Chapman, 2020; Aziz et al., 2019). However, these differences are yet to be experimentally tested.

Further, such experiments to examine substrate promiscuity also should include known FAAH substrates (e.g., unsaturated, saturated or hydroxylated NAEs).

2.8 NAE oxidation via lipoxygenase pathways

Alternative to the traditional NAE signaling termination mediated by FAAH, polyunsaturated 18C NAE species like NAE18:2 or NAE18:3 also can be oxidized by lipoxygenase enzymes (LOX) to generate hydroperoxides that can be reduced nonenzymatically to hydroxides (Fig. 5) (Keereetaweep et al., 2013, 2015; Kilaru et al., 2011). There are two broad

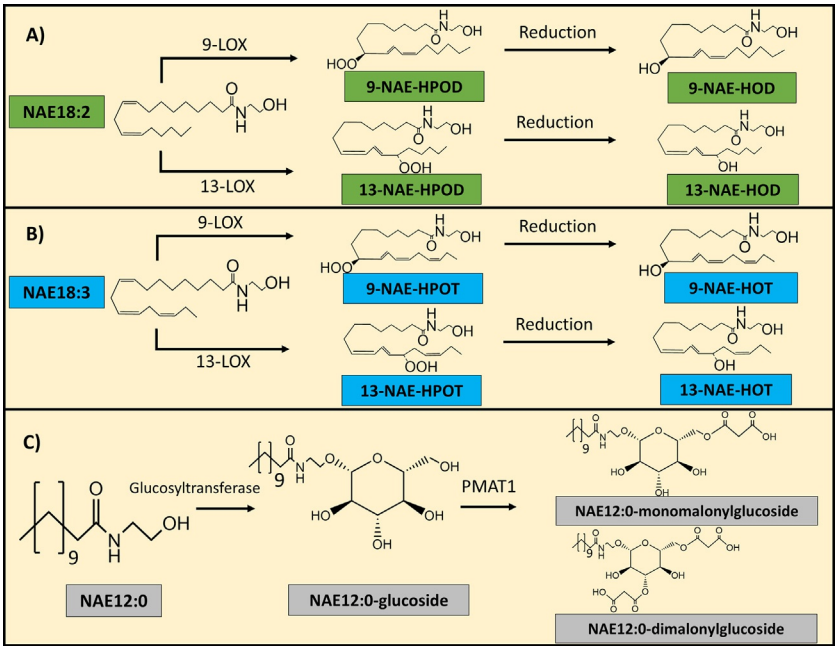

Fig. 5 Summary of selected NAE metabolic fates. Formation of hydroperoxides and hydroxides from (A) NAE18:2, (B) NAE18:3 and (C) NAE12:0-conjugates. Abbreviations: 9-NAE-HPOD, (9S,12Z,10E)-9-hydroperoxy-10,12-octadecadienoylethanolamide; 13-NAE-HPOD, (13S,9Z,11E)-13-hydroperoxy-9,11-octadecadienoylethanolamide; 9-NAE-HOD, (9S,12Z,10E)-9-hydroxy-10,12-octadecadienoylethanolamide; 13-NAE-HOD, (13S,9Z,11E)-13-hydroxy-9,11-octadecadienoylethanolamide; 9-NAE-HPOT, (9S,12Z,10E,15Z)-9-hydroperoxy-10,12,15-octadecatrienoylethanolamide; 13-NAE-HPOT, (13S,9Z,11E,15Z)-13-hydroperoxy-9,11,15-octadecatrienoylethanolamide; 9-NAE-HOT, (9S,12Z,10E,15Z)-9-hydroxy-10,12,15-octadecatrienoylethanolamide; 13-NAE-HOT, (13S,9Z,11E,15Z)-13-hydroxy-9,11,15-octadecatrienoylethanolamide; PMAT, glucoside malonyltransferase 1; 9-LOX, 9-lipoxygenase; 13-LOX, 13-lipoxygenase.

families of LOX enzymes in plants based on their position of peroxidation, namely, 9-LOX and 13-LOX (Hayward, Cilliers, & Swart, 2017). They introduce molecular oxygens at the C9 or C13 positions of the acyl chain (Andreou & Feussner, 2009). These same LOX enzymes can act on polyunsaturated NAEs, free fatty acids or acyl groups of glycerolipids (Chechetkin, Osipova, Antsygina, Gogolev, & Grechkin, 2011; Gabbs & Leng, 2015; Kilaru et al., 2011).

Evidence of oxylipin formation from NAEs in plants was first described in experiments with imbibed cottonseed extracts, where a 13-LOX activity mediated the oxidation of NAE18:2 (Shrestha, Noordermeer, van der Stelt, Veldink, & Chapman, 2002). Experiments with recombinant Arabidopsis LOX enzymes also demonstrated activity toward NAEs, where two 9-LOX genes (AtLOX1 and AtLOX5) and four 13-LOX genes (AtLOX2, AtLOX3, AtLOX4, and AtLOX6) have been reported (Bannenberg, Martínez, Hamberg, & Castresana, 2009). 9-LOX or 13-LOX enzymes act upon NAE18:2 to generate (9S,12Z,10E)-9-hydroperoxy-10,12-octadecadienoylethanolamide (9-NAE-HPOD) or (13S,9Z,11E)-13-hydroperoxy-9,11-octadecadienoylethanolamide (13-NAE-HPOD) (Kilaru et al., 2011). Subsequently, these hydroperoxides may be reduced to (9S,12Z,10E)-9-hydroxy-10,12-octadecadienoylethanolamide (9-NAE-HOD) or (13S,9Z,11E)-13-hydroxy-9,11-octadecadienoylethanolamide (13-NAE-HOD). Interestingly, 9-NAE-HOD rather than 13-NAE-HOD acts as a potent bioactive inhibitor of seedling growth (Keereetaweep et al., 2015), pointing to a selective lipid mediator function for specific NAE metabolites. Furthermore, the action of 9-NAE-HOD requires an intact abscisic acid (ABA) signaling pathway and works synergistically with ABA to modulate growth in Arabidopsis seedlings (Keereetaweep et al., 2015).

Similarly, NAE18:3 also can be used as lipophilic substrate by 9-LOX or 13-LOX to form (9S,12Z,10E,15Z)-9-hydroperoxy-10,12,15-octadecatrienoylethanolamide (9-NAE-HPOT) or (13S,9Z,11E,15Z)-13-hydroperoxy-9,11,15-octadecatrienoylethanolamide (13-NAE-HPOT) (Keereetaweep et al., 2013). These intermediates are then reduced to (9S,12Z,10E,15Z)-9-hydroxy-10,12,15-octadecatrienoylethanolamide (9-NAE-HOT) or (13S,9Z,11E,15Z)-13-hydroxy-9,11,15-octadecatrienoylethanolamide (13-NAE-HOT), and both exert negative regulatory effects in seedling growth. Alternatively, 13-NAE-HPOT could undergo additional metabolic steps that lead to (13S,9Z,10E,15Z)-12-oxo-10,15-phytodienoic acid ethanolamide (NAE-OPDA) formation. Overall, these 9-LOX and 13-LOX mediated routes indicate a previously unrecognized versatility in NAE oxidative metabolism.

