Trends in Plant Science

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Opinion

Fatty Acid Amide Hydrolases: An Expanded Capacity for Chemical Communication?

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Fatty acid amide hydrolase (FAAH) is an enzyme that belongs to the amidase signature (AS) superfamily and is widely distributed in multicellular eukaryotes. FAAH hydrolyzes lipid signaling molecules – namely, *N*-acylethanolamines (NAEs) – which terminates their actions. Recently, the crystal structure of *Arabidopsis thaliana* FAAH was solved and key residues were identified for substrate-specific interactions. Here, focusing on residues surrounding the substrate-binding pocket, a comprehensive analysis of FAAH sequences from angiosperms reveals a distinctly different family of FAAH-like enzymes. We hypothesize that FAAH, in addition to its role in seedling development, also acts in an *N*-acyl amide communication axis to facilitate plant–microbe interactions and that structural diversity provides for the flexible use of a wide range of small lipophilic signaling molecules.

Highlights

Fatty acid amide hydrolase (FAAH) is the signal-terminating enzyme of the *N*-acylethanolamine signaling pathway with an established role in seedling development.

The crystal structure of *Arabidopsis thaliana* FAAH was recently solved, revealing for the first time the structural features of FAAH from plants and explaining the enzyme's promiscuity toward *N*-acyl amide substrates.

A second group of FAAH enzymes in angiosperms has been identified with conserved substitutions in the substrate-binding pocket altering the size, shape, and physicochemical properties for substrate recognition.

FAAH Is the Signal-Terminating Enzyme of the NAE Signaling Pathway

The AS superfamily is a large group of hydrolytic enzymes that catalyze the hydrolysis of the C–N bond of various amides to their corresponding carboxylic acid and amine (or ammonia) products. The AS enzymes share strong sequence homology over a serine- and glycine-rich stretch of ≈130 amino acids, designated as the AS sequence, which includes a highly conserved Ser-*cis*Ser-Lys catalytic triad [1,2]. This family of enzymes is distinct from the 'classical' serine proteases, as well as some esterases and lipases, wherein the active site is a Ser-His-Asp catalytic triad [3]. Members of the AS superfamily are distributed throughout prokaryotes and eukaryotes and have diverged widely in terms of substrate specificity and function. For instance, in addition to **FAAH** (see Glossary), which hydrolyzes **NAEs**, this group of enzymes includes the following: subunit A of the heteromeric enzyme glutamyl-tRNA^{Gln} amidotransferase, involved in the formation of correctly charged Gln-tRNA^{Gln} through the transamidation of misacylated Glu-tRNA^{Gln} [4]; allophanate hydrolase, which converts allophanate to ammonium and carbon dioxide and is essential for the utilization of urea as a nitrogen source by many organisms [5]; peptide amidase (PAM), which selectively catalyzes the hydrolysis of the C-terminal amide bond of peptides [6]; the bacterial malonamidase E2 (MAE2) from *Bradyrhizobium japonicum*, which catalyzes the hydrolysis of malonamate to malonate and ammonia and is involved in the transport of fixed nitrogen from bacteroids to plant cells in symbiotic nitrogen metabolism [7]; and the plant amidase 1 (AMI1), involved in the biosynthesis of the plant growth hormone auxin from indole-3-acetamide [8].

FAAH is unique among the AS enzymes in that it is an integral membrane protein, a feature that is essential for the enzyme to have access to its lipophilic acylethanolamide substrates that are often derived from membrane phospholipid precursors. FAAH hydrolysis of NAEs into corresponding free fatty acid and ethanolamine products is a key step in NAE signaling, a mechanism that in all organisms studied to date terminates the regulatory functions of NAE [2]. However, there are interesting similarities and differences between animals and plants in the interaction between FAAH and its substrates.

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NAE signaling is a ubiquitous lipid signaling pathway that regulates a wide array of physiological processes in multicellular eukaryotes [9]. There are structural differences in the types of NAE molecules employed for signaling among different groups of organisms, but FAAHs from all organisms appear to be promiscuous in their ability to hydrolyze NAEs with a wide range of acyl moieties (varying chain lengths and degrees of unsaturation). For example, in mammalian systems the ethanolamide conjugate of arachidonic acid (known as anandamide; Figure 1) is primarily the bioactive molecule of the endocannabinoid signaling pathway. Anandamide binds to membrane-bound cannabinoid receptors (CB1 and CB2) to regulate many behavioral and physiological processes in vertebrates, including pain, cognition, memory, inflammation, and others [10]. However, in addition to anandamide, mammalian FAAH also hydrolyzes NAE 16:0 (see Glossary for the **numerical designation of acyl chains**), NAE 18:1, and NAE 18:2 (Figure 1), all of which are CB receptor inactive and occur at much higher endogenous concentrations than anandamide (NAE 20:4). This leads to an intriguing 'entourage effect' in cells whereby anandamide levels are influenced by the overall competing pool of NAEs available to FAAH [11]. By contrast, in higher plants there is essentially no arachidonic acid and NAEs with 18C acyl chains are the most abundant. In addition, the bioactive NAEs that influence seedling development are **oxylipin** metabolites of polyunsaturated NAEs [e.g., hydro(pero)xy derivatives of NAE 18:2 and NAE 18:3; Figure 1] rather than their parent, unsubstituted NAEs [12,13]. Surprisingly, *Arabidopsis thaliana* FAAH hydrolyzes both hydroxylated and unsubstituted NAE 18:2 with equal efficiency [14], but the unsubstituted NAE 18:2 occurs at much higher endogenous concentrations than the oxylipin derivative. Thus, in plants, as in animals, an entourage effect of competing NAE types may influence NAE signaling. Nonetheless, it is clear that plants and animals utilize different types of NAE molecules for signaling activity [15,16].

Structural and Functional Differences in Plant and Mammalian FAAHs

The 3D structures of mammalian [17] and plant [14] FAAHs demonstrate how organizational differences in the acyl-binding pocket account for both the differences in the types of bioactive NAEs in plants and animals and the substrate promiscuity of both plant and mammalian FAAHs.

Structures of both animal and plant FAAHs include five key regions that are important for the function of the enzyme: (i) the membrane-binding cap, which is formed by a helix-turn-helix motif that is presumed to anchor the enzyme into the cytoplasmic leaflet of the lipid bilayer; (ii) the membrane-access channel (MAC), where the lipophilic substrates access the active site from the membrane; (iii) the substrate-binding pocket or acyl-binding channel (ABC), where the acyl chain of the substrate resides during catalysis; (iv) the active site, which is located deep at the bottom of the substrate-binding pocket with the highly conserved catalytic triad of residues (Ser-*cis*Ser-Lys); and (v) the cytosolic access channel, which is a putative exit path for the polar head group of the substrate as it leaves the active site after hydrolysis and an entry path for the water molecule required for the deacylation of the FAAH-acyl intermediate (see [14,17] for specific details).