Moreover, the interplay between LOX and FAAH enzymes is key in determining NAE oxylipin production. Side by side experiments comparing the NAE-derived oxylipin profiles of Arabidopsis wild-type vs *atfaah*-knockout or *AtFAAH* overexpressing lines fed with micromolar concentrations of NAE18:2 or NAE18:3, demonstrated that *atfaah*-knockouts produced an increase of NAE-HODs or NAE-HOTs contents, whereas, NAE18:2- or NAE18:3-oxylipin levels in *FAAH* overexpressing lines were much lower than in wild-type controls (Keereetaweep et al., 2013, 2015). It is reasonable to assume that the suppression of FAAH leads to an increase of the NAE pool content (especially of 18C polyunsaturated NAEs), which can be further metabolized by LOX enzymes (hence the elevated NAE-oxylipin content). Conversely, in the overexpressing lines, an increase of *FAAH* expression and hydrolytic activity reduces the bioavailability of polyunsaturated NAE substrates that could be used by LOX enzymes to form NAE-HODs or NAE-HOTs (Keereetaweep et al., 2013, 2015; Kilaru et al., 2011). Overall, LOX and FAAH interactions for NAE processing provide new layers of complexity and fine tuning for these lipid molecules that represent opportunities for further research in other plant systems and physiological conditions.

2.9 NAE12:0 glyco-conjugates

The study of NAE-metabolic fates led to somewhat of a surprise when using untargeted analytical tools. Khan et al. demonstrated the presence of NAE12:0 conjugates in Arabidopsis seedlings treated with micromolar concentrations of NAE12:0 (Fig. 5) (Khan et al., 2016). Data from mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy identified detectable levels of NAE12:0-glucoside. In this reaction, a yet-to-be determined glycosyltransferase is responsible for adding a sugar moiety to the free hydroxyl group of NAE12:0. In addition, NAE12:0-monomalonylglucoside and NAE12:0-dimalonylglucoside were identified, representing two additional previously unknown NAE conjugates. *In vivo* and *in vitro* experiments showed that the enzyme phenolic glucoside malonyltransferase 1 (PMAT1) attaches malonic acid to the carbon 3 or/and 6 of NAE12:0-glucoside conjugates (Khan et al., 2016). The identification of these novel NAE derivatives suggest additional metabolic pathways that plants might use to process and transport NAEs to different cellular compartments, thus expanding the potential routes for NAE metabolism (Fig. 5). However, the biological effects or bioactivity of these NAE12:0-glyco-conjugates remain to be elucidated in future research.

2.10 Some NAEs untilize G-proteins for signal transduction

The propagation of NAE signal(s) in animal systems depends upon G protein-coupled receptors (GPCRs) and associated G proteins. This is the topic of intense investigations in vertebrates where these receptors are the same as those that are activated by cannabinoids. As such, specific NAEs are considered endogenous, or "endocannabinoids," and regulate numerous physiological processes as part of the endocannabinoid signaling system (Kilaru & Chapman, 2020). For example, in mammals, postsynaptic neurons produce NAE20:4 which binds to CB receptors, and this leads to the activation of heterotrimeric G proteins (Howlett, Blume, & Dalton, 2010). Activated trimeric G-proteins undergo dismantling of the Gαβγ trimeric state, which results in the release of Gα-GTP and Gβγ dimer, and these units are free to interact with downstream factors to regulate multiple neurological processes, thus, propagating NAE signaling (Mahavadi, Sriwai, Huang, Grider, & Murthy, 2014; Samson et al., 2003).

In Arabidopsis, NAE18:3-induced chloroplast responses have been linked to G-protein activity (Cannon & Chapman, 2021; Cannon et al., 2020; Yan et al., 2020). For instance, the de-greening of chloroplasts induced by NAE 18:3 application to seedlings, was inhibited in G-protein mutants. Exogenous applications of NAE18:3 also led to differential expression of many genes associated with G-protein activity, stress responses, senescence, autophagy, and chlorophyll anabolic and catabolic pathways (Cannon et al., 2020; Yan et al., 2020). This and other similar experiments support a model in which G-proteins are required, at least in part, for NAE18:3 action in plants. Altogether, studies from animals and plants suggest a partially-conserved mechanism for NAE signal transduction requiring G-proteins, and also suggest different components that may have arisen during the course of evolutionary modification of NAE-signaling.

3. Functions of NAEs

3.1 Plant growth

Pharmacological approaches have revealed multiple effects of NAEs on plant growth (Blancaflor et al., 2014; Wang et al., 2006). For example, NAE12:0-treated Arabidopsis seedlings showed a dose-dependent, reversible inhibition of primary root elongation, abnormal root apices with disrupted cell walls, and reduced root hair length and numbers compared to controls (Blancaflor et al., 2003). Also, NAE18:2- and NAE18:3-derived oxylipins (but not the parent NAEs) inhibited seedling growth (Keereetaweep

et al., 2013, 2015). ABA seems to be required for the action of NAE18:2-derived oxylipin (9-NAE-HOD) on overall seedling growth, thus providing evidence of cross talk between NAE and ABA pathways (Keereetaweep et al., 2015). These examples together have suggested a link between NAE metabolism and seedling development.

To gain insights of the physiological role of FAAH-mediated NAE hydrolysis in Arabidopsis, several genetic approaches also have been taken. Different FAAH overexpression and knockout lines were compared to wild-type plants to assess phenotypic changes. Studies showed that overexpression of Arabidopsis FAAH results in an increase in plant growth, a notion derived from the enhanced seedling growth and early flowering phenotypes found in the *FAAH* overexpressors compared with wild-type seedlings (Teaster et al., 2012, 2007; Wang et al., 2006). Interestingly, the quantities of endogenous NAE levels were reduced significantly in seedlings of *FAAH* overexpressing lines compared to wild-type. When genetic and pharmacological approaches were combined, it was evident that the growth effects were even more pronounced when seedlings were challenged with NAEs. For instance, exogenous applications of NAE12:0 or NAE18:2 did not inhibited growth in FAAH overexpressing plants to the same extent as wild-type or knockouts. By contrast, *atfaah*-knockouts showed only a modest reduction in seedling growth and minor discernible impacts on endogenous NAE levels during seedling growth, but these growth effects were much more pronounced in the presence of added NAEs than those of wild-type seedlings (Cotter et al., 2011; Teaster et al., 2007; Wang et al., 2006). Altogether, work derived from these studies support an important function(s) for NAEs in plant development.

Alkamides are a group of NAE-like structures known to be hydrolyzed by Arabidopsis FAAH (Faure et al., 2015). Experiments have shown that alkamides are negative regulators of plant development (Ramírez-Chávez, López-Bucio, Herrera-Estrella, & Molina-Torres, 2004). Exogenous applications of affinin (one type of alkamide) greatly impacted Arabidopsis seedling growth, and such effects were more noticeable in the context of FAAH altered lines. For instance, *atfaah*-knockout plants exposed to exogenous alkamides showed reduced root and seedling growth compared with wild-type seedlings, whereas *AtFAAH* overexpressing lines were more tolerant to the exposure (Faure et al., 2015; Ramírez-Chávez et al., 2004). Similarly, *N*-acyl L-homoserine lactones (another type of NAE-like structures) have been associated with the alteration of root architecture, and overall seedling growth. Like NAEs and alkamides, *N*-acyl L-homoserine

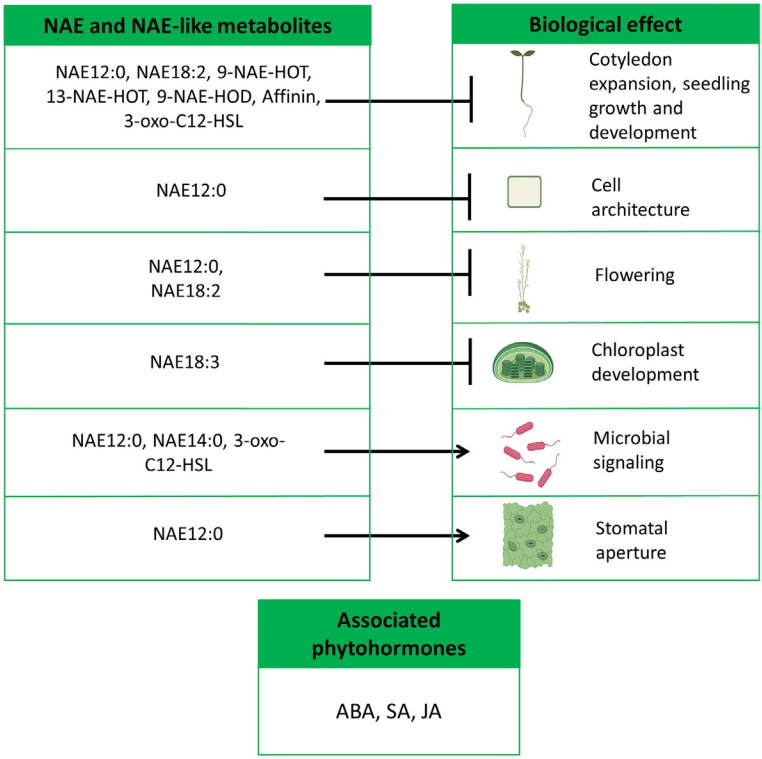

Fig. 6 Selected plant NAE, and NAE-like metabolites and their corresponding biological effects, and associated phytohormones. *Figure created with BioRender.com and Power Point 2016.*

lactone-induced effects are influenced in a FAAH-depended manner (Ortíz-Castro, Martínez-Trujillo, & López-Bucio, 2008; Palmer et al., 2014). These examples support the notion that NAE and NAE-like molecules can influence plant development in a FAAH-dependent manner (Fig. 6).

3.2 Flowering

NAEs have been implicated in the modulation of flowering time in Arabidopsis plants (Teaster et al., 2012). *FAAH* overexpressing lines had the tendency to flower up to 10 days earlier than wild-type and *atfaah*-knockouts plants under noninductive short-day conditions. This phenotype coincided with 30% reduction of NAE12:0 and NAE18:2 contents, and elevated expression of genes associated with flowering time. For example, *FLOWERING LOCUS* (*FT*) and *SEPTELLA3* (*SEP3*) genes were

elevated in *FAAH* overexpressing plants. *FT* is an important transcription factor which activates or represses flowering gene regulators (e.g., *SEP3*). Given that NAE12:0 was one of the NAEs reduced in *FAAH* overexpressing plants, micromolar concentrations of NAE12:0 were irrigated to the base of wild-type plants every 3 days to assess phenotypes. Data showed a decrease in the number of rosette leaves, shorter inflorescent stems, and flowering delay of 6 days compared to DMSO controls. Consistent with these findings, NAE12:0-treated plants had reduced *FT* and *SEP3* expression levels (Teaster et al., 2012). These pieces of evidence point to NAE12:0 capacity to delay the onset of flowering in plants (Fig. 6), maybe in association with flowering gene networks, and that this can be modulated by FAAH.

3.3 Chloroplast degradation

Destabilization of chlorophyll components are also an effect associated with NAEs. Exogenous applications of NAE18:3 to Arabidopsis wild-type seedlings led to reduced growth and an evident cotyledon bleaching phenotype. The de-greening effect was even more severe in *atfaah*-knockout seedlings whereas *AtFAAH* overexpressing lines were unaffected and did not undergo de-greening in the presence of NAE (Keereetaweep et al., 2013). In this context, a FAAH-suppressed plant would be unable to efficiently hydrolyze NAEs which renders it more susceptible to NAE18:3. By contrast, FAAH-overexpressing plants tolerate NAE negative effect (s) due to an enhanced FAAH activity and capacity for NAE18:3 turnover. Further, analysis at the transcriptome level, revealed that exogenous applications of NAE18:3 to Arabidopsis wild-type seedlings result in down-regulation of multiple genes associated with chlorophyll biosynthesis and development (Cannon et al., 2020). These data show that exogenous application of NAE18:3 triggers detrimental effect (s) through molecular networks associated with chlorophyll metabolism, and retards the normal chloroplast development and cotyledon expansion associated with seedling growth.

Further observations of NAE18:3-treated seedlings with confocal laser scanning microscopy and by transmission electron microscopy revealed a disruption of thylakoid membranes and an overall dismantling of chloroplasts over a 3-day time course (Keereetaweep et al., 2013). The same study also showed that the loss of chlorophyll observed upon NAE18:3-treatment

was reversible, and stage-specific. Experiments showed that removal of NAE18:3 resulted in the restoration of chloroplast architecture and a re-greening of cotyledons over a 4-day time course. Interestingly, addition of micromolar concentrations of NAE12:0 prevented NAE18:3-seedling bleaching effect (Keereetaweep et al., 2013; Keereetaweep, Kilaru, Feussner, Venables, & Chapman, 2010). Thus, this may suggest that certain NAEs can interact with other NAE types to influence the ultimate physiological outcome (Khan, Chapman, & Blancaflor, 2021), perhaps by competing for the active site of FAAH or LOX enzymes. Overall, these experiments suggest that levels of NAE18:3 or metabolites thereof can impact chloroplast homeostasis in plants (Fig. 6).