Plant FAAHs have evolved several structural modifications to accommodate the hydrolysis of a wide range of NAEs, including both unsubstituted and oxygenated substrates as a means of regulating NAE signaling in plants. In particular, this includes a more open and polar substrate-binding pocket, that also functions as a MAC, with a marked degree of flexibility to support a 'squeeze and lock' substrate-binding mechanism [14]. These features are structurally and functionally different from that of mammalian FAAH, which possesses a less open and more nonpolar substrate-binding pocket that is separated from the MAC by a large aromatic/hydrophobic 'dynamic paddle' [17]. These structural alterations and diversification in substrate specificity in plant FAAHs represent an important evolutionary adaptation of NAE signaling in plants [14] and

Glossary

***N*-Aryl L-homoserine lactones (aryl HLs):** a group of HLs that are produced by some photosynthetic bacteria and contain an aromatic tail instead of the 'canonical' aliphatic acyl chains in AHLs.
Fatty acid amide hydrolase (FAAH): enzymes that are best known for their role in terminating NAE signaling pathways in multicellular eukaryotes.
Gating residues: group of amino acid residues located at the N terminus of the arabidopsis FAAH, where they define the opening of the MAC and were shown to undergo conformational changes and close the MAC of AtFAAH on ligand binding.
***N*-Acylethanolamines (NAEs):** a family of functionally diverse signaling lipids. They comprise a fatty acid linked by an amide bond to ethanolamine and are classified based on the chain length and degree of unsaturation of their acyl chain.
***N*-Acyl L-homoserine lactones (AHLs):** a class of QS signals produced by Gram-negative bacteria to coordinate their group activity based on the population density. They comprise an acyl chain linked by an amide bond to a homoserine lactone head group.
Numerical designation of acyl chains: the NAE abbreviation includes two numbers *x*:*y*, where *x* indicates the number of carbons and *y* indicates the number of double bonds in the corresponding acyl chain. For example, NAE 16:0 is an NAE with a 16-carbon acyl chain that has no double bonds, while NAE 18:2 is an NAE with an 18-carbon acyl chain that has two double bonds.
Oxylipins: bioactive lipid metabolites that are derived from polyunsaturated fatty acids or polyunsaturated NAEs (e.g., NAE 18:2, NAE 18:3) via the actions of a family of dioxygenases named lipoxygenases (9-LOX and 13-LOX), which introduce hydro(pero)xy functional groups at the C9 or C13 position of the acyl chain.

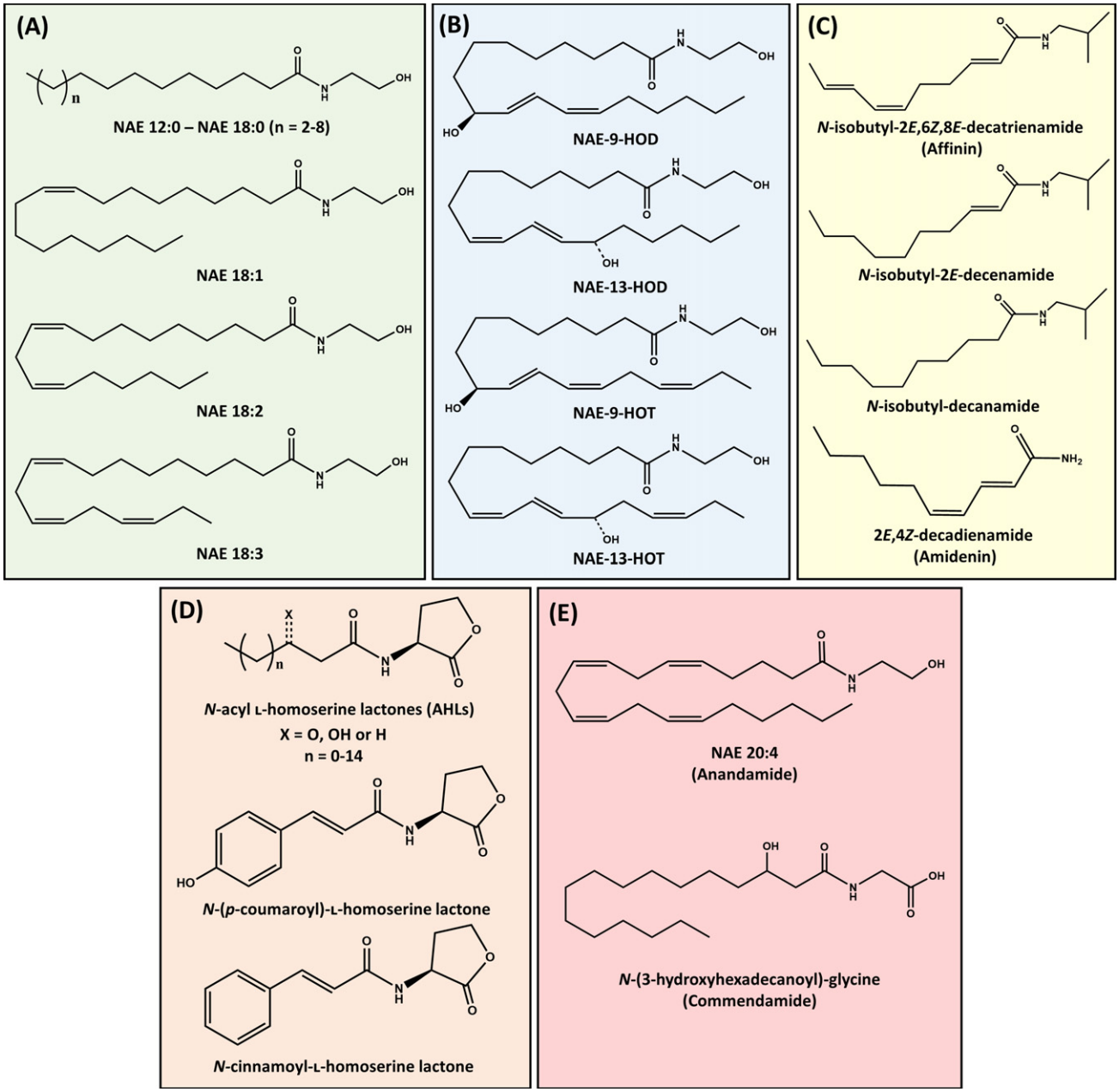

Figure 1. Chemical Structures of the Different Classes of Lipophilic Amides. (A) The common *N*-acylethanolamines (NAEs) that occur in angiosperms differ in the length and degree of unsaturation of their acyl chain. (B) Chemical structures of the oxylipin derivatives of NAE 18:2 and NAE 18:3 in plants. These hydroxide derivatives are reduced from the corresponding hydroperoxides generated by lipoxygenases. NAE-9-HOD, (9*S*,12*Z*,10*E*)-9-hydroxy-10,12-octadecadienoylethanolamide; NAE-13-HOD, (13*S*,9*Z*,11*E*)-13-hydroxy-9,11-octadecadienoylethanolamide; NAE-9-HOT, (9*S*,12*Z*,10*E*,15*Z*)-9-hydroxy-10,12,15-octadecatrienoylethanolamide; NAE-13-HOT, (13*S*,9*Z*,11*E*,15*Z*)-13-hydroxy-9,11,15-octadecatrienoylethanolamide (adapted from [12,13]). (C) Structures of selected alkamides with plant-growth-modulating effects. The first three alkamides with isobutyl head groups are of plant origin (adapted from [23]), while amidenin is from the actinomycete *Amycolatopsis* sp. [24]. (D) The aliphatic and aryl homoserine lactones (HLs). The aliphatic HLs have an acyl chain that ranges from four to 18 carbons in length and with different substitutions at position 3 (adapted from [18]). The aryl HLs include the *p*-coumaroyl and cinnamoyl derivatives isolated from the photosynthetic bacteria *Rhodopseudomonas palustris* [31] and *Bradyrhizobium* ORS278 [32], respectively. (E) Anandamide (NAE 20:4) is the major bioactive component of the endocannabinoid signaling pathway in animal systems, while commendamide is an *N*-(3-hydroxypalmitoyl)glycine derivative that is produced by human commensal bacteria and was shown to mimic the endogenous *N*-acyl amides and activate G protein-coupled receptors (GPCRs) in humans [43].

appear to have allowed expanded substrate recognition by AtFAAH to include the bacterial signals ***N*-acyl L-homoserine lactones (AHLs)** [18] (Figure 1).