3.4 Stomatal closure

As previously mentioned, there appears to be crosstalk between NAE and ABA signaling pathways (Cotter et al., 2011). Another piece of information that supports such a notion is the finding that NAE12:0 blocks PLDα activity and its ability to modulate ABA-induced closure of stomata. Indeed, tobacco guard cells treated with ABA alone led to reduction of the diameters of the stomatal pores, whereas treatments at low micromolar concentrations of both NAE12:0 and ABA led to greater pore diameters over a 60-min course (Austin-Brown & Chapman, 2002). These results indicated that NAE12:0 can modulate stomatal aperture in a dose- and time-dependent manner (Fig. 6).

3.5 Cellular reorganization

The link between cell architecture and NAEs is an area that has received attention (Blancaflor et al., 2014). Pharmacological applications of NAE 12:0 to different types of hypocotyl and cotyledon cells caused a dramatic impact on cellular organization and integrity. Data showed that long-term exposures of NAE 12:0 led to transverse alignment of the microtubules and an aggregation of F-actin bundles (Blancaflor et al., 2003; Motes et al., 2005). Furthermore, these changes compromise other cellular processes such as endomembrane trafficking and cell expansion. Together these support the notion that NAE12:0 interferes with regular cell expansion, which may be responsible, in part, for the overall reduced growth phenotypes observed in NAE-treated seedlings.

3.6 Microbial interactions

One of the first indications that NAE could participate in plant–pathogen interactions came from studies in tobacco cells and leaves, where NAE14:0 levels were dramatically increased in the presence of fungal elicitors (Tripathy et al., 2003, 1999). This NAE-profile coincided with increased expression of defense genes (e.g., phenylalanine ammonia lyase) and the inhibition of extracellular alkalinization in a time- and dose-dependent manner (Tripathy et al., 1999). The influence of NAEs on plant–fungal interactions also has been examined in cotton (Zhang et al., 2019). Research revealed that defoliating strains (DS) of the plant pathogenic fungus *Verticillium dahliae* were capable of producing NAE12:0 during infection of cotton seedlings. Furthermore, the leaf defoliation phenotype seemed to depend on ABA induction. Notably, in these studies, neither *FAAH* overexpressors nor *atfaah*-knockouts were tested for sensitivity to *V. dahliae* (Zhang et al., 2019). Nevertheless, the study found that many *FAAH* transcripts increased upon *V. dahliae* infection or NAE 12:0 treatment. These findings support a model in which FAAHs and NAEs modulate leaf defoliation phenotypes, suggesting a role for NAEs in plant–microbe interactions (Fig. 6).

NAE modulation in plant–bacterial interactions has been addressed as well. *AtFAAH* overexpressing or knockout lines with altered NAE profiles have been used to understand their link to plant–pathogen interactions (Kang et al., 2008; Kim et al., 2009). *FAAH* overexpressing plants were found to be more susceptible to different strains of the bacterial plant pathogen *Pseudomonas syringae* compared to wild-type. The enhanced bacterial growth rate in Arabidopsis leaves also was associated with a reduction of multiple phytohormone levels. Free salicylic acid (SA) and conjugated forms were decreased by several fold compared to wild-type controls. Concordantly, SA-associated genes were also significantly downregulated in *FAAH* overexpressors. Similarly, jasmonic acid (JA) and several of its biosynthetic associated genes were reduced in *FAAH* overexpressing tissues. Abscisic acid (ABA) was another phytohormone that was found to be reduced in *FAAH* overexpressing lines. These indicate a potential interaction of NAE signaling in plant hormone regulation during biotic stress and that NAE metabolism by FAAH may participate in the modulation of plant immunity (Kang et al., 2008; Kim et al., 2009).

Although, the role(s) of NAEs in the influence of endogenous plant microbial populations remains unknown, promising evidence for NAE modulation of microbiomes has been recently described in mammals

(Fornelos & Franzosa, 2020). Tissues from inflammatory bowel disease patients had elevated amounts of NAE18:2 and NAE20:4, and this misregulation was shown to shift the composition of bacterial communities in humans. Further, metagenomic analysis revealed that *ex vivo* treatments with NAE18:2, NAE20:4, or a combination of both NAEs enhanced the growth of Proteobacteria (especially of Enterobacteriaceae) while depleting Bacteroidetes. While the exact mechanism by which bacteria process these host-derived NAEs is still unknown, transcriptomic analysis in Enterobacteriaceae communities revealed the need for elements of the respiratory and electron transfer chains for NAE metabolism (Fornelos & Franzosa, 2020). In this context, it is reasonable to speculate that an altered NAE content or composition may be associated with host-microbe communications in plants.

4. Future prospectives

While the advances in NAE-related research have produced meaningful insights regarding NAE-metabolism and regulation in plants, many open questions are yet to be addressed. For instance, does the discovery of structurally and phylogenetically different group I and II FAAHs in vascular plants greatly expand the types of lipophilic acyl amides (NAE-like structures) that could be potential substrates for FAAHs? Efforts to characterize these two distinct FAAH groups should be studied in systems beyond Arabidopsis. Extending the scope of research toward crops of agricultural impotence could lead to the discovery of previously unrecognized functions. Further, the notion that NAEs may also participate in endogenous microbial communications is an intriguing possibility that remains to be addressed. Certainly, the ability of AtFAAH to hydrolyze *N*-acyl-L-homoserine lactones (a quorum sensing molecule) supports a link between NAE and NAE-like structures and the potential for endogenous microbial signaling. Understanding the connection between plant-microbial communication and endogenous plant growth regulation is likely to provide much needed insights into the functional roles of NAEs, acylamides and their potential regulatory hydrolases—the FAAHs. Finally, the evolutionary conservation of NAE signaling in nonvascular plants and algae compared to vascular plants and nonplant systems seems to be an area open for exploration, especially with recent studies in *P. patens* for the occurrence of mammalian type NAE and FAAHs (Haq & Kilaru, 2020).

Acknowledgments

Work in the senior author's laboratory was supported initially by the US Dept of Energy, Office of Science under DE-FG02-05ER15647, and more recently by the National Science Foundation—Division of Integrative and Organismal Sciences (IOS-1656263).

References

Alharthi, N., Christensen, P., Hourani, W., Ortori, C., Barrett, D. A., Bennett, A. J., et al. (2018). n − 3 polyunsaturated N-acylethanolamines are CB2 cannabinoid receptor-preferring endocannabinoids. *Biochimica et Biophysica Acta (BBA)—Molecular and Cell Biology of Lipids*, *1863*, 1433–1440.

Alhouayek, M., Stafberg, L., Karlsson, J., Bergström, S. H., & Fowler, C. J. (2020). Effects of orthotopic implantation of rat prostate tumour cells upon components of the N-acylethanolamine and monoacylglycerol signalling systems: An mRNA study. *Scientific Reports*, *10*, 6314.

Andreou, A., & Feussner, I. (2009). Lipoxygenases—Structure and reaction mechanism. *Phytochemistry*, *70*, 1504–1510.

Austin-Brown, S. L., & Chapman, K. D. (2002). Inhibition of phospholipase D alpha by N-acylethanolamines. *Plant Physiology*, *129*, 1892–1898.

Aziz, M., & Chapman, K. D. (2020). Fatty acid amide hydrolases: An expanded capacity for chemical communication? *Trends in Plant Science*, *25*, 236–249.

Aziz, M., Wang, X., Tripathi, A., Bankaitis, V. A., & Chapman, K. D. (2019). Structural analysis of a plant fatty acid amide hydrolase provides insights into the evolutionary diversity of bioactive acylethanolamides. *The Journal of Biological Chemistry*, *294*, 7419–7432.

Balsevich, G., Sticht, M., Bowles, N. P., Singh, A., Lee, T. T. Y., Li, Z., et al. (2018). Role for fatty acid amide hydrolase (FAAH) in the leptin-mediated effects on feeding and energy balance. *Proceedings of the National Academy of Sciences of the United States of America*, *115*, 7605–7610.

Bannenberg, G., Martínez, M., Hamberg, M., & Castresana, C. (2009). Diversity of the enzymatic activity in the lipoxygenase gene family of Arabidopsis thaliana. *Lipids*, *44*, 85.

Blancaflor, E. B., Hou, G., & Chapman, K. D. (2003). Elevated levels of N-lauroylethanolamine, an endogenous constituent of desiccated seeds, disrupt normal root development in Arabidopsis thaliana seedlings. *Planta*, *217*, 206–217.

Blancaflor, E. B., Kilaru, A., Keereetaweep, J., Khan, B. R., Faure, L., & Chapman, K. D. (2014). N-Acylethanolamines: Lipid metabolites with functions in plant growth and development. *The Plant Journal*, *79*, 568–583.

Bluett, R. J., Gamble-George, J. C., Hermanson, D. J., Hartley, N. D., Marnett, L. J., & Patel, S. (2014). Central anandamide deficiency predicts stress-induced anxiety: Behavioral reversal through endocannabinoid augmentation. *Translational Psychiatry*, *4*, e408.

Bracey, M. H., Hanson, M. A., Masuda, K. R., Stevens, R. C., & Cravatt, B. F. (2002). Structural adaptations in a membrane enzyme that terminates endocannabinoid signaling. *Science*, *298*, 1793–1796.

Bruijnzeel, A. W., Qi, X., Guzhva, L. V., Wall, S., Deng, J. V., Gold, M. S., et al. (2016). Behavioral characterization of the effects of cannabis smoke and anandamide in rats. *PLoS One*, *11*, e0153327.

Bulat, E., & Garrett, T. A. (2011). Putative N-acylphosphatidylethanolamine synthase from Arabidopsis thaliana is a lysoglycerophospholipid acyltransferase. *The Journal of Biological Chemistry*, *286*, 33819–33831.

Cannon, A. E., & Chapman, K. D. (2021). Lipid signaling through G proteins. *Trends in Plant Science*, *26*, 720–728.

Cannon, A. E., Yan, C., Burks, D. J., Rao, X., Azad, R. K., & Chapman, K. D. (2020). Lipophilic signals lead to organ-specific gene expression changes in Arabidopsis seedlings. *Plant Direct*, *4*, e00242.

Chapman, K. D. (2004). Occurrence, metabolism, and prospective functions of N-acylethanolamines in plants. *Progress in Lipid Research*, *43*, 302–327.

Chapman, K. D., Lin, I., & DeSouza, A. D. (1995). Metabolism of cottonseed microsomal N-acylphosphatidylethanolamine. *Archives of Biochemistry and Biophysics*, *318*, 401–407.

Chapman, K. D., & Moore, T. S., Jr. (1993). Catalytic properties of a newly discovered acyltransferase that synthesizes N-acylphosphatidylethanolamine in cottonseed (Gossypium hirsutum L.) microsomes. *Plant Physiology*, *102*, 761.

Chapman, K. D., & Sprinkle, W. B. (1996). Developmental, tissue-specific, and environmental factors regulate the biosynthesis of N-acylphosphatidyl-ethanolamine in cotton (Gossypium hirsutum L.). *Journal of Plant Physiology*, *149*, 277–284.

Chapman, K. D., Tripathy, S., Venables, B., & Desouza, A. D. (1998). N-acylethanolamines: Formation and molecular composition of a new class of plant lipids. *Plant Physiology*, *116*, 1163–1168.

Chapman, K. D., Venables, B., Markovic, R., Blair, R. W., Jr., & Bettinger, C. (1999). N-acylethanolamines in seeds. Quantification of molecular species and their degradation upon imbibition. *Plant Physiology*, *120*, 1157–1164.

Chechetkin, I. R., Osipova, E. V., Antsygina, L. L., Gogolev, Y. V., & Grechkin, A. N. (2011). Oxidation of glycerolipids by maize 9-lipoxygenase and its A562G mutant. *Chemistry and Physics of Lipids*, *164*, 216–220.

Chilufya, J., Devaiah, S., Sante, R. R., & Kilaru, A. (2015). *Endocannabinoid-like lipids in plants*. John Wiley & Sons, Ltd.

Clapper, J. R., Moreno-Sanz, G., Russo, R., Guijarro, A., Vacondio, F., Duranti, A., et al. (2010). Anandamide suppresses pain initiation through a peripheral endocannabinoid mechanism. *Nature Neuroscience*, *13*, 1265–1270.

Cotter, M. Q., Teaster, N. D., Blancaflor, E. B., & Chapman, K. D. (2011). N-acylethanolamine (NAE) inhibits growth in Arabidopsis thaliana seedlings via ABI3-dependent and -independent pathways. *Plant Signaling & Behavior*, *6*, 671–679.