Discovery of a New Group of FAAH-like Sequences in Plant Genomes

Expanded *in silico* analysis of a large group of FAAH amino acid sequences (*ca.* 88 sequences) reveals that FAAHs in angiosperms can be divided into two distinct groups, one group that includes arabidopsis FAAH (named group I here) and another group of FAAH-like enzymes (named group II here) (Figure 2). These two FAAH groups are delineated by conserved substitutions in amino acid residues surrounding the substrate-binding pocket and the cytosolic access channel, whereas the other structural features remain unchanged between the two groups (Figure 3). In general, the residues in group II FAAHs that surround the substrate-binding pocket are mostly nonpolar (as opposed to polar residues in group I) and include several bulkier aromatic residues in the ligand-binding site. This more hydrophobic substrate-binding pocket of group II FAAHs is more reminiscent of mammalian FAAH. Also different from group I FAAHs, residues predicted to form the cytosolic access channel are more nonpolar in group II FAAHs. These conserved differences between the two groups of FAAHs suggest that this new family of enzymes (group II) is likely to have unique substrate profiles, different from group I.

The phylogenetic analysis of these plant FAAHs shows that the Brassicaceae plants (e.g., *A. thaliana*, *Brassica napus*, *Camelina sativa*) and castor (*Ricinus communis*) have only group I FAAHs. By contrast, members of both groups of FAAH are found in all of the other dicot and monocot species examined, including *Amborella trichopoda*, which has been suggested to be the evolutionary ancestor of all flowering plants [19]. The presence of both FAAH groups in the *A. trichopoda* genome suggests that this bifurcation in plant FAAHs predates angiosperms, and probably *A. thaliana* and its relatives have lost the orthologs of group II FAAHs during evolution. Notably, group I FAAHs are more represented in some plant taxa (e.g., solanaceous plants), whereas other taxa (e.g., *Gossypium*, the leguminous plants) have more group II FAAHs than group I (Figure 2).

Detailed analysis of these FAAH sequences shows that this new group of FAAHs (group II) retains the highly conserved catalytic triad (Ser-*cis*Ser-Lys) of the AS superfamily but differs in several specific aspects from AtFAAH (group I). To gain comparative insights into the structural features of group II FAAHs, homology modeling was conducted for both group I and group II FAAHs from soybean (*Glycine max*) using the AtFAAH crystal structure as a template (PDB: 6DHV [14]; Figure 3A). The 3D structural models of both GmFAAH-I and GmFAAH-II retain the overall structural features of AtFAAH, including the membrane-binding cap, the MAC, the substrate-binding pocket, and the cytosolic access channel (Figure 3A). The structural models do not show much difference in the membrane-binding cap and the membrane access regions between the two groups, which was expected from the primary sequence analyses since the groups share the long N-terminal region and conserved **'gating' residues** [14]. However, the residues that line the substrate-binding pocket and the cytosolic access channel reveal some key differences between the two groups. For example, the two polar residues Ser^{471} and Thr^{534} in the substrate-binding pocket of GmFAAH-I are substituted by nonpolar residues (Ala^{473} and Gly^{536}, respectively) in GmFAAH-II (Figure 3A). These substitutions impart less polarity to the substrate-binding pocket of group II FAAHs at these two positions, which correspond to Ser^{472} and Thr^{535}, respectively, in AtFAAH; these two residues in AtFAAH were shown in computational docking experiments and molecular dynamics simulations to be involved in the interaction with NAE-oxylipins [14]. Another set of substitutions in the substrate-binding pockets of the two groups include the presence of three tyrosine residues (Tyr^{444}, Tyr^{477}, and Tyr^{533}) in GmFAAH-II in place of Val^{441}, Phe^{475}, and Met^{531}, respectively, in GmFAAH-I (Figure 3A). Comparison of the

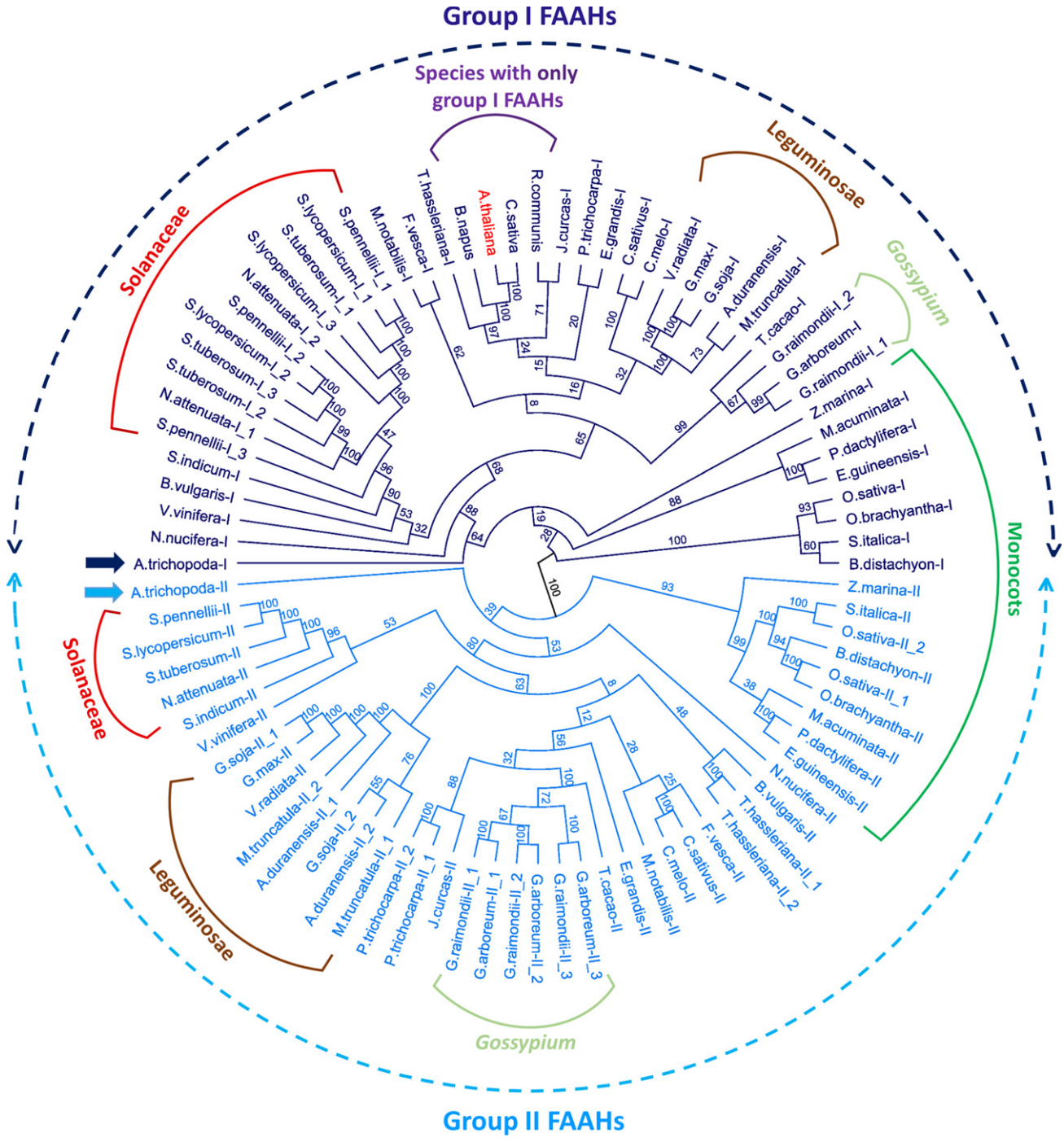

Figure 2. Phylogenetic Analysis of Fatty Acid Amide Hydrolase (FAAH) Proteins in Angiosperms. The enzymes are divided into two groups; AtFAAH (highlighted in red) is in the original group of FAAHs (group I; dark blue), while the new group was named group II (light blue). The FAAHs from *Amborella trichopoda* (the evolutionary ancestor of all angiosperms [19]) are marked with arrows. Eighty-eight FAAH amino acid sequences from angiosperms were obtained from GenBank and included in the phylogenetic analysis. The phylogenetic tree was generated using Geneious 10.0.3 software with the PhyML plugin, with the following settings: JTT substitution model, 100 bootstraps, four substitution rate categories, estimated gamma distribution parameter, estimated proportion of invariable sites, and optimized tree topology, branch length, and substitution rate, with best of nearest neighbor interchanges (NNI) and subtree pruning and regrafting (SPR) topology search.

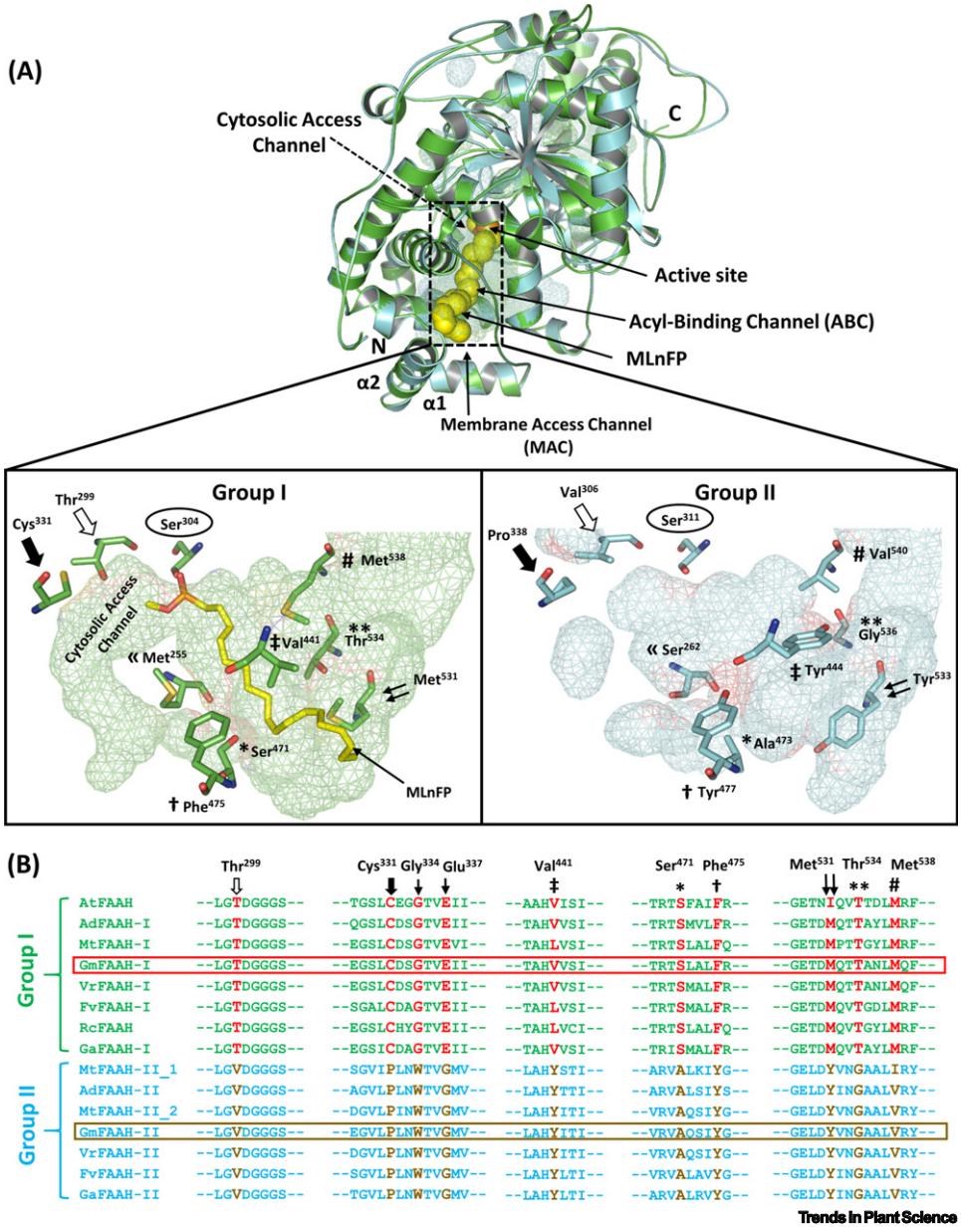

Figure 3. Structural Models of Group I and Group II Fatty Acid Amide Hydrolases (FAAHs) from Soybean (*Glycine max*). (A) An overlay of the overall structures of GmFAAH-I (green) and GmFAAH-II (cyan) with the N and C termini indicated. The models were generated using the Swiss-Model server [46] with one subunit of the Apo-AtFAAH crystal structure (PDB: 6DHV [14]) as a template. The coordinates of the irreversible inhibitor (substrate analog) methyl α-linolenyl fluorophosphonate (MLnFP) were introduced from the ligand-bound AtFAAH crystal structure (PDB: 6DII [14]). The mesh rendering indicates the protein cavity surface. The hydrophobic helices α1 and α2 at the N terminus are predicted to form the membrane-binding cap of plant FAAHs. The two main cavities that represent the substrate-binding pocket and the cytosolic access channel are enlarged with the surface rendered partially transparent to demonstrate the key differences between the two groups of FAAHs. The Ser-*cis*Ser-Lys catalytic triad residues are conserved in the two groups of FAAHs, but only the nucleophilic serine is shown for simplification (Ser^{304} and Ser^{311} in groups I and II, respectively). Each different amino acid in group II is marked with the same symbol as its counterpart in group I for clarification. (B) Alignment of the amino acid sequences of several FAAHs from group I (green) and group II (light blue),

(Figure legend continued at the bottom of the next page.)

amino acid sequences indicates that most of these amino acid substitutions are conserved among FAAHs in each group (Figure 3B). Importantly, these overall amino changes result in differently shaped substrate-binding cavities with different physicochemical properties in the two groups of FAAH (Figure 3A), which are likely to accommodate different potential substrates.