Dainese, E., & Oddi, S. (2020). The endocannabinoid hydrolase FAAH is an allosteric enzyme. *Scientific Reports*, *10*, 2292.

Dlugos, A., Childs, E., Stuhr, K. L., Hillard, C. J., & de Wit, H. (2012). Acute stress increases circulating anandamide and other N-acylethanolamines in healthy humans. *Neuropsychopharmacology: Official Publication of the American College of Neuropsychopharmacology*, *37*, 2416–2427.

Dosoky, N. S., Guo, L., Chen, Z., Feigley, A. V., & Davies, S. S. (2018). Dietary fatty acids control the species of N-acyl-phosphatidylethanolamines synthesized by therapeutically modified Bacteria in the intestinal tract. *ACS Infectious Diseases*, *4*, 3–13.

Faure, L., Cavazos, R., Khan, B. R., Petros, R. A., Koulen, P., Blancaflor, E. B., et al. (2015). Effects of synthetic alkamides on Arabidopsis fatty acid amide hydrolase activity and plant development. *Phytochemistry*, *110*, 58–71.

Faure, L., Coulon, D., Laroche-Traineau, J., Le Guedard, M., Schmitter, J. M., Testet, E., et al. (2009). Discovery and characterization of an Arabidopsis thaliana N-acylphosphatidylethanolamine synthase. *The Journal of Biological Chemistry*, *284*, 18734–18741.

Fornelos, N., & Franzosa, E. A. (2020). Growth effects of N-acylethanolamines on gut bacteria reflect altered bacterial abundances in inflammatory bowel disease. *Nature Microbiology*, *5*, 486–497.

Gabbs, M., & Leng, S. (2015). Advances in our understanding of oxylipins derived from dietary PUFAs. *Advances in Nutrition*, *6*, 513–540.

Gachet, M. S., Schubert, A., Calarco, S., Boccard, J., & Gertsch, J. (2017). Targeted metabolomics shows plasticity in the evolution of signaling lipids and uncovers old and new endocannabinoids in the plant kingdom. *Scientific Reports*, *7*, 41177.

Ghosh, A. K., Chauhan, N., Rajakumari, S., Daum, G., & Rajasekharan, R. (2009). At4g24160, a soluble acyl-coenzyme A-dependent lysophosphatidic acid acyltransferase. *Plant Physiology*, *151*, 869–881.

Gómez-Boronat, M., Isorna, E., Armirotti, A., Delgado, M. J., Piomelli, D., & de Pedro, N. (2019). Diurnal profiles of N-acylethanolamines in goldfish brain and gastrointestinal tract: Possible role of feeding. *Frontiers in Neuroscience*, *13*, 450.

Hansen, H. S., Kleberg, K., & Hassing, H. A. (2015). Chapter 1—Non-endocannabinoid N-acylethanolamines and monoacylglycerols: Old molecules new targets. In V. Di Marzo, & J. Wang (Eds.), *The endocannabinoidome* (pp. 1–13). Boston: Academic Press.

Haq, I., & Kilaru, A. (2020). An endocannabinoid catabolic enzyme FAAH and its paralogs in an early land plant reveal evolutionary and functional relationship with eukaryotic orthologs. *Scientific Reports*, *10*, 3115.

Hayward, S., Cilliers, T., & Swart, P. (2017). Lipoxygenases: From isolation to application. *Comprehensive Reviews in Food Science and Food Safety*, *16*, 199–211.

Howlett, A. C., Blume, L. C., & Dalton, G. D. (2010). CB(1) cannabinoid receptors and their associated proteins. *Current Medicinal Chemistry*, *17*, 1382–1393.

Hussain, Z., Uyama, T., Tsuboi, K., & Ueda, N. (2017). Mammalian enzymes responsible for the biosynthesis of N-acylethanolamines. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids*, *1862*, 1546–1561.

Iqbal, S., Ali, U., Fadlalla, T., Li, Q., Liu, H., Lu, S., et al. (2020). Genome wide characterization of phospholipase A & C families and pattern of lysolipids and diacylglycerol changes under abiotic stresses in Brassica napus L. *Plant Physiology and Biochemistry*, *147*, 101–112.

James, C. N., Horn, P. J., Case, C. R., Gidda, S. K., Zhang, D., Mullen, R. T., et al. (2010). Disruption of the Arabidopsis CGI-58 homologue produces Chanarin-Dorfman-like lipid droplet accumulation in plants. *Proceedings of the National Academy of Sciences of the United States of America*, *107*, 17833–17838.

Janfelt, C., Wellner, N., Leger, P. L., Kokesch-Himmelreich, J., Hansen, S. H., Charriaut-Marlangue, C., et al. (2012). Visualization by mass spectrometry of 2-dimensional changes in rat brain lipids, including N-acylphosphatidylethanolamines, during neonatal brain ischemia. *The FASEB Journal*, *26*, 2667–2673.

Kang, L., Wang, Y. S., Uppalapati, S. R., Wang, K., Tang, Y., Vadapalli, V., et al. (2008). Overexpression of a fatty acid amide hydrolase compromises innate immunity in Arabidopsis. *The Plant Journal*, *56*, 336–349.

Keereetaweep, J., Blancaflor, E. B., Hornung, E., Feussner, I., & Chapman, K. D. (2013). Ethanolamide oxylipins of linolenic acid can negatively regulate Arabidopsis seedling development. *Plant Cell*, *25*, 3824–3840.

Keereetaweep, J., Blancaflor, E. B., Hornung, E., Feussner, I., & Chapman, K. D. (2015). Lipoxygenase-derived 9-hydro(pero)xides of linoleoylethanolamide interact with ABA signaling to arrest root development during Arabidopsis seedling establishment. *The Plant Journal*, *82*, 315–327.

Keereetaweep, J., & Chapman, K. D. (2016). Lipidomic analysis of endocannabinoid signaling: Targeted metabolite identification and quantification. *Neural Plasticity*, *2016*, 2426398.

Keereetaweep, J., Kilaru, A., Feussner, I., Venables, B. J., & Chapman, K. D. (2010). Lauroylethanolamide is a potent competitive inhibitor of lipoxygenase activity. *FEBS Letters*, *584*, 3215–3222.

Khan, B. R., Chapman, K. D., & Blancaflor, E. B. (2021). Chemical genetics to uncover mechanisms underlying lipid-mediated signaling events in plants. *Methods in Molecular Biology*, *2213*, 3–16.

Khan, B. R., Wherritt, D. J., Huhman, D., Sumner, L. W., Chapman, K. D., & Blancaflor, E. B. (2016). Malonylation of glucosylated N-lauroylethanolamine: A new pathway that determines N-acylethanolamine metabolic fate in plants. *The Journal of Biological Chemistry*, *291*, 27112–27121.