In addition to the differences between group I and II FAAHs in the substrate-binding pocket, other differences are observed in the cytosolic access channel. The cytosolic access channel in AtFAAH (group I) is mainly hydrophilic, presumably to facilitate the release of the ethanolamine head group after hydrolysis, and this includes Thr^{300} and Cys^{332}. These two polar residues are conserved in other group I FAAHs (shown as Thr^{299} and Cys^{331} in GmFAAH-I), but substituted by conserved hydrophobic amino acids, valine and proline, respectively, in group II FAAHs (shown as Val^{306} and Pro^{338} in GmFAAH-II) (Figure 3). In the crystal structures of arabidopsis and rat FAAH, the threonine residue is the first residue adjacent to the active site (i.e., it is located at the junction of the substrate-binding pocket and the cytosolic channel; Figure 3A). This residue has been suggested to hydrogen bond with the ethanolamine head group of the substrate and is important for the head group to exit the active site after hydrolysis [20]. The substitution of this threonine residue with valine imparts a less hydrophilic nature to the cytosolic channel of group II FAAHs, which is likely to accommodate substrates with a more hydrophobic head group. This assumption is further supported by other conserved polar-to-hydrophobic amino acid substitutions in the cytosolic access channel of group II FAAHs (e.g., Cys^{331} vs Pro^{338} and Glu^{337} vs Gly^{344} in group I and II FAAHs, respectively; Figure 3B).

Prospective Substrates for Group II Enzymes in Plants

The predicted, conserved structural differences between group I and group II FAAHs suggest different potential substrates for group II FAAHs. In addition to NAEs, there is a wide array of naturally occurring fatty acid amides of plant or microbial origin with signaling functions in plants, providing an extensive list of potential substrates for group II FAAHs (Figure 1). Evidence in the literature points to two groups of small molecules in particular that may serve as additional substrates for FAAHs, the alkamides and the AHLs, both of which facilitate plant–microbe communication. In some cases *in vitro* enzyme activity assays support this broader substrate utilization by FAAH [18,21]. In addition, here, docking studies provide further support for these substrate interactions with group I and II FAAH isoforms. For examples, a representative alkamide and an ***N*-aryl L-homoserine lactone (aryl HL)** are shown docked in the substrate-binding pockets of GmFAAH-I and GmFAAH-II (Figure 4).

Alkamides

Alkamides are a group of fatty acid amides that are widely distributed in several plant species covering ≈30 families, as well as in some plant-associated microbes. They are structurally similar to NAEs with C8 to C18 saturated or unsaturated acyl chains connected to a head group that is an aliphatic, a cyclic, or an aromatic amine. The nature of the acid and amine components is family and genus dependent [22]; however, the most common alkamides in plants contain an acyl chain with a 2*E* double bond and an *N*-isobutyl head group (e.g., affinin; Figure 1 [23]). Alkamides have been well known for their pharmacological and medicinal properties, but their function in plants is

showing some of the conserved residues that differ between the groups (colored red and brown in group I and II, respectively). The residues are named and numbered based on their identity and position in GmFAAH-I and marked with the same symbols employed in (A) for clarification. Other residues that contribute to the predicted cytosolic access channel [not shown in (A)] are Gly^{334} and Glu^{337}, which are conserved in group I FAAHs but are substituted by conserved Trp^{341} and Gly^{344}, respectively, in group II. The sequences of GmFAAH-I and GmFAAH-II are marked with red and brown boxes, respectively. At, *Arabidopsis thaliana*; Ad, *Arachis duranensis*; Mt, *Medicago truncatula*; Gm, *G. max*; Vr, *Vigna radiata*; Fv, *Fragaria vesca*; Rc, *Ricinus communis*; Ga, *Gossypium arboreum*.

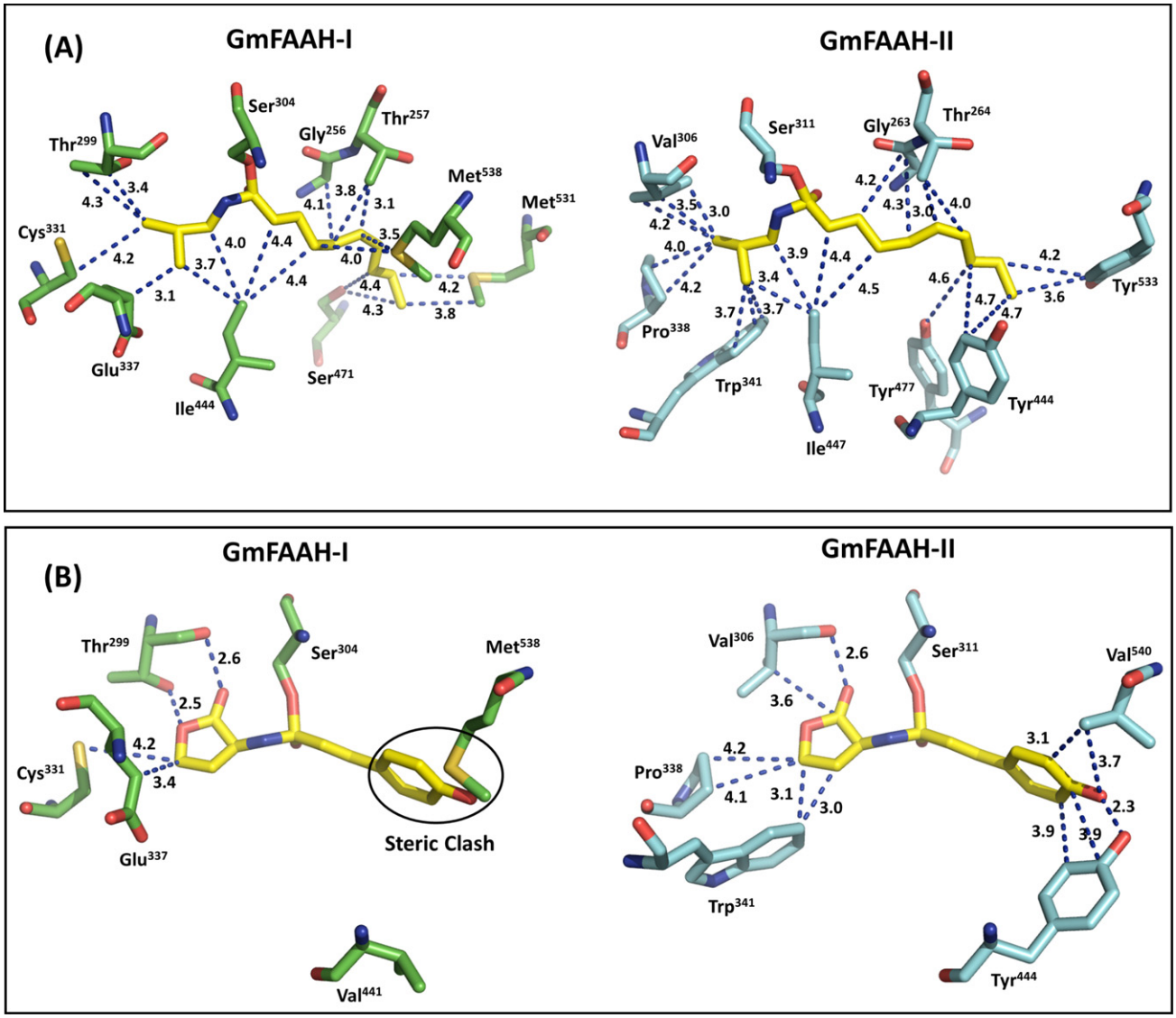

Figure 4. Computational Docking of an Alkamide and an Aryl Homoserine Lactone (HL) in the Substrate-Binding Pocket of Group I and Group II Fatty Acid Amide Hydrolases (FAAHs). (A) *N*-Isobutyl-2*E*,6*Z*,8*E*-decatrienamide (affinin; yellow sticks) docked into the substrate-binding pocket of GmFAAH-I (green sticks) and GmFAAH-II (cyan sticks). As expected, several hydrophobic and aromatic amino acids in the substrate-binding pockets of both enzymes accommodate the binding of the acyl chain of the substrate mainly via hydrophobic interactions. The main difference between the two enzymes is in the accommodation of the hydrophobic (isobutyl) head group of the substrate. In GmFAAH-I, the cytosolic access channel is mainly lined with polar amino acids (e.g. Thr^{299}, Cys^{331}, Glu^{337}), allowing only a few hydrophobic (carbon–carbon) interactions with the isobutyl head group of the substrate. By contrast, the cytosolic channel of GmFAAH-II includes several hydrophobic amino acids (e.g., Val^{306}, Pro^{338}, Trp^{341}), providing a more accommodating environment that supports the binding of the hydrophobic isobutyl head group of the substrate via a network of hydrophobic interactions, suggesting that alkamides could be better substrates for group II FAAHs, relative to the group I enzymes. (B) *N*-(*p*-Coumaroyl)-L-homoserine lactone (yellow sticks) docked into the substrate-binding pocket of GmFAAH-I (green sticks) and GmFAAH-II (cyan sticks). In this case, not much difference is observed in the accommodation of the HL head group by the two enzymes, where the binding of the head group is supported by hydrogen-bonding and hydrophobic interactions. However, in the GmFAAH-I substrate-binding pocket, a steric clash between the *p*-coumaroyl tail of the substrate and Met^{538} prohibits the binding of the substrate, providing a possible explanation for why group I FAAHs could not utilize this aryl HL as a substrate [18]. Conversely, this Met^{538} is substituted by a less bulky residue (Val^{540}) in GmFAAH-II, allowing accommodation of the aromatic ring of the substrate. Moreover, the presence of the aromatic amino acid Try^{444} in the substrate-binding pocket of GmFAAH-II further supports the binding of the substrate via H-bonding and π–π interactions, suggesting that the aryl HLs could be potential substrates for group II FAAHs. The ligand-bound AtFAAH crystal structure (PDB: 6DII [14]) was used as a reference to guide the substrate docking experiments and the carbonyl carbon of the amide group of the substrates is placed at a covalent-bond distance from the nucleophilic serine (Ser^{304} and Ser^{311} in GmFAAH-I and II, respectively), to account for the catalytic mechanism of FAAHs.

not well understood. Several studies have suggested that alkamides could have a role in plant growth and differentiation as well as in defense. For example, treatment of rice with an alkamide isolated from the actinomycete *Amycolatopsis* sp. was shown to promote or inhibit plant growth at low and high concentrations, respectively [24]. Similar results were observed when arabidopsis seedlings were treated with three plant alkamides, where the seedlings exhibited concentration-dependent alterations in primary root growth, lateral root formation, and root hair elongation [23]. More recently, the plant alkamide *N*-isobutyl decanamide (Figure 1) was shown to activate jasmonic acid (JA) biosynthesis and signaling pathways in arabidopsis, resulting in higher resistance to the necrotic fungus *Botrytis cinerea*, suggesting a role of alkamides in the induction of plant immunity [25]. In another systematic study, a series of alkamides were synthesized to contain the common acyl chains found in plant NAEs (e.g., 12:0, 16:0, 18:2) and each of the four common amino-alkyl head groups found in alkamides (i.e., ethyl-, propyl-, isopropyl-, and isobutyl-amine). These 'hybrid' synthetic alkamides were tested for activity toward arabidopsis FAAH, and although these compounds were hydrolyzed to some degree by AtFAAH, they were decidedly inferior substrates relative to NAEs [21]. As described above, the cytosolic port of group I FAAHs is mainly hydrophilic and may not efficiently accommodate the binding of hydrophobic head groups of alkamides, while the cytosolic access channel of group II FAAHs is relatively more hydrophobic, which could favor the binding of alkamides as substrates (Figure 4A). While alkamides may be potential substrates, the group II FAAHs extend to plant species that do not produce their own endogenous alkamides, so it may be that alkamides of microbial origin are also substrates for plant FAAHs.

AHLs

N-Acyl amides of microbial origin include the AHLs (Figure 1). AHLs are a well-known class of quorum-sensing (QS) signals that are produced and perceived by Gram-negative bacteria for cell-to-cell communications to monitor population density and coordinate group activities, such as the release of virulence factors, biofilm formation, antibiotic resistance, and motility, among others [26]. They also play a role in interkingdom interactions between bacteria and eukaryotic hosts, including animals, and plants [27]. An example that bacterial AHLs are involved in plant–microbe interactions was demonstrated for the interaction between the plant pathogen *Pseudomonas aeruginosa* and *A. thaliana*, where mutation of the AHL synthase-encoding gene *lasI* disrupted bacterial QS for virulence and promoted the biosynthesis of compounds (known as cyclodipeptides) that mimic the plant growth hormone auxin [28]. Consequently, root inoculation with wild-type *P. aeruginosa* PAO1 strongly inhibited plant growth, while *P. aeruginosa lasI* mutants, which are defective in the production of the virulence factor pyocyanin, promoted root branching and increased phytostimulation [29]. AHLs are structurally similar to NAEs and alkamides, with a C4–C18 acyl chain that is amide linked to a homoserine lactone (HL) head group, and are classified based on their acyl chain length as short- (C4–C8), medium- (C8–C12), and long- (≥C12) chain AHLs (Figure 1); their specific activities depend on the acyl chain length, the degree of unsaturation, and the substitution at position 3 [30]. Interestingly, some photosynthetic bacteria can also produce aryl HLs with an aromatic tail instead of the 'classical' aliphatic chain [31,32] (Figure 1). The soil zone in close contact with the plant roots, known as the rhizosphere, is a nutrient-rich region that is essential for plant–microbe interactions. AHLs can diffuse through bacterial membranes and distribute within the rhizosphere, representing specific 'signatures' of the rhizosphere-inhabiting bacteria [33]. Plants can recognize QS signals to regulate growth, development, and defense, and in turn release an array of compounds with signaling or nutritional functions for bacteria [33]. The first study that demonstrated the ability of plants to perceive AHL signals was performed on medicago (*Medicago truncatula*), where treatment with two different AHLs from two different bacterial species triggered significant changes in the accumulation of over 150 proteins with diverse functions related to plant defense,

stress response, transcriptional regulation, plant hormone responses, energetics and primary metabolism, and cytoskeletal activities [34]. Moreover, exposure to AHLs was found to induce changes in the secretion of compounds by plants that mimic QS signals, suggesting that plants have the ability to interact with or disrupt QS in root-associated bacteria [34]. Extensive studies have now established the effects of several synthetic and naturally occurring AHLs from various symbiotic or pathogenic bacteria on different plant systems, including arabidopsis, tomato (*Solanum lycopersicum*), medicago, barley (*Hordeum vulgare*), and wheat (*Triticum aestivum*), exhibiting a broad range of activities, such as enhancement of plant growth, modulation of hormone responses, priming and induction of plant defense and disease resistance, increase of nodule number, and others (see recent reviews in [33,35]). In general, the effects of AHLs on plants can vary, depending on the plant species and the AHL type [35]. Also, some plant responses can be triggered by different AHLs regardless of their structure, while other responses are distinct to specific AHLs, suggesting that plants may be able to recognize the frequently encountered bacterial symbionts or pathogens based on the organism-specific formation of particular AHLs [34].