Kilaru, A., & Chapman, K. D. (2020). The endocannabinoid system. *Essays in Biochemistry*, *64*, 485–499.

Kilaru, A., Herrfurth, C., Keereetaweep, J., Hornung, E., Venables, B. J., Feussner, I., et al. (2011). Lipoxygenase-mediated oxidation of polyunsaturated N-acylethanolamines in Arabidopsis. *The Journal of Biological Chemistry*, *286*, 15205–15214.

Kilaru, A., Tamura, P., Isaac, G., Welti, R., Venables, B. J., Seier, E., et al. (2012). Lipidomic analysis of N-acylphosphatidylethanolamine molecular species in Arabidopsis suggests feedback regulation by N-acylethanolamines. *Planta*, *236*, 809–824.

Kim, S. C., Kang, L., Nagaraj, S., Blancaflor, E. B., Mysore, K. S., & Chapman, K. D. (2009). Mutations in Arabidopsis fatty acid amide hydrolase reveal that catalytic activity influences growth but not sensitivity to abscisic acid or pathogens. *The Journal of Biological Chemistry*, *284*, 34065–34074.

Kotel'nikova, I. M. (2011). The content of N-acylphosphatidylethanolamines in the seeds of cultivated plants and grain products. *Russian Journal of Plant Physiology*, *58*, 364–369.

Lin, L., Metherel, A. H., Jones, P. J., & Bazinet, R. P. (2017). Fatty acid amide hydrolase (FAAH) regulates hypercapnia/ischemia-induced increases in n-acylethanolamines in mouse brain. *Journal of Neurochemistry*, *142*, 662–671.

Liu, J., Wang, L., Harvey-White, J., Osei-Hyiaman, D., Razdan, R., Gong, Q., et al. (2006). A biosynthetic pathway for anandamide. *Proceedings of the National Academy of Sciences of the United States of America*, *103*, 13345–13350.

Long, J. Z., LaCava, M., Jin, X., & Cravatt, B. F. (2011). An anatomical and temporal portrait of physiological substrates for fatty acid amide hydrolase. *Journal of Lipid Research*, *52*, 337–344.

Lowin, T., Apitz, M., Anders, S., & Straub, R. H. (2015). Anti-inflammatory effects of N-acylethanolamines in rheumatoid arthritis synovial cells are mediated by TRPV1 and TRPA1 in a COX-2 dependent manner. *Arthritis Research & Therapy*, *17*, 321.

Maccarrone, M. (2017). Metabolism of the endocannabinoid anandamide: Open questions after 25 years. *Frontiers in Molecular Neuroscience*, *10*, 166.

Magotti, P., Bauer, I., Igarashi, M., Babagoli, M., Marotta, R., Piomelli, D., et al. (2015). Structure of human N-acylphosphatidylethanolamine-hydrolyzing phospholipase D: Regulation of fatty acid ethanolamide biosynthesis by bile acids. *Structure*, *23*, 598–604.

Mahavadi, S., Sriwai, W., Huang, J., Grider, J. R., & Murthy, K. S. (2014). Inhibitory signaling by CB1 receptors in smooth muscle mediated by GRK5/β-arrestin activation of ERK1/2 and Src kinase. *American Journal of Physiology. Gastrointestinal and Liver Physiology*, *306*, G535–G545.

McKinney, M. K., & Cravatt, B. F. (2003). Evidence for distinct roles in catalysis for residues of the serine-serine-lysine catalytic triad of fatty acid amide hydrolase. *The Journal of Biological Chemistry*, *278*, 37393–37399.

McKinney, M. K., & Cravatt, B. F. (2005). Structure and function of fatty acid amide hydrolase. *Annual Review of Biochemistry*, *74*, 411–432.

Motes, C. M., Pechter, P., Yoo, C. M., Wang, Y. S., Chapman, K. D., & Blancaflor, E. B. (2005). Differential effects of two phospholipase D inhibitors, 1-butanol and N-acylethanolamine, on in vivo cytoskeletal organization and Arabidopsis seedling growth. *Protoplasma*, *226*, 109–123.

Neelamegan, D., Schoenhofen, I. C., Richards, J. C., & Cox, A. D. (2012). Identification and recombinant expression of anandamide hydrolyzing enzyme from Dictyostelium discoideum. *BMC Microbiology*, *12*, 124.

Ogura, Y., Parsons, W. H., Kamat, S. S., & Cravatt, B. F. (2016). A calcium-dependent acyltransferase that produces N-acyl phosphatidylethanolamines. *Nature Chemical Biology*, *12*, 669–671.

Ortíz-Castro, R., Martínez-Trujillo, M., & López-Bucio, J. (2008). N-acyl-L-homoserine lactones: A class of bacterial quorum-sensing signals alter post-embryonic root development in Arabidopsis thaliana. *Plant, Cell & Environment*, *31*, 1497–1509.

Pacioni, G., Rapino, C., Zarivi, O., Falconi, A., Leonardi, M., Battista, N., et al. (2015). Truffles contain endocannabinoid metabolic enzymes and anandamide. *Phytochemistry*, *110*, 104–110.

Palermo, G., Bauer, I., Campomanes, P., Cavalli, A., Armirotti, A., Girotto, S., et al. (2015). Keys to lipid selection in fatty acid amide hydrolase catalysis: Structural flexibility, gating residues and multiple binding pockets. *PLoS Computational Biology*, *11*, e1004231.

Palmer, A. G., Senechal, A. C., Mukherjee, A., Ané, J. M., & Blackwell, H. E. (2014). Plant responses to bacterial N-acyl L-homoserine lactones are dependent on enzymatic degradation to L-homoserine. *ACS Chemical Biology*, *9*, 1834–1845.

Pappan, K., Austin-Brown, S., Chapman, K. D., & Wang, X. (1998). Substrate selectivities and lipid modulation of plant phospholipase Dα, −β, and -γ. *Archives of Biochemistry and Biophysics*, *353*, 131–140.

Park, S., Gidda, S. K., James, C. N., Horn, P. J., Khuu, N., Seay, D. C., et al. (2013). The α/β hydrolase CGI-58 and peroxisomal transport protein PXA1 coregulate lipid homeostasis and signaling in Arabidopsis. *Plant Cell*, *25*, 1726–1739.

Qin, W., Pappan, K., & Wang, X. (1997). Molecular heterogeneity of phospholipase D (PLD). Cloning of PLDgamma and regulation of plant PLDgamma, −beta, and -alpha by polyphosphoinositides and calcium. *The Journal of Biological Chemistry*, *272*, 28267–28273.