Due to the structural similarities between NAEs and AHLs, Ortiz-Castro *et al.* [36] studied the effect of C10-HL on seedling growth in *AtFAAH* overexpressors and knockout mutants. These lines exhibited modified sensitivity to C10-HL relative to wild type, suggesting that AHLs can be degraded by FAAH *in vivo*. Further studies using *A. thaliana* and *M. truncatula*, Palmer *et al.* [18] demonstrated that AHLs elicit a biphasic growth response, where they enhance seedling growth at low concentrations, via increases in transpiration and nutrient uptake, and inhibit growth at high concentrations by stimulating ethylene production. These responses were acyl chain-length dependent, with the effects more pronounced for long-chain AHLs (≥12 carbons). Interestingly, on exposure to the hydrolytic products of AHLs, the free L-homoserine, and not the acyl chain tail, elicited the same responses as the intact AHLs, indicating that L-homoserine was the bioactive agent in plants in this case. Moreover, *Atfaah* knockout mutants were insensitive to the growth-modulating effects of AHLs but not to the free L-homoserine, suggesting that the activity of AHLs in plants is dependent on their hydrolysis by FAAH to generate free L-homoserine [18]. Furthermore, AtFAAH was shown to utilize AHLs as substrate *in vitro* and the level of the amidohydrolase activity toward AHLs was correlated with the acyl chain length [18], which was consistent with the more pronounced growth-modulating effects observed for the long-chain AHLs in the seedlings growth assays.

These overall results establish that FAAH plays a key role in plant responses to AHLs, and hydrolysis by FAAH is essential for plants to perceive these QS signals. Moreover, the *in vitro* enzymatic activity of arabidopsis FAAH toward AHLs [18] showed that AtFAAH (group I) could efficiently utilize the long-chain AHLs, but exhibited lower activity toward the C10-HLs and had no activity toward the short-chain AHLs and the aryl HLs. In the same study [18], the short-chain AHLs and aryl HLs did not show significant growth-modulating effects in arabidopsis, probably because arabidopsis lacks the enzymatic machinery to hydrolyze these specific HLs. These findings raise the question of whether group II FAAHs in other plant systems expand the HL-hydrolyzing activity of group I to short-chain AHLs or aryl HLs to mediate interactions with additional specific, beneficial or pathogenic, bacteria that utilize these particular HLs for QS and/or communication with their host plants. Some plant pathogenic bacteria, such as *Burkholderia glumae* that causes rice (*Oryza sativa*) seedling and grain rots and *Erwinia carotovora* that causes soft-rot disease in several plants [e.g., potato (*Solanum tuberosum*), tomato], rely on short-chain AHLs to coordinate the activation of genes encoding their main virulence determinants [37,38]. Also, aryl HLs were recently discovered to be produced by some photosynthetic bacteria, such as *Rhodopseudomonas palustris* (*p*-coumaroyl HL; Figure 1 [31]) and the stem-nodulating bacterium *Bradyrhizobium* ORS278 (cinnamoyl HL; Figure 1 [32]). Both of these bacteria commonly live in association with plants [18], and interestingly *R. palustris* was found to rely on

environmental *p*-coumaric acid to produce *p*-coumaroyl HL. Since *p*-coumarate is produced by plants as a constituent of lignin and its synthesis is stimulated by tissue damage and other stresses, it was suggested that plants are the source of the *p*-coumaroyl moiety for *p*-coumaroyl HL synthesis, which further suggests an 'intimate' relationship between *p*-coumaroyl HL-producing bacteria and specific plants, and that this relationship involves *p*-coumaroyl HL signaling [31]. Although aryl HLs and their effects on plants are not well studied, perhaps their perception by plants is also dependent on FAAH hydrolysis (as in the case of aliphatic HLs), which may be mediated by group II FAAHs. However, a recent study by Palmer *et al.* [39] showed that some synthetic (non-native) analogs of aryl HLs (referred to as SAHLAs), and not the native molecules, can produce auxin-like effects in arabidopsis. Since only minor amounts of the cleavage products of the tested compounds (<1% of the starting material) accumulated in the treated arabidopsis seedlings, the authors proposed that the observed auxin-like effects of these SAHLAs were more likely to be due to the intact molecules, rather than their hydrolytic products [39]. These findings further support that arabidopsis lacks the enzymatic machinery that can efficiently hydrolyze the aryl HLs and suggest that aryl HLs may be perceived by the plants differently from the aliphatic AHLs (i.e., in case of aryl HLs, the intact structures, and not the cleavage products, are the bioactive molecules that produce the growth-modulating effects in plants). However, it remains unclear why arabidopsis responded to certain SAHLA scaffolds and not to the others and, more importantly, why the native aryl HLs did not produce an effect in arabidopsis. It will be interesting to determine which plant systems can perceive the signaling activities of the naturally occurring aryl HLs and to see whether it is dependent on the occurrence of group II FAAHs in these plant systems. If it turns out to be ubiquitous that the intact aryl HLs are the bioactive molecules, and not their hydrolytic products, group II FAAHs may be involved in their signal termination/tuning (as in the case for endogenous NAEs) as opposed to activation (as in the case for aliphatic AHLs). The substrate-binding pocket of group II FAAHs is lined with more aromatic amino acid residues relative to group I, where it includes two tyrosine residues in place of Val and Met (Figure 3). These aromatic amino acids support the binding of substrates with aromatic moieties (e.g., aryl HLs) through π–π interactions (Figure 4B) in addition to reducing the cavity size, which may favor shorter-chain AHLs.

Hypothesis: A FAAH-Dependent *N*-Acyl Amide Communication Axis Supports Plant–Microbe Interactions

Bringing together recent information in the literature on acyl amides, the observed promiscuity of AtFAAH for a range of *N*-acyl amide substrates, and the structural divergence of plant FAAHs, we propose that there are likely to be expanded functional roles for FAAH in angiosperms that include plant–microbe interactions. It is our hypothesis that a FAAH-dependent acyl amide communication axis supports beneficial and/or pathogenic plant–microbe interactions and that the substrate-binding pockets of two groups of FAAH enzymes provide the flexible use of a wide range of small lipophilic signaling molecules that determine the outcome of these interactions. In addition to the aliphatic and aryl HLs discussed above, there are additional lines of evidence that support this hypothesis. For example, it was recently shown that the defoliating strains of the plant pathogenic fungus *Verticillium dahliae* produce high levels of NAE 12:0, which is transported into the host plant (cotton) inducing the expression of several FAAHs that in turn disrupt endogenous NAE metabolism and abscisic acid signaling and cause severe leaf defoliation symptoms [40]. NAE 12:0 is a minor NAE component of the endogenous NAE pool in plants [41], but its overproduction by pathogenic microorganisms to achieve pathogenesis via induction of plant FAAHs emphasizes the concept that FAAH in plants play a key role in plant–microbe interactions. Also, of particular relevance here, is the previous research showing that overexpression of *AtFAAH* in *A. thaliana* resulted in compromised immunity toward bacterial pathogens [42]. This strengthens the assumption that FAAHs may be involved in the hydrolysis of lipophilic amides produced by plant symbionts or pathogens to communicate with their host plants.