Ramírez-Chávez, E., López-Bucio, J., Herrera-Estrella, L., & Molina-Torres, J. (2004). Alkamides isolated from plants promote growth and alter root development in Arabidopsis. *Plant Physiology*, *134*, 1058–1068.

Rawyler, A. J., & Braendle, R. A. (2001). N-Acylphosphatidylethanolamine accumulation in potato cells upon energy shortage caused by anoxia or respiratory inhibitors. *Plant Physiology*, *127*, 240–251.

Rettori, E., De Laurentiis, A., Zorrilla Zubilete, M., Rettori, V., & Elverdin, J. C. (2012). Anti-inflammatory effect of the endocannabinoid anandamide in experimental periodontitis and stress in the rat. *Neuroimmunomodulation*, *19*, 293–303.

Samson, M.-T., Small-Howard, A., Shimoda, L. M. N., Koblan-Huberson, M., Stokes, A. J., & Turner, H. (2003). Differential roles of CB1 and CB2 cannabinoid receptors in mast cells. *The Journal of Immunology*, *170*, 4953–4962.

Sandoval, J. A., Huang, Z. H., Garrett, D. C., Gage, D. A., & Chapman, K. D. (1995). N-acylphosphatidylethanolamine in dry and imbibing cottonseeds (amounts, molecular species, and enzymatic synthesis). *Plant Physiology*, *109*, 269–275.

Shrestha, R., Dixon, R. A., & Chapman, K. D. (2003). Molecular identification of a functional homologue of the mammalian fatty acid amide hydrolase in Arabidopsis thaliana. *The Journal of Biological Chemistry*, *278*, 34990–34997.

Shrestha, R., Kim, S. C., Dyer, J. M., Dixon, R. A., & Chapman, K. D. (2006). Plant fatty acid (ethanol) amide hydrolases. *Biochimica et Biophysica Acta*, *1761*, 324–334.

Shrestha, R., Noordermeer, M. A., van der Stelt, M., Veldink, G. A., & Chapman, K. D. (2002). N-acylethanolamines are metabolized by lipoxygenase and amidohydrolase in competing pathways during cottonseed imbibition. *Plant Physiology*, *130*, 391–401.

Simon, G. M., & Cravatt, B. F. (2006). Endocannabinoid biosynthesis proceeding through glycerophospho-N-acyl ethanolamine and a role for alpha/beta-hydrolase 4 in this pathway. *The Journal of Biological Chemistry*, *281*, 26465–26472.

Simon, G. M., & Cravatt, B. F. (2008). Anandamide biosynthesis catalyzed by the phosphodiesterase GDE1 and detection of glycerophospho-N-acyl ethanolamine precursors in mouse brain. *The Journal of Biological Chemistry*, *283*, 9341–9349.

Strittmatter, F., Gandaglia, G., Benigni, F., Bettiga, A., Rigatti, P., Montorsi, F., et al. (2012). Expression of fatty acid amide hydrolase (FAAH) in human, mouse, and rat urinary bladder and effects of FAAH inhibition on bladder function in awake rats. *European Urology*, *61*, 98–106.

Sun, Y. X., Tsuboi, K., Okamoto, Y., Tonai, T., Murakami, M., Kudo, I., et al. (2004). Biosynthesis of anandamide and N-palmitoylethanolamine by sequential actions of phospholipase A2 and lysophospholipase D. *The Biochemical Journal*, *380*, 749–756.

Teaster, N. D., Keereetaweep, J., Kilaru, A., Wang, Y. S., Tang, Y., Tran, C. N., et al. (2012). Overexpression of fatty acid amide hydrolase induces early flowering in Arabidopsis thaliana. *Frontiers in Plant Science*, *3*, 32.

Teaster, N. D., Motes, C. M., Tang, Y., Wiant, W. C., Cotter, M. Q., Wang, Y. S., et al. (2007). N-Acylethanolamine metabolism interacts with abscisic acid signaling in Arabidopsis thaliana seedlings. *Plant Cell*, *19*, 2454–2469.

Tripathy, S., Kleppinger-Sparace, K., Dixon, R. A., & Chapman, K. D. (2003). N-Acylethanolamine signaling in tobacco is mediated by a membrane-associated, high-affinity binding protein. *Plant Physiology*, *131*, 1781.

Tripathy, S., Venables, B. J., & Chapman, K. D. (1999). N-Acylethanolamines in signal transduction of elicitor perception. Attenuation of alkalinization response and activation of defense gene expression. *Plant Physiology*, *121*, 1299–1308.

Ueda, N., Tsuboi, K., & Uyama, T. (2013). Metabolism of endocannabinoids and related N-acylethanolamines: Canonical and alternative pathways. *The FEBS Journal*, *280*, 1874–1894.

Venables, B. J., Waggoner, C. A., & Chapman, K. D. (2005). N-acylethanolamines in seeds of selected legumes. *Phytochemistry*, *66*, 1913–1918.

Wang, Y. S., Shrestha, R., Kilaru, A., Wiant, W., Venables, B. J., Chapman, K. D., et al. (2006). Manipulation of Arabidopsis fatty acid amide hydrolase expression modifies plant growth and sensitivity to N-acylethanolamines. *Proceedings of the National Academy of Sciences of the United States of America*, *103*, 12197–12202.

Wellner, N., Diep, T. A., Janfelt, C., & Hansen, H. S. (2013). N-acylation of phosphatidylethanolamine and its biological functions in mammals. *Biochimica et Biophysica Acta (BBA)—Molecular and Cell Biology of Lipids*, *1831*, 652–662.

Yan, C., Cannon, A., Watkins, J., Keereetaweep, J., Khan, B. R., Jones, A. M., et al. (2020). Seedling chloroplast responses induced by N-linolenoylethanolamine require intact G-protein complexes. *Plant Physiology*, *184*, 01552.02019.

Zhang, C., & Tian, S. (2010). Peach fruit acquired tolerance to low temperature stress by accumulation of linolenic acid and N-acylphosphatidylethanolamine in plasma membrane. *Food Chemistry*, *120*, 864–872.

Zhang, B., Wang, Y., & Liu, J. Y. (2018). Genome-wide identification and characterization of phospholipase C gene family in cotton (Gossypium spp.). *Science China. Life Sciences*, *61*, 88–99.

Zhang, D. D., Wang, J., Wang, D., Kong, Z. Q., Zhou, L., Zhang, G. Y., et al. (2019). Population genomics demystifies the defoliation phenotype in the plant pathogen Verticillium dahliae. *The New Phytologist*, *222*, 1012–1029.

Zhu, J., Zhou, Y., Li, J., & Li, H. (2020). Genome-wide investigation of the phospholipase C gene family in Zea mays. *Frontiers in Genetics*, *11*, 611414.