Such hydrolysis by FAAH could be for either activation or inactivation of these signals, depending on the plant–microbe system and the nature of the signal molecule. Along the same lines, it was recently shown that human commensal bacteria produce small molecules [*N*-acyl-3-hydroxyglycines; e.g., commendamide (Figure 1)] that resemble the endogenous long-chain *N*-acyl amides and were able to activate the human G protein-coupled receptor (GPCR), which has important implications for human health and disease [43]. The occurrence of similar signaling molecules (e.g., amino acid conjugates of long-chain fatty acids) and their contribution to FAAH-mediated plant–microbe interactions should be investigated further, especially with regard to well-known amino acid conjugates of fatty acid derivatives like JA-Ile.

Actinomycetes and other actinobacteria represent a large group of plant-growth promoting (PGP) bacteria with diverse beneficial effects on plants, including phosphate solubilization, iron acquisition, phytohormone production, induction of systemic resistance against pathogens, and nitrogen fixation in nonleguminous plants. They also act as 'helper' bacteria that promote symbiotic interactions, such as legume–rhizobia symbiosis and mycorrhizal symbiosis [44]. As mentioned above, an alkamide isolated from the actinomycete *Amycolatopsis* sp. was shown to modulate plant growth in rice [24], raising the question of whether the interaction of these PGP actinobacteria with their host plants involves the production of lipophilic amide signaling molecules (e.g., alkamides) that require the enzymatic activity of group II FAAHs for signal regulation.

Although it is unclear why arabidopsis and other Brassicaceae plants, as well as castor, lack group II FAAHs, it may be that these groups of plants during evolution have lost orthologs of putative genes that mediate some microbial interactions. For instance, 29% of vascular plants,

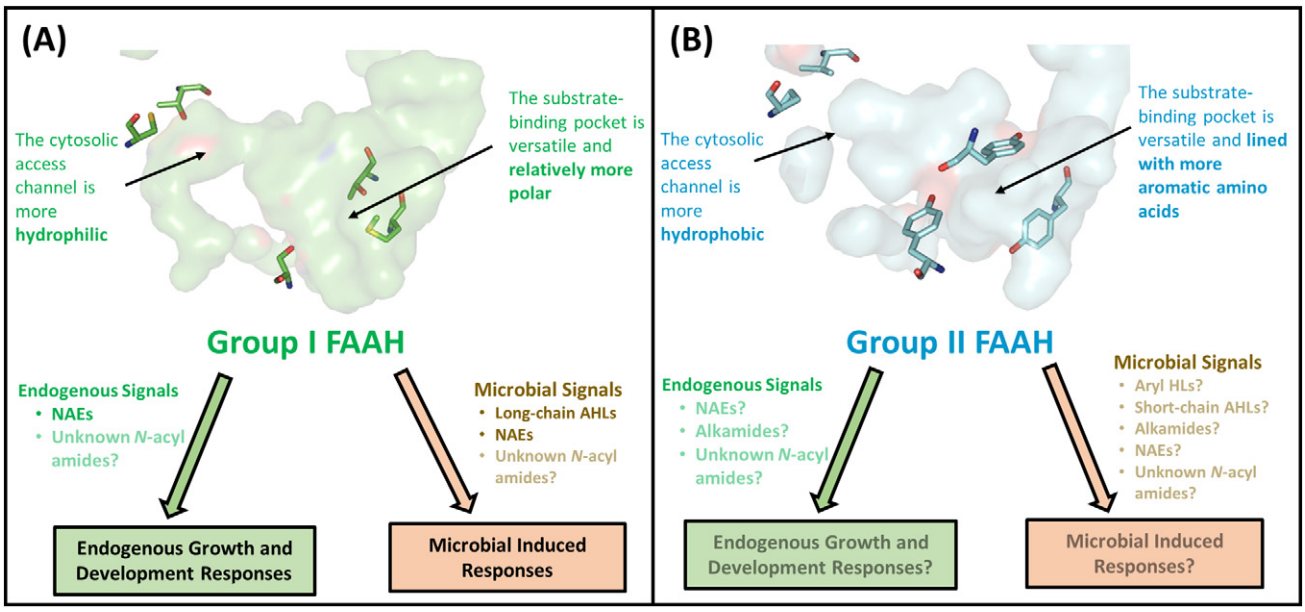

Figure 5. Summary of the Different Potential Roles of Fatty Acid Amide Hydrolase (FAAH) in Angiosperms. The established and proposed functions of group I (A) and group II (B) FAAHs are outlined. The unknown, proposed substrates/functions are indicated by lighter lettering and question marks. The main established role of group I FAAHs is in bolded font – the regulation of *N*-acylethanolamine (NAE) signaling in plants (mainly studied in *Arabidopsis thaliana*), which in turn regulates several physiological processes during seedling growth and establishment [15]. Also, AtFAAH (group I) was shown to utilize long-chain *N*-acyl L-homoserine lactones (AHLs) as substrates and therefore mediate their perception by arabidopsis to produce their growth-modulating effects [18]. A potential role of FAAH in *Verticillium dahliae* pathogenesis in cotton, which involves the production of NAEs as microbial signals, was reported [40] and suggests that both groups of FAAHs may be involved; however, it is still unknown whether group II FAAHs can utilize NAEs as substrates, so it remains an open question for group II FAAHs. The other proposed substrates/functions of group II FAAHs are presented.

including those of the Brassicaceae, have lost or are suppressed in their ability to host symbioses with arbuscular mycorrhizal (AM) fungi; these plants are called nonmycorrhizal or nonhost plants [45], and perhaps without a group II FAAH these plants are 'blind' to some sets of microbially derived chemical messages.

Concluding Remarks and Future Perspectives

The structural elucidation of FAAH from arabidopsis has helped to reveal two distinct groups of structurally related enzymes in angiosperms. Substitutions in the substrate-binding pocket conserved between the two groups of proteins suggest divergence in substrate recognition, and an emerging literature from diverse areas suggests that a promiscuous FAAH-substrate platform may represent a previously underappreciated chemical communication system for plants and their interacting microbiota. Continued studies in this area will help to test the hypothesis that FAAH proteins hydrolyze a broader range of lipophilic substrates than previously recognized and consequently play a pivotal role in *N*-acyl amide-mediated plant–microbe interactions, a function beyond the established role for FAAH in seedling development in arabidopsis (Figure 5) (see Outstanding Questions).

Outstanding Questions

What is the complete inventory of *N*-acyl amides recognized and hydrolyzed by plant FAAH enzymes?

Do the amino acid substitutions in group II FAAH proteins support the selective preference for alkamides and/or short-chain and aryl HLs?

Are there additional, novel *N*-acyl amides (e.g., amino acid conjugates of long-chain fatty acids) of plant or microbial origin with signaling functions in plants that have yet to be identified?

What specific combinations of *N*-acyl amides and FAAH proteins define selective plant–microbe communication pathways for interaction?

